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ABSTRACT BOOK

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EHA aims to promote excellence in clinical practice, research and education in European hematology.

Today, EHA – with over 3200 active members from 100 countries – is a consolidated organization that pursues a large and growing number of projects and programs. An Executive Board and Councilors elected by the membership form the governmental body and are responsible for the strategy and organization of the Association.

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1. Promote scientific research in hematology
2. Promote education and training in hematology
3. Promote optimal clinical care in hematology
4. Advance the exchange and dissemination of knowledge and scientific information in hematology
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14TH CONGRESS
JUNE 4 - 7, 2009
BERLIN

Word of welcome

On behalf of the EHA Board and the Scientific Program Committee we would like to welcome you to Berlin for the 14th Congress of the European Hematology Association.

This year, over 2000 abstracts have been submitted – a new record – showing the growing significance of the EHA Congress to hematologists in Europe and abroad. Moreover, 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 (5 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 Berlin and hope that this Abstract Book will serve you as an important reference for recent advances in hematology research.

Radek Skoda
Chair Scientific Program Committee

Rüdiger Hehlmann
Congress President

EUROPEAN HEMATOLOGY ASSOCIATION

EHA FELLOWSHIP PROGRAM

One of EHA's goals is to promote the career development of young scientists involved in basic, clinical, and experimental research in hematology. EHA funds research fellowships, including the long-established EHA – José Carreras Young Investigator Fellowship, for researchers in the field of malignant and non-malignant hematology. Each grant is for a two-year period. The next call for applications will be announced on the EHA website in October 2009. Awards will be made at the 15th Congress of EHA in Barcelona and will be payable from January 2011.

EHA PARTNER FELLOWSHIP PROGRAM

A partner fellowship program was established last year to support training in centers of excellence in Western Europe for the career development of young scientists involved in basic and clinical research in hematology in new accession and EU candidate countries. The goal of this program is to build up and create inter-institutional networks of collaboration between hematology institutes in Western Europe focusing on research and clinical hematology and similar institutes in new accession and EU candidate countries. The call for applications is announced on the EHA website and the deadline for the letter of interest is June 30, 2009. Deadline for full application is August 15, 2009 and notification of the award will be November 1, 2009.

EHA – ASH RESEARCH EXCHANGE AWARD

The EHA-ASH International Research Fellowship Award was established in 2006. This award has been developed as a partnership between the European Hematology Association (EHA) and the American Society of Hematology (ASH) to provide hematologists in training or early in their careers the opportunity to conduct research in another country. The purpose of the program is to give both clinical and laboratory-based researchers an opportunity to establish new collaborations and experience research in a different environment. This program will benefit not only the individual participants, but also each host institution, as it will build stronger ties between the North American and European scientific community. The call for applications is announced on EHA and ASH websites and the deadline for the letter of intent was May 15, 2009. Deadline for full application is September 25, 2009. Awards will be made on July 1, 2010.

Submission: Detailed information on the EHA and EHA/ASH fellowship programs and submission is available in the Fellowships section at the EHA website: www.ehaweb.org or contact fellowships.grants@ehaweb.org

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POSTER SESSION I

Developmental hematopoiesis, stem cells and microenvironment

0001

RESCUE OF HEMATOPOIETIC FAILURE INDUCED BY THE GATA 1(LOW) MUTATION IS MEDIATED BY A PERMISSIVE MICROENVIRONMENT IN THE SPLEEN THAT INDUCES DIFFERENTIATION OF GATA -1(LOW) PROGENITORS THROUGH THE ALTERNATIVE ENHANCER HS2

A.R. Migliaccio,[1] F. Martelli,[2] M. Verrucci,[2] M.E. Fabucci,[2] M. Sanchez,[2] G. Migliaccio,[2] A.M. Vannucchi,[3] M. Zingariello,[4] A. Di Baldassarre,[4] B. Ghinassi,[4] R.A. Rana,[4] Y. Van Hensbergen,[5] W. Fibbe[5]

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Background. Recently, progress has been made in understanding the relationship existing between stem cells and their marrow niches under steady state conditions. By contrast, little is known on the relationship between these cells and the niches in extramedullary sites under stress. Targeted deletion of the HS1 enhancer of Gata1 (Gata1low mutation) induces in C57BL/6 mice a lethal phenotype because of severe anemia and thrombocytopenia at birth. The mutation is not lethal in those backgrounds (CD1 and DBA2) that recover from anemia by developing hematopoiesis in the spleen (Martelli *et al.,* Blood 106:4102, 2006), suggesting the existence of a *unique* relationship between Gata1low stem cells and the niches present in extramedullary sites. *Aim.* To identify the relationship between Gata1low stem cells and the extramedullary niche in the spleen, that rescues the Gata1low mutation in the CD1 strain. *Methods.* The phenotype of Gata1low/0 males and heterozygous Gata1low/+ females (Gata1 is on the X chromosome) after splenectomy was determined. In addition, tracking experiments of cKitpos cells in Gata1low/0 males carrying a reporter gene under the control of an alternative Gata 1 enhancer (HS2) spared by the mutation (-2.7kbGata1GFP), and transplantation experiments with Gata1low/0 male bone marrow cells into NOD/SCID females were performed. *Results.* After splenectomy, hemizygous males died of severe anemia within 1 month while heterozygous females survived. Moreover, while in untreated heterozygous females hematopoiesis was driven by both stem cells expressing the wild-type allele and those expressing the Gata1low allele, hematopoiesis in spenectomized heterozygous females was wild type, suggesting that spleen and marrow drive maturation of wild-type and Gata1low stem cells, respectively. This hypothesis was confirmed by the localization of -2.7kbGata1GFPcKitpos cells within the architecture of the marrow and spleen of Gata1+/0 and Gata1low/0 males. Rare GFPnegcKitpos cells were detected along the endosteum of -2.7kbGata1GFPGata1+/0 males but numerous cKitpos cells were detected within their medulla. Rare cKitpos cells were also detected in the spleen of these males. By contrast, clusters of GFPposcKitpos cells were detected along the endosteum of -2.7kbGata1GFPGata1low/0 males while cKitpos cells detectable within their medulla were rare. Numerous cKitpos cells were in stead detected in the spleen of these mice. Therefore, Gata1low stem/progenitor cells are associated in greater numbers with the osteoblast niches than with the marrow vascular niche, consistently with the low levels of CXCR4, the receptor necessary for interaction with the vascular niche, expressed by these cells. -2.7kbGata1GFPGata1low/0 stem/progenitor cells from the spleen, but not those from the marrow expressed high GFP levels and were functional in colony assay. In addition, Gata1low bone marrow cells engrafted NOD/SCID females and donor-derived cells were mainly detected in blood, spleen and liver, but not in the marrow of the recipients two-four months after transplantation, indicating that Gata1low stem/progenitor cells contribute to hematopoiesis by engrafting preferentially the extramedullary sites of the hosts. *Summary and conclusions.* These data suggest that Gata1low hematopoiesis is supported by the splenic microenvironment that favors maturation of those stem/progenitor cells which activate Gata1 expression through the alternative HS2 enhancer.

0002

COMPARISON OF HEMATOPOIETIC STEM CELL ENGRAFTMENT ON SYNGENEIC AND ALLOGENEIC STROMAL TERRITORIES IN THE ABSENCE OF IMMUNE REACTION

I. Resnick, T. Prigozhina, G. Elkin, B. Avni, M. Shapira, R. Or, P. Stepensky, O. Gurevich

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Background. In clinical practice of bone marrow transplantation (BMT) there is a set of cases with compromised HSC engraftment which is apparently not a consequence of immunological rejection but most likely due to recipient stromal microenvironment which they encounter. We evaluate hypothesis that matching between stromal and hematopoietic cells is important for adequate support of hematopoietic development. Aim of this study was to demonstrate that hematopoietic stem cell (HSC) can discriminate between *self* and *non self* stromal territories. *Methods.* An animal model of two genetically different types of functioning stroma in the common recipient was worked out. Step 1: bone marrow plugs from C57Bl/6 and BALB/c donor mice were ectopically transplanted to F1(C57Bl/6xBALB/c) recipients. Within one month the newly formed ectopic osteo-hematopoietic complex displayed all properties of a skeletal hematopoietic area. Forty days later F1 recipients having additional stromal territories of C57Bl/6 and BALB/c genotypes got total body irradiated with 1150 cGr, a the lethal dose for the cells of hematopoietic but not mesenchymal stromal origin. Step 2: the first group of irradiated mice was injected with the mixture (1:1) of embryonic liver precursor cells from C57Bl/6 and BALB/c fetuses while second received hematopoietic cells from C57Bl/6 embryonic liver only. Step 3: histological examination and evaluation of competitive repopulation of C57Bl/6 and BALB/c stromal territories with C57Bl/6 and BALB/c HSC using FACS analysis of harvested hematopoietic cell populations on days +14 and +28 after transplantation of HSC were performed. *Clinical trial design.* A single pediatric patient with severe Wiscott-Aldrich syndrome who had neither sibling nor matched unrelated donor was admitted for haploidentical HSC transplantation. Treatment was approve by IRB. After myeloablative conditioning patient got intravenous transplantation of positively selected $CD34^+$ cells combined with intraosseous injection of pre-expanded mesenchymal stromal cells (MSC) from the same donor. *Results.* Histological analysis of ectopic ossicles developed from hematopoietic inoculum of C57Bl/6 embryonic cells on C57Bl/6 and BALB/c ectopic territories showed young, active and very dense hematopoietic tissue on syngeneic (C57Bl/6) stroma while on the allogeneic (BALB/c) stroma was observed aplastic hematopoiesis accompanied with numerous fat containing cells. Both 2 and 4 weeks after HSC transplantation proportion of C57Bl/6 and BALB/c HSC was 1.5 to 2.5 times higher on syngeneic compare to allogeneic compartment. The same effect was clearly seen in clinical setting. After engraftment, morphological findings in the bone marrow demonstrate 50% cellularity with 3 lineage hematopoiesis in the site of donor type MSC injection compare to aplastic bone marrow in different place. *Interpretation.* Taking into consideration that immunological conditions for inoculated HSC of C57Bl/6 and BALB/c genotypes present in the common F1 recipient are similar, different colonization of *self* and *non self* stroma is not mediated by immunocompetent cells. This effect can be due to discrepancy between hematopoietic tissue and misfitting stromal territories. Our pilot clinical data confirm that presence of genetically identical niche can significantly influence on HSC engraftment.

0003

THE EFFECT OF THE TEL-TRKC ONCOGENE ON THE HAEMATO-ENDOTHELIAL DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELLS

R. Malladi, J. Sloane-Stanley, R. Gupta, N. Rodrigues, W. Wood, T. Enver

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Background. A fusion between the pointed domain of TEL and the tyrosine kinase domain of TRKC (TEL-TRKC) causes tumours presenting in utero and in early childhood. We hypothesised that a cellular intermediate in the mesoderm-derived haemato-endothelial hierarchy is a potential target for the TEL-TRKC oncogene. The human ES cell system can generate haemato-endothelial cells from mesoderm precursors and may provide a suitable *in vitro* model for investigating the oncogenic properties of TEL-TRKC during ontogeny. *Aims.* We sought to identify a cellular target for TEL-TRKC during human embryonic stem (ES) cell differentiation into haemato-endothelial lineages as well as to understand the impact of TEL-TRKC on cell fate decisions within this hierarchy. *Methods.* Haemato-endothelial differentiation of human ES cells was achieved by co-culture with the murine OP9 stroma according to the protocol of Vodyanik *et al.* Blood 108, 2006. In this model, $CD34^+$, KDR^+, $CD43^-$ endothelial cells and $CD34^+$, $CD43^+$ haematopoietic cells were prospectively isolated for functional analysis. Enforced expression of TEL-TRKC in human ES cells was achieved by lentiviral gene delivery with a green fluorescent protein reporter. The equivalent lentiviral vector without the TEL-TRKC was used as a control. *Results.* Normal human ES cells co-cultured with OP9 give rise to a population of $CD34^+$ cells which can differentiate into either blood or endothelium. TEL-TRKC expression was compatible with the generation of normal numbers of these $CD34^+$ precursors derived from mesoderm. TEL-TRKC, however, promoted the expansion of $CD43^+$ haematopoietic cells from these $CD34^+$ precursor cells without affecting the capacity of this $CD34^+$ population to differentiate into endothelial cells. The function of these endothelial cells was normal, as judged by uptake of Dil-Ac-LDL and the matrigel tube formation assay. We next investigated the function of the expanded $CD43^+$ haematopoietic cells from TEL-TRKC transduced hES cells and found that this population acquired self-renewal properties as assessed by its ability to replate on OP9 stroma. Strikingly, these $CD43^+$ haematopoietic cells demonstrated an endothelial phenotype as evidenced by inappropriate co-expression of the endothelial marker, VE-Cadherin. This, together with the reduced ability of these $CD43^+$ cells to form colony forming units in methylcellulose assays, suggests a block or arrest in haematopoietic commitment or differentiation. *Summary and Conclusion.* TEL-TRKC expression in human ES cell derived haemato-endothelial cells is consistent with the trapping of a population of $CD43^+$ haematopoietic cells with acquired self-renewal properties and endothelial potential. We conclude that TEL-TRKC transforms mesoderm derivatives and speculate that these derivatives are a target for TEL-TRKC in the provenance of developmental cancers in children.

0004

INTERFERON-ALPHA ACTIVATES 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-α/β receptor (IFNAR), STAT1 or Sca-1 are insensitive to IFNα stimulation, demonstrating that STAT1 and Sca-1 mediate IFNα induced HSC proliferation. We will present our newest data on the examination of the molecular basis of these striking effects of IFNa signalling on HSCs, both during homeostasis as well as during stress. Moreover, 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.

0005

FURTHER CHARACTERIZATION OF COMMITTED NK PROGENITORS IN ADULT BONE MARROW

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Natural killer cells, members of the innate immune system, represent a third lymphoid lineage distinct from T and B lymphocytes. Although significant progress has been made in understanding the regulation and the function of mature NK cells, the cellular and molecular pathways of the earliest stages of NK lineage commitment and development remain largely unknown. Committed NK progenitors (NKPs) have been recently identified in adult bone marrow (BM) as having the $Lin^-CD122^+NK1.1^-DX5^-$ phenotype; however NKPs lineage potential has not been studied at the single cell level. In adult mice, BM is the main site of NK cell production and it harbours both progenitors and mature NK cells. Recent studies demonstrated the existence of another and seemingly distinct, thymus dependent NK pathway and it remains to be established whether BM- and thymic- dependent NK cells can be generated from the same NK committed progenitor. In agreement with the previous studies, FACS phenotypic analysis revealed that NKP population is heterogeneous: small fraction of NKPs expressed tyrosine kinase receptor FLT3 (5-10%) or KIT (5-10%), while majority of NKPs were positive for B220 (40-50%) and Interleukin 7 (IL-7) receptor (IL-7R) (50-60%). In addition, analysis using Rag-1-GFP reporter mouse, confirmed that 50-60% of NKPs expressed Rag-1. Limiting dilution culture assay revealed that NKPs lack myeloid and B cell potentials, while 60% of single sorted NKPs cultured on OP9DL cell line for 14 days generated clones containing $CD3^-NK1.1^+DX5^+$ NK cells (72%), $TCR\beta^+NK1.1^+DX5^+$ NK T cells (21%) and $TCR\beta^+NK1.1$-DX5-$CD4^+CD8^+$ T cells (9%). In agreement with *in vitro* data, NKPs after transplantation into $Il2rg^{-/-}$ recipients showed NK cell ($CD3^-NK1.1^+DX5^+$), T cell ($CD3^+CD4^+CD8^+$) and NK T cell ($CD3^+NK1.1^+$) donor-derived lineage reconstitution, but failed to generate B, myeloid as well as thymic NK cells. Taken together these data suggest that NKPs maintains significant T cell potential that will be further addressed both *in vitro* as well as in intra-thymic transplantation assays.

0006

TYROSINE KINASE INHIBITION FAILS TO ERADICATE LEUKEMIC STEM/PROGENITOR CELLS IN FLT3-ITD+ ACUTE MYELOID LEUKEMIA: ROLE OF THE STEM CELL NICHE

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Background and Aims. Acute myeloid leukemia (AML) is a clonal disease originating from a leukemic stem/progenitor cell. Activating mutations of the FLT3 receptor by internal tandem duplication (FLT3-ITD) are present in 30% of all cases of AML and are associated with poor prognosis. Although inhibition of mutant FLT3 leads to clearance of leukemic blasts in the periphery, the bone marrow often remains unchanged and remissions are usually short-lived, suggesting a protective effect of the marrow niche on leukemic stem cells. We studied the effect of tyrosine kinase inhibition on $CD34^+$ leukemic stem/progenitor cells from patients with newly diagnosed normal karyotype AML with wild-type FLT3 or mutated FLT3-ITD receptor in the presence or absence of stromal support. *Methods.* $CD34^+$ cells were isolated from bone marrow of AML patients at diagnosis by density gradient centrifugation and magnetic bead isolation. Cells were cultured for four days in serum-free medium with growth factors in the presence or absence of the tyrosine kinase inhibitor SU5614 in suspension culture or stroma-contact cultures with the stromal cell line EL08-1D2. Cell division was measured by flow cytometry after labelling of cells with CFSE. Analysis of cell cycle and apoptosis was performed by flow cytometry after staining with propidium iodide (PI) and annexin V. Hematopoietic activity was assessed

in short-term and long-term colony-forming assays. PCR for FLT3 WT and ITD products was performed to determine colony origin. Results. CD34+ cells from FLT3-ITD samples divided at an intrinsically slower pace over four days *in vitro* than CD34+ cells from FLT3-WT samples. Treatment with SU5614 resulted in reduced cell division of both FLT3-WT and FLT3-ITD progenitor cells. However, FLT3 mutated progenitor cells differed from FLT3 WT cells in their behavior on stroma. Whereas stromal contact induced cell division of FLT3-WT progenitors in the presence of SU5614, no such effect was observed with FLT-ITD progenitors. Apoptosis induced by tyrosine kinase inhibition was more pronounced in FLT3-ITD than FLT3-WT progenitors. Stromal contact protected both FLT3-WT and FLT3-ITD CD34+ progenitor cells from apoptosis. Colony assays revealed that FLT3-ITD committed progenitors were effectively reduced by SU5614 treatment in suspension culture while stroma contact exerted a significant protective effect. In contrast, committed progenitors from FLT3-WT AML were less susceptible to tyrosine kinase inhibition but also protected by adhesion to stroma. More importantly, primitive LTC-IC from FLT3-ITD AML were selectively spared from tyrosine kinase inhibition. Additional stromal contact led to significant expansion of LTC-IC in the presence of SU5614. PCR from single hematopoietic colonies of long-term cultures revealed both WT and ITD FLT3 products, indicating LTC-IC were of leukemic origin. Cell signaling studies revealed independent activation of AKT in the presence of stroma in FLT-ITD cells treated with SU5614. Inhibition of downstream ERK and STAT5 by SU5614 in FLT3-ITD cells was not affected by stromal support. *Conclusions.* Our data demonstrate that tyrosine kinase inhibition fails to eradicate leukemic stem/progenitor cells in FLT3-ITD AML. Interaction with stromal cells additionally promotes selective survival of leukemic stem/progenitor cells in the presence of tyrosine kinase inhibitor, resulting in net expansion of malignant cells. Independent activation of AKT through alternate pathways mediated by stromal contact in FLT3-ITD cells allows escape from dependence on FLT3 signalling. This data highlights the fact that molecular therapy may have unpredicted effects on leukemic stem cells and underlines the importance of developing strategies to selectively eliminate the malignant stem cell clone.

0007

ARYL HYDROCARBON RECEPTOR ACTIVATION PERTURBS MYELOID SUBLINEAGE DIFFERENTIATION BY INHIBITING PU.1 UPREGULATION

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The transcription factor aryl hydrocarbon receptor (AhR) is a ligand inducible transcription factor. AhR activation by different ligands has been been shown to influence lymphopoiesis as well as myelopoiesis and in line with this human hematopoietic stem cells express AhR and are affected by AhR ligands. Furthermore, dysregulated AhR function can be found in certain hematopoietic malignancies (e.g. high expression of AhR in adult T cell lymphomas). Besides its role in hematopoeisis, the AhR represents a promising therapeutic target in allergy and autoimmunity. AhR signalling induced by the newly described ligand VAF347 inhibits allergic lung inflammation as well as suppresses pancreatic isl*et al.*lograft rejection. These effects are mediated likely via alterations in dendritic cell (DC) function. Moreover, VAF347 induces tolerogenic DCs. The mechanisms underlying functional DC alteration by AhR remained undefined. Furthermore, data on human DCs were lacking. Langerhans cells (LCs) are immediate targets of exogenous AhR ligands at epithelial surfaces; how they respond to AhR ligands remained undefined. We studied AhR expression and function in human LCs and myelopoietic cell subsets using a lineage differentiation and gene transduction model of human CD34+ hematopoietic progenitors. We found that AhR is highly regulated during differentiation. LCs expressed highest AhR levels followed by monocytes. Conversely, granulocytes lacked AhR expression. AhR ligands including VAF347 arrested the differentiation of monocytes and LCs at an early precursor cell stage, whereas progenitor cell expansion or granulopoiesis remained unimpaired. AhR expression was co-regulated with the transcription factor PU.1 during myeloid subset differentiation. VAF347 inhibited PU.1 induction during initial monocytic differentiation, and ectopic PU.1 restored monocyte and LC generation in the presence of this compound. AhR ligands failed to interfere with cytokine receptor signalling (TGF-β1 or TNFα) during LC differentiation and failed to impair LC activation/maturation. In conclusion, activation of AhR signalling interferes with transcriptional processes leading to monocyte/DC lineage commitment of human myeloid progenitor cells.

0008

ULTRACONSERVED GENOMIC REGIONS EXPRESSION IN HEMATOPOIESIS

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Background. Ultraconserved genomic regions (UCRs) are a subset of conserved sequences (100% between orthologous regions of human, rat and mouse genomes) located in both intra- and intergenic regions. There are 481 UCRs described and more than 50% of all the UCRs have been classified as non exonic (with no evidence of coding protein), while the other 47% have been designated either exonic (overlap mRNAs of known genes), or possible exonic. *Aims*. Since hematopoiesis is regulated by signaling pathways and transcription factors that are highly conserved throughout phylogeny, we hypothesize that UCRs may be differentially expressed in hematopoietic tissues and may play a role in the regulation of this process. *Methods*. To explore the UCRs expression in normal hematopoiesis we performed microarray analysis of hematopoietic precursors (HPCs) obtained by culturing human bone marrow (non-mobilized) CD34+ selected cells with different cytokine combinations for 2-3 weeks to stimulate differentiation to the erythrocyte (E), megakaryocyte (MK), monocyte (M) and granulocyte lineages (G). The following cytokine combinations were used: E (TPO/SCF/IL-3); G (G-CSF, GM-CSF, SCF, IL-3, IL-3); MK (TPO, SCF, IL-3) and M (M-CSF, GM-CSF, IL-6, IL-3 and SCF). Differentiation to the selected lineages was monitored every 3 days after 1 week of culture, using morphology, special staining (benzidine) and flow cytometry analysis using appropriate lineage specific antibodies. Human bone marrow CD34+ and peripheral blood CD3+ (pan-T cells) and CD-19+ (B-lymphocytes) selected cells were obtained from 3 different donors. Total RNA was obtained from the E and M cultures at days 7, 9, 11 and 14; G (days 10 and 14) and MK at day 14 and was hybridized in duplicate to the UCRs/miRNA microarray chip. After normalization of the array data with quantiles and exclusion of genes with less than 20 % of expression data with at least a 1.5 -fold change in either direction from gene's median value, we proceeded with further analysis using the BRB tools. *Results.* Unsupervised analysis of the differentiated CD34+ HPCs and peripheral blood lymphocytes revealed that the samples cluster according to the hematopoietic lineage of origin. To detect the minimum number of UCRs that are able to distinguish differentiated HPCs from BM CD34+ cells with high accuracy we performed class prediction analysis. As expected, very few numbers of UCR were able to predict the lineage with no error (mean percentage of correct classification: 100%). These UCRs were overlapping with the ones identified later by using two class analysis where we compared the UCRs expression of CD34+ HPCs Vs. differentiated cells (E, G, MK, MO, T and B-lymphocytes) one at a time (i.e. CD34+ Vs. E; CD34+ Vs. MK) Table 1. We were able to validate the results for uc.283+, uc.285+, uc.73+ in a panel of CD34+ derived hematopoietic precursors using qRT-PCR. *Conclusions*. We characterized the UCRs expression at different stages of hematopoietic differentiation and identified distinctive signatures associated with particular lineages. This research has the potential to identify novel regulators of hematopoiesis and may give insights into basic biology of gene expression and cell fate determination.

Table 1. UCRs differentially expressed between CD34+HPCs and *in vitro* differentiated CD34+ cells/peripheral blood selected lymphocytes.

	Eryth.	Megak.	Monocyte	Granul.	Pan T	CD19+
Up	uc.283+	uc.285+	uc.132-	uc.262-	uc.419-	uc.469-
	uc.285+	uc.10-	uc.33+	uc.161+	uc.145-	uc.132-
	uc.132-	uc.350+	uc.420-	uc.145-	uc.382-	uc.145-
	uc.204+	uc.420+	uc.5+	uc.132-	uc.338+	uc.183+
	uc.477+	uc.95+	uc.262-	uc.43+	uc.160+	uc.420-
Down	uc.188-	uc.356-	uc.269-	uc.170-	uc.170-	uc.170-
	uc.43-	uc.309+	uc.188-	uc.160+	uc.356-	uc.331+
	uc.183-	uc.43-	uc.160+	uc.356-	uc.477+	uc.183-
	uc.8+	uc.298-	uc.170-	uc.110-	uc.233-	uc.134-
	uc.309	uc.182+	uc.356-	uc.248+	uc.298-	uc.281-

0009

THE ROLE OF CYCLIN A1 AND APOPTOSIS IN MURINE HEMATOPOIETIC STEM CELLS

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The cell cycle regulating protein cyclin A1 is found at elevated levels in several malignancies, including acute leukemia. In normal tissues cyclin A1 expression is restricted to meiotic cells, brain and hematopoietic progenitor cells. Several reports have suggested a potential role of cyclin A1 in the regulation of the apoptotic pathways. The studies indicate that cyclin A1 may either protect or promote cells for undergoing apoptosis independently of the origin of the tissue. Given that cyclin A1 is restricted to early hematopoietic cells it was of interest to define the physiological function of cyclin A1 in the regulation of apoptosis in hematopoietic stem (HSC) and progenitor cells. We used mice engineered with knockout alleles on the cyclin A1 gene, CCNA1, and characterized the effect of cyclin A1 deficiency on apoptosis in hematopoietic stem cells (HSC) and progenitor cells. This was determined by staining Lin^- cells with the cell surface markers, Sca-1 and c-kit, combined with the apoptotic markers, AnnexinV/7AAD. There was a significantly higher level of apoptosis in the HSC compartment of cyclin A1 deficient mice compared to wild type mice. However, the Lin^- cells from the different genotypes showed only a minor difference in the rate of apoptosis. These results suggest that the role of cyclin A1 in apoptosis may be more crucial for stem cells than for more developed hematopoietic cells. With these data, we are currently elucidating the pathways by which cyclin A1 is involved in modulating apoptosis in the hematopoietic compartments.

0010

INTEGRIN ALPHA9 CONTRIBUTES TO ADHESION AND DIFFERENTIATION OF HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS IN THE HUMAN BONE MARROW

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Background. Hematopoietic stem cells can interact with their microenvironment via integrins which are adhesion receptors consisting of α and β subunits. Current knowledge suggests that the integrin subunits α4 and α6 expressed on hematopoietic stem and progenitor cells (HSPCs) have distinct roles in retaining stem cells in the bone marrow. *Aim*. The aim of our study was to gain insight into the expression and functions of the integrin subunits α7-α11, which have not been studied so far in the human bone marrow, within the endosteal stem cell niche. *Methods.* Human osteoblasts isolated from trabecular bone and HSPCs purified from umbilical cord blood or bone marrow aspirates were analyzed for the expression of integrin α7-α11 chains by RT-PCR. The involvement of the integrin α9β1 in HSPC cell adhesion, proliferation and differentiation was analyzed in functional assays. *Results*. Transcripts for all investigated integrin chains were found in primary osteoblasts. Highly purified $CD34^+$ HSPCs, however, expressed only transcripts encoding integrin subunits α7 and α9. Flow cytometric analysis verified extracellular expression of the integrin α9β1on HSPCs. Cell-cell adhesion assays with osteoblasts and dye-labeled $CD34^+$ HSPCs in the presence of function-blocking antibodies revealed a role for integrin α9 in HSPC adhesion to osteoblasts. Furthermore, the addition of anti-integrin α9 antibodies significantly inhibited proliferation and *in vitro* differentiation of $CD34^+$ HSPCs. *Summary*. The integrin α9β1 has been identified as a new member of the integrin β1-subfamily expressed on human hematopoietic stem cells. The functional studies strongly suggest that integrin α9β1 contributes to adhesion and differentiation of HSPCs in the endosteal stem cell niche.

0011

DIFFERENTIAL LOCALIZATION OF P-SELECTIN AND VON WILLEBRAND FACTOR DURING MEGAKARYOCYTE MATURATION

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Background. An important step in megakaryocyte maturation is the appropriate assembly of at least two distinct subsets of α-granules, containing either pro- or anti-angiogenic factors. In addition, the α-granules contain hemostatically active factors such as fibrinogen, von Willebrand factor (VWF) and P-selectin. Since P-selectin and VWF mediate different platelet functions during wound healing (VWF binds to exposed collagen and P-selectin mediates neuthrophil attachment), we hypothesized that these two proteins may be routed to different α-granule subsets. In apparent contrast with this hypothesis, the megakaryocytes from Gata1low mice, mice harboring a hypomorphic mutation that decreases Gata1 expression and blocks megakaryocyte maturation, are characterized by reduced expression of VWF and abnormal localization of P-selectin, suggesting that correct P-selectin transport is dependent on VWF and that the two proteins may be routed together to the α-granules. This Gata1low megakaryocyte phenotype is partially rescued by treatment with thrombopoietin. Whether thrombopoietin rescues P-selectin localization to the α-granules is not known. *Aims.* To investigate the localization of P-selectin and VWF during megakaryocyte maturation induced in wild-type and Gata1low mice by treatment with thrombopoietin. *Methods.* Gata1low and wild-type mice were injected with recombinant murine thrombopoietin (100 microgram thrombopoietin/Kg of body weight/daily for 5 days) and sacrificed at day 7 after the first injection. Their spleens were sterilely removed and analyzed by transmission electron microscopy and double immuno-electro microscopy using goat-anti-P-selectin and rabbit-anti-VWF as primary antibodies and either anti-goat or anti-rabbit secondary antibodies coupled with 20 or 15 nm gold-particles, as appropriate. *Results.* The megakaryocytes present in spleen sections from untreated wild-type mice were scarce (0.33 megakaryocytes/field) and mostly mature. By contrast, spleen sections of thrombopoietin-treated wild-type mice were hyper-cellular (6.3 megakaryocytes/field) and contained both immature and mature megakaryocytes. Sections from the spleen of untreated Gata1low mice were also hyper-cellular (5.1 megakaryocytes/field) but did not contain detectable mature megakaryocytes. However, after thrombopoietin-treatment, mature megakaryocytes became detectable in the spleen of Gata1low mice (3.2 mature vs. 1.2 immature megakaryocytes/field, p=0.003). Double gold-immuno-labelling for P-selectin (isolated dots) and VWF (punctuate pattern) in the α-granules (arrow heads) and cytoplasm/DMS (arrows) of megakaryocytes at different stages of maturation is presented in Figure 1. In mature (stage III) megakaryocytes from untreated wild-type mice, P-selectin and VWF gold-particles were not co-localized. By contrast, in thrombopoietin-treated wild-type mice, the two factors were frequently associated in immature (stage I) megakaryocytes, but became localized in separate α-granules in mature cells. Also in immature megakaryocytes from the spleen of untreated Gata1low mice, P-selectin and VWF were found in close proximity, while in mature megakaryocytes from thrombopoietin-treated Gata1low animals they were found in separate cellular compartments. *Summary and conclusions*. This study shows that VWF and P-selectin are associated in the cytoplasm at early stages of megakaryocyte maturation but that their localization diverges to separate α-granules with maturation. The separate localization of the two proteins supports the hypothesis that the two proteins, by binding sequentially to their respective ligand, may time the release of the content of different α-granule subtypes during the process of wound healing.

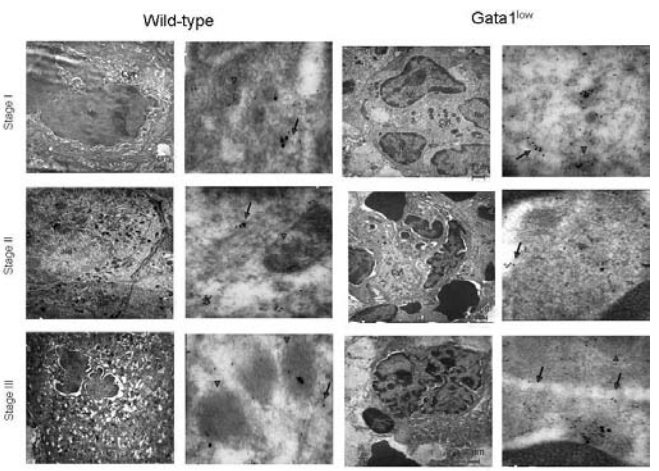

Figure 1.

0012

ASSESSMENT OF INDUCED BONE MARROW SUPPRESSION AND HEMATOPOIETIC GROWTH FACTORS ON HEPATIC REGENERATION AFTER PARTIAL HEPATECTOMY IN ALBINO RATS

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Introduction. The remarkable capacity of hepatic regeneration after partial hepatectomy or hepatic injury had been frequently documented, however; the role of the hematopoietic stem cells and the hematopoietc growth factors is still controversal. Aim of the study: to evaluate the effect of bone marrow suppression & the hematopoietic growth factors (Erythropoietin and Granulocyte colony stimulating factor) on hepatic regeneration after partial hepatectomy in albino rats. Material & methods; 36 adult male albino rats were included & they were divided into six groups each group included 6 rats: the 1st group: the control group, the 2nd group: bone marrow suppression was induced by benzene subcutaneous injection for three weeks, the 3rd group: rats were subjected to 70% surgical partial hepatectomy, the 4th group: rats were subjected to bone marrow suppression by benzene followed by 70% surgical partial hepatectomy, the 5th group: rats were subjected to 70% surgical partial hepatectomy and injection of (Erythropoietin and Granulocyte colony stimulating factor) for five days starting after partial hepatectomy, The 6th group; rats were subjected to bone marrow suppression by benzene followed by 70% surgical partial hepatectomy with injection of (Erythropoietin and Granulocyte colony stimulating factor) daily for five days post hepatectomy. Cytological changes during regeneration were assessed in all groups by quantitative assessment of binucleated cells, the restored number of hepatocytes and mitotic index. Histological evaluation & immunohistochemical study for $CD34^+$ cells in the hepatic tissues were assessed. *Results.* There were no regenerative changes in both control & benzene treated groups, a statistically significant changes was detected regarding the regenerative changes in the partial hepatectomy group compared to bone marrow suppressed group, in the same time a statistically significant changes was detected regarding the regenerative changes in the partial hepatectomy group compared to bone marrow suppressed both groups were treated simalteously with hematopoietic growth factors, Immunostaining of CD34 expression as marker of HSCs in liver sections showed the following; the normal group, benzene treated group and partially hepatectomized group after benzene treatment showed no expression, but partially hepatectomized group show focally positive expression. Both groups treated with hematopoietic growth factors either after partial hepatectomy or after partial hepatectomy and bone marrow suppression by benzene show diffusely positive expression. *Conclusion.* the present study had shown that the hematopoietic growth facors (Erythropoietin and Granulocyte colony stimulating factors) stimulated the hepatic regenerative process after partial hepatectomy only, while a reduced effect was detected after partial hepatectomy with bone marrow suppression.

0013

ISOLATION, DIFFERENTIATION AND NEUROPROTECTIVE PROPERTIES OF NEURONAL PROGENITORS FROM HUMAN UMBILICAL CORD BLOOD

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Since the availability of human neuronal stem cells derived from early embryos is extremely limited, progenitors of other origins, such as human umbilical cord blood (HUCB), a novel source of hematopoietic stem cells for allogeneic transplantation in hematological disorders, are being considered for cell therapy of neurodegenerative disorders. For this purpose, populations of collagen-adherent, nestin-positive (94.8±2.9%) progenitors expressing $\alpha 1/2$ integrin receptors HUCB-derived neuronal progenitors (HUCBNP), were isolated from the mononuclear fraction. *In vitro* differentiation of the HUCBNP was achieved by treatment with 10% human SH-SY5Y neuroblastoma cell-conditioning media (CM) supplemented with 10 ng/mL nerve growth factor (NGF) and characterized by analysis of neurite outgrowths (83±8.2%), MAPK kinases activation (ERK2-36-fold vs control; p38α-nine-fold vs control; p38β-23-fold vs. control) and neuronal markers expression such as microtubule-associated protein 2 (MAP-2; 98.5±2%), neurotrophin receptor (TrkA; 98.5±0.06%), neurofillament-160 (NF-160; 94.2±1%), β-tubulin III (89.8±4.2%) and neuron specific enolase (NSE). Since the growth factors requirement for neuronal differentiation *in vitro* is poorly understood, we explored interferon-γ (IFN-γ) effects on HUCBNP and demonstrated IFN-γ-induced neuronal differentiation alone and in a cooperative manner with NGF. IFN-γ was detected in the CM and transcriptome analysis of CM-differentiated HUCBNP, identified 79 genes highly up-regulated, among them 26 genes interferon-induced. These findings propose IFN-γ and NGF use in future protocols of neuronal differentiation of HUCB-derived progenitors towards cell therapy. The neuroprotective potential of HUCBNP was evaluated using a neuronal ischemic *in vitro* model. In this model, HUCBNP conferred ~30% neuroprotection towards apoptotic and necrotic neuronal cell death. HUCBNP decreased by 95% the level of free radicals in the insulted-neuron, in correlation with the appearance of antioxidants in the medium. An increased level of NGF, VEGF and FGF-2 protein and mRNA modulation were temporally correlated with the neuroprotection effect. These findings indicate a "bystander" effect of HUCBNP-induced neuroprotection involving antioxidant(s) and neurotrophic factors, which, by paracrine and/or autocrine interactions between the insulted-neuron and the HUCBNP, conferred neuroprotection. Altogether, these studies contribute a novel understanding on human umbilical cord blood-derived progenitors for future cell therapy approaches.

Transcriptional control, epigenetics, cell cycle regulation and apoptosis

0014

MAINTENANCE OF NORMAL HEMATOPOIETIC PROGENITOR CELLS BY THE DEACETYLASE INHIBITOR LAQ824 IS ASSOCIATED WITH INDUCTION OF NOTCH TARGET GENES

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Background. Deacetylase inhibitors (DACi) are known to induce cell cycle arrest and apoptosis in AML blasts. However, we have recently demonstrated stimulation of leukemic hematopoietic progenitor cells (HPC) by the selective class I DACi valproic acid (VPA), which may contribute to its rapid clinical failure in spite of promising antileukemic activity in the bulk of AML blasts. Here, we describe the effect of the novel and more potent hydroxamic acid LAQ824 on phenotype and function of leukemic $CD34^+$ HPC in comparison to VPA. In addition, we show that LAQ824 enhances maintenance of normal HPC and evaluate the mechanisms involved by analysing LAQ824-induced gene expression in the most primitive $CD34^+CD38^-$ normal HPC population. *Methods*. Differentiation and proliferation of $CD34^+$ cells of bone marrow of healthy donors and peripheral blood samples of newly diagnosed AML patients were evaluated after one week of culture in presence of SCF, FLT3 ligand, TPO, IL-3 ± LAQ824. The effect of LAQ824 on gene expression profiles in normal $CD34^+CD38^-$ cells was assessed in three independent cell samples following incubation with cytokines ± LAQ824 for 48 hours using Affymetrix GeneChip Human Genome U133 Plus 2.0 and Gene Spring Software. Serial replating of murine Sca1+Lin- HPC was performed in the presence of SCF, G-CSF, GM-CSF, IL-3, IL-6 ± LAQ824. *Results*. Treatment of murine Sca1+Lin- HPC with LAQ824 (10 nM) significantly augmented colony numbers ($p<0.01$; n=3), and supported colony growth after four cycles of replating whereas no colonies developed in its absence beyond the second plating indicating preservation of functionally active multipotent progenitor cells. LAQ824 (10-20 nM) mediated acetylation of histone H3 in human normal and leukemic HPC. In normal HPC, LAQ824 (0-20 nM) lead to a dose-dependent increase in the proportion of $CD34^+$ cells (20% w/o LAQ824 vs. 36% with LAQ824 20nM, $p=0.07$) and a significant reduction of $CD14^+$ monocytes (18% vs. 3%, $p=0.02$; n=3). The total number of $CD34^+$ cells remained stable up to 10 nM and decreased at 20 nM. Gene expression analysis showed, that LAQ824 (20 nM) lead to an at least 3-fold up-regulation of 221 genes in all three HPC samples tested including HDAC11 and the cell cycle inhibitor p21waf1/cip1 known to be induced by most DACi in HPC. We identified several members of the notch pathway such as mastermind-like protein 2 (MAML2, a component of the active notch transcriptional complex) and notch target genes including the transcription factors HES1, HEY1 and HOXA10 and confirmed increase of protein levels by Western blotting. Reduced gene expression of mini-chromosome-maintenance (MCM) protein family members was observed which - in addition to up-regulation of p21 - has previously been associated with notch-mediated cell cycle arrest. To compare the effect of LAQ824 (20 nM) with VPA (150 ng/mL) on leukemic HPC, cells were cultured for one week with or w/o DACi. Of note, LAQ824 resulted in a 20% reduction of $CD34^+$ leukemic HPC, while VPA expanded this population to 220% compared with cytokine-treated controls ($p=0.03$; n=12). CFU numbers growing from $CD34^+$ leukemic HPC in presence of LAQ824 did not differ significantly from controls (n=9). *Conclusion*. LAQ824 seems to diminish, but not eliminate normal as well as leukemic HPC as determined by phenotypic and functional *in vitro* analyses. Our gene expression analysis suggested an association with coactivator and target genes of the notch pathway and cell cycle arrest-inducing genes. In contrast to VPA, LAQ824 does not seem to support growth of leukemic HPC which may contribute to its more potent antileukemic effect.

0015

THE TUMOR SUPPRESSOR GENE DISABLED-2 IS EPIGENETICALLY DOWNREGULATED IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a B-cell neoplasm characterized by the accumulation of malignant plasma cells in the bone marrow. Aberrant DNA methylation of CpG islands in the promoter region is the best studied epigenetic mechanism and is associated with dysregulation of cancer-related genes. Wnt signalling is a key factor in regulation of cell proliferation, hematopoietic stem cell maintenance and B-cell proliferation. Disabled-2 (DAB2) is a tumor suppressor gene and acts a negative regulator of Wnt signalling. *Aims.* In this study, we have analyzed the occurrence and the possible impact of epigenetic dysregulation of DAB2 in MM cell lines and samples from patients with malignant plasma cell disorders. Furthermore, correlations between epigenetic dysregulation of DAB2 and clinical parameters were investigated. Methods: The methylation status of the DAB2 promoter region was analyzed by methylation-specific PCR (MSP) and pyrosequencing. Furthermore, the expression of DAB2 in cell lines was examined by real-time RT-PCR before and after treatment with the demethylating agent 5-aza-2'-deoxycytidine (DAC) for 96 hours at a 1.0 µmol/L concentration. Additionally, correlations between the methylation status of the DAB2 promoter and clinical parameters were investigated. *Results.* By MSP, DAB2 promoter hypermethylation was observed in the MM cell line OPM-2, in the human Burkitt lymphoma cell line Raji, as well as in the lymphoma cell line L-540. Aberrant methylation of DAB2 was associated with low expression in these hematopoietic cell lines. Treatment of the cell lines with DAC induced higher DAB2 expression and partial promoter demethylation. By pyrosequencing, we confirmed the high methylation density in the promoter region of DAB2 in the hematopoietic cell lines OPM-2, Raji and L540, and we observed partial demethylation after treatment with DAC. We then analyzed the DAB2 methylation status in 95 samples from patients with plasma cell disorders by MSP. The frequency of hypermethylation among the patient cohort was 7.4 % (7/95). Patients with DAB2 hypermethylation showed significant shorter overall survival ($p=0.0045$). Median survival time of the seven patients with methylated DAB2 promoter was 18 months, whereas patients without methylation had an overall survival time of 57 months. In patient samples, we confirmed by pyrosequencing the hypermethylation of the DAB2 promoter region observed by MSP. *Summary.* We conclude that DAB2 downregulation is a novel epigenetic event in MM and may contribute to dysregulation of the Wnt pathway. The functional consequences of aberrant Wnt signalling in the pathogenesis of MM have to be elucidated. The prognostic impact of DAB2 hypermethylation needs to be confirmed in prospective trials. The increasing evidence for the important role of DNA methylation changes in MM may serve as a basis for epigenetically targeted approaches in MM.

0016

A DNASE I HYPERSENSITIVE SITE IN THE DISTAL PROMOTER REGION OF THE BCL-6 LOCUS BINDS ZEB1 AND IS RESPONSIBLE FOR TRANSCRIPTIONAL REPRESSION

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Background. The B-cell transcription factor, BCL-6, is expressed at a high level in germinal centre B-cells and prevents terminal differentiation to plasma cells whilst allowing proliferation. BCL-6 is essential for high affinity antibody production. Translocation of the BCL-6 locus (especially with the IgH locus) is associated with diffuse large B-cell lymphoma, and expression from an undisrupted locus occurs in Burkitt lymphoma, follicular lymphoma and lymphocyte predominant Hodgkin's lymphoma. Understanding the regulation of BCL-6 transcription is, therefore, important for normal immunity and also some non-Hodgkin's and Hodgkin's lymphomas. *Aims.*We have taken a systematic approach to mapping possible cis-acting transcriptional control regions at the BCL-6 locus. *Methods.* We employed DNase I hypersensitive site mapping across 50kb of the locus. Transcription factor binding to the strongest sites were identified and further characterized by gel shift assays and reporter assays. *Results.* We identified strong DNase I hypersensitive sites at -4.4kb, +0.3kb, +0.5kb and +3.8kb (relative to the tran-

scription start site) at the BCL-6 locus in human tonsillar B cells and EBV(-) Burkitt cell lines. We noted that there was a site of human/mouse DNA sequence homology at HSS-4.4 and focused subsequent work on this site. Gel shift assays showed a strong shift with a probe containing an E-box sequence (CAGGTG). Anti-E2A and anti-ZEB1 antibodies both produced super-shifts suggesting specific binding of these proteins, which are known to compete for binding to the same sequence. We confirmed specific E2A and ZEB1 binding by chromatin immunoprecipitation, and also that of CtBP, a known ZEB1 co-repressor. Transient transfection of ZEB-1 cDNA downregulated levels of the BCL-6 mRNA and protein in two EBV(-) Burkitt lymphoma cell lines and reduced luciferase activity of a BCL-6 promoter construct when transfected into 293T cells. *Conclusions.* Our study revealed a novel DNase I hypersensitive site (HSS-4.4) containing binding elements for E2A and ZEB1. We implicate ZEB1 as a transcriptional repressor of BCL-6 acting in concert with CtBP, and competing with E2A. Future work will characterize the function of ZEB1 in normal immunity and lymphomagenesis.

0017

GENOME-WIDE IDENTIFICATION OF BINDING SITES OF EVI1 IN HUMAN MYELOID CELL LINES

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The ecotropic viral integration site-1 (EVI1) is an oncogenic transcription factor in murine and human acute myeloid leukemia (AML). Overexpression of EVI1 in AML is one of the worst prognostic factors in patients with or without 3q26 rearrangements. The effect of EVI1 involves several pathways through the interaction with proteins with important functions in transcriptional control; however, the role of EVI1 as a transcription factor is not well known. In mice, several EVI1 target genes have been identified, including Gadd45g, Gata2, Fog2, Skil, Klf5, and Map3k14; nevertheless, no EVI1 target genes have been described in human models. Our aim was to investigate the pathways and direct target genes of EVI1 by studying the differential expression profile of cell lines before and after the gene knockdown, by a bioinformatics approach to identify hypothetical EVI1 binding sites, and by chromatin immunoprecipitation (ChIP). Differential expression profiles after EVI1 knockdown allowed us to identify 125 genes involved in cell growth, differentiation and signal transduction that could be related to EVI1. Moreover, we looked for potential EVI1 binding sites within the region 1000pb upstream of the transcription start sites of all human genes, and selected the genes found in both the Transfac and Jaspar databases. We selected a total of 69 genes from the bioinformatics search, genes related to EVI1 by literature, and genes differentially expressed in the TF1 cell line expression array. We performed ChIP in the TF1 and HEL cell lines with two EVI1 antibodies, and demonstrate that EVI1 binds to the proximal promoter regions of 12 of these genes, confirming the important role of EVI1 in GATA2, and SKIL transcription. Interestingly, EVI1 seems to regulate its own transcription. To extend this study, we analyzed 3000pb upstream of the transcription start site of the reference form of EVI1, and we found 3 more EVI1 binding sites. Further functional studies are in progress. In conclusion, we have identified 12 EVI1 target genes, most of them involved in important differentiation and proliferation pathways. Interestingly, EVI1seems to have binding sites on its promoter, suggesting that it could be regulating its own transcription. These data provide a starting point for further studies aimed at uncovering the mechanism for EVI1-induced transformation.

0018

MICRORNA EXPRESSION PROFILES IN UMBILICAL CORD BLOOD CELL LINEAGES

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Background. MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression either by inhibiting protein translation or by cleavage of mRNA targets. miRNAs are key regulators of many cellular processes such as differentiation, proliferation and apoptosis. It has been demonstrated that miRNAs show characteristic expression signatures in different cell lineages and stages of hematopoietic stem cell (HSC) differentiation, indicating their importance in hematopoiesis. Because neonatal blood displays various features of cellular immaturity compare to adult blood, we could expect differential miRNA expression in neonatal blood cells. *Aims.* To understand molecular basis of phenotypic differences between neonatal and adult blood, we studied variations in miRNA expression between umbilical cord blood (UCB), peripheral blood (PB) and bone marrow (BM) cells with emphasis on HSC. METHODS: Samples of UCB, PB and BM were collected from healthy adult donors (10 samples of PB and 3 samples of BM) or Cord Blood Bank of the Czech Republic (10 samples of UCB). All samples were obtained with donor's written informed consent. Using qRT-PCR low-density arrays, we determined miRNA expression profiles of UCB cell lineages (CD34+ progenitor/stem cells, $CD3^+$ T-lymphocytes, $CD14^+$ monocytes, and granulocytes) and compared them to those of BM and PB cell counterparts. Further, in the same samples we determined mRNA expression profiles using whole-genome microarrays. An approach combining bioinformatic prediction of miRNA targets with mRNA expression profiling was used to search for putative targets of miRNAs with potential functions in UCB. *Results.* We pointed out several differentially expressed miRNAs in particular cell lineages and associated their expression with their putative or previously verified target gene levels. miR-148a expression was suppressed in HSCs and its level inversely correlated with its previously verified target DNA methyltransferase 3B (DNMT3B), suggesting dependence of *de novo* DNA methylation in HSCs on miR-148a. Prolonged cell survival of HSCs may be associated with low expression of miR-143 and miR-145 and up-regulation of their down-stream targets (high expression of c-MYC and miR-17-92 oncogenes and following repression of tumour suppressor TGFBR2). In HSCs, we monitored significant up-regulation of 8 miRNAs (miR-10a miR-99 miR-126, miR-130a, miR-181d, miR-196b), which were previously verified as regulators of HOX genes. Further, miR-146b may be associated with immaturity of neonatal immune system because it is strongly up-regulated in UCB granulocytes and T-lymphocytes compared to PB cells. Comparative analysis revealed 13 miRNAs significantly altered between UCB and BM $CD34^+$ cells (up-regulation of miR-517c, miR-518a, miR-519d, and miR-520h and down-regulation of let-7b, miR-1, miR-34a, miR-195, miR-203, miR-214, miR-545, and miR-548d in UCB $CD34^+$ cells). As has been previously suggested, miR-520h might promote differentiation of HSCs into progenitor cells (possibly due to ID1 and ID3 suppression) and miR-214 expression might support survival of BM HSCs. *Conclusions.* UCB shows specific miRNA expression pattern, demonstrating different regulation in these cells compared to adult PB and BM.

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0019

RESTORATION OF RETINOIC ACID-TARGET GENE TRANSCRIPTION BY EPIGENETIC THERAPY IN ACUTE MYELOID LEUKEMIA CELL LINES

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Background. Retinoic acid (RA) plays a major role in control of cell growth, differentiation, and apoptosis via RA receptors (RARs), which act as transcription factors to regulate the expression of RA-target genes. All-trans RA (ATRA) is the first example of targeted therapy in PML-RAR+ acute myeloid leukemia (AML). Repression of RA signalling pathways through aberrant epigenetic mechanisms may explain ATRA resistance in others AMLs. Thus, combination of a DNA hypomethylating agent (5-azacytidine, AZA) with a histone deacetylase inhibitor (valproic acid, VPA) might sensitize RA-resistant AMLs to ATRA therapy. Promising results of clinical trial with the combination of these drugs confirm the rational of epigenetic therapy with ATRA in AML patients. AIM: The objective of this *in vitro* study was to define molecular and cellular parameters of epigenetic drug response in AMLs through a candidate gene approach on RA-target genes and a wider global methylome analysis. METHODS: All cellular analyses were performed in five AML cell lines (NB4, UF1, U937, KG1, Kasumi1). Epigenetic status of RARs promoters, as representative of RA-target genes, was studied by quantitative methylation specific PCR and chromatin immunoprecipitation experiments. Quantitative DNA methylation of 808 genes (1536 CpG sites) involved in cancer was realized using the GoldenGate Assay for Methylation with BeadArray® from Illumina. Gene expression was measured by quantitative PCR. *Results.* Unlike ATRA alone, AZA/VPA/ATRA induced apoptosis and inhibition of cell growth in all cell lines except KG1 ($p<0.05$). Differentiation was achieved only in ATRA-sensitive cell lines (NB4 and U937), with no significant improvement with AZA/VPA/ATRA compared to ATRA alone. On the other hand, increase of RARα, β and γ gene expression was more potent with AZA/VPA/ATRA than with ATRA alone in ATRA-sensitive cell lines

(p<0.05), as well as in ATRA-resistant cell lines (UF1, Kasumi1) except KG1. Expression was associated with a prolonged and stable DNA demethylation of RARs promoters while increased levels of acetylated H3 histones followed a more time-dependant pattern. Interestingly, increased levels of acetylation induced by AZA/VPA/ATRA occurred in the same time interval as with ATRA alone, suggesting a synergistic effect on ATRA-induced chromatin modification. Methylome analysis showed that median methylation level before treatment across all sites was different according to each cell line (UF1 76.6%, NB4 71.8%, U937 65.9%, KG1 53.2%; p<0.0001). In sites with methylation level >50% before treatment (n=806), AZA/VPA/ATRA led to significant decreases of methylation values of CpG sites in 542 sites (p<0.05), while no significant difference was found with ATRA alone. Overall, the mean decrease in methylation level with AZA/VPA/ATRA ranged between 3 and 33% (median 10%). The number of sites showing more than 20% reduction was different in each cell line (UF1: 133 sites; NB4: 48 sites; U937: 68 sites; KG1: 4 sites). *Conclusions.* Epigenetic modifications induced by AZA/VPA/ATRA combination, involve some RA-target gene promoters. These modifications might thus participate in restoring ATRA sensitivity in some ATRA-resistant AML cells.

0020

SMALL MOLECULE XIAP INHIBITORS SENSITIZE CHILDHOOD ACUTE LEUKEMIA CELLS FOR DEATH RECEPTOR- OR CHEMOTHERAPY-INDUCED APOPTOSIS IN VITRO AND EXERT ANTI-LEUKEMIC ACTIVITY IN A NOD/SCID MOUSE MODEL *IN VIVO*

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Children with high risk acute lymphoblastic leukemia (ALL) do not respond well to current treatments. This failure is, at least in part, due to defects in apoptosis programs. Therefore, new strategies are required that counter apoptosis resistance in order to improve the poor prognosis of high risk pediatric acute leukemia. Since XIAP, a member of *Inhibitor of Apoptosis Proteins* (IAPs), is expressed at high levels in acute leukemia and blocks apoptosis at a central point of the apoptotic machinery, XIAP may present a suitable molecular target for therapeutic intervention. Here, we report that neutralizing XIAP by small molecule inhibitors is a novel and effective approach to sensitize childhood acute leukemia cells for death receptor- or chemotherapy-induced apoptosis. XIAP inhibitors at subtoxic concentrations, but not a structurally related control compound, synergize with TRAIL, agonistic anti-CD95 antibodies or various anti-leukemic drugs, e.g. cytarabine, doxorubicin, etoposide and 6-mercaptopurine, to induce apoptosis in acute lymphoblastic leukemia cells. Further, XIAP 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 XIAP inhibitors enhance death receptor- or chemotherapy-induced activation of caspases, loss of mitochondrial membrane potential and cytochrome c release in a caspase-dependent manner, indicating that they promote a caspase-dependent feedback mitochondrial amplification loop. Intriguingly, XIAP 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, XIAP 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, XIAP 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, XIAP 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, XIAP inhibitors significantly reduce leukemic burden *in vivo* in a mouse model of pediatric ALL engrafted in NOD/SCID mice. Thus, small molecule XIAP inhibitors present a promising novel approach for apoptosis-based therapy of childhood acute leukemia, which warrants further exploitation.

0021

CASPASE INHIBITION BLOCKS APOPTOSIS CAUSED BY MLL-AF4 DEPLETION IN T(4;11)-POSITIVE ALL CELL LINES, BUT CANNOT ABROGATE SUBSEQUENT NECROPTOSIS-LIKE CELL DEATH

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Chromosomal rearrangements involving the 11q23 locus are recurring cytogenetic aberrations in acute leukaemias. One of the common chromosomal abnormalities in acute lymphoblastic leukaemia (ALL) is the reciprocal translocation t(4;11)(q21;q23). This translocation generates the fusion oncogene MLL-AF4, and cell lines carrying this translocation show a remarkable resistance to chemotherapy-related stress and cell death. Accordingly, MLL-AF4-positive paediatric ALL is associated with poor prognosis. In order to further elucidate the role of MLL-AF4 in leukaemogenesis, we employ RNA interference (RNAi) to functionally analyse MLL-AF4. We have shown that RNAi-mediated depletion of the MLL-AF4 oncogene induces apoptosis in cell lines in a caspase-dependent manner. In this work we investigated whether apoptosis in response to MLL-AF4 down-regulation could be prevented by inhibiting caspase activity. The t(4;11)-positive pro-B ALL cell line SEM was transfected with siMLL-AF4, a siRNA specifically directed against the fusion breakpoint of the endogenous MLL-AF4 transcript and which has no off-target activity in t(4;11)-negative leukaemic cell lines. As control, SEM cells were transfected with an active siRNA targeting a fusion transcript absent in our cell line. In an attempt to block apoptosis, we cultured siRNA transfected cells simultaneously with the pancaspase inhibitor zVAD-FMK or with its solvent DMSO. Immunoblotting and a luciferase-based caspase-3 and caspase-7 activity assay demonstrate that, in contrast to the corresponding siMLL-AF4/DMSO control, the addition of zVAD to siMLL-AF4 transfected SEM cells blocks effector caspase activation and functional activity. Concomitantly, other apoptotic markers such as PARP1 cleavage, ANNEXIN V-single positive staining and DNA fragmentation are absent in siMLL-AF4/zVAD treated cells, and induced in siMLL-AF4/DMSO cells. The siRNA controls show no apoptotic activation and a viable, non-necrotic phenotype when cocultured with or without zVAD. However, zVAD-FMK does not abrogate siMLL-AF4-mediated cell death, as SEM cells transfected with siMLL-AF4 and zVAD-FMK show an increase in ANNEXIN V/PI double-positive population. This finding implies that caspase-inhibition directs SEM cells to an alternative non-canonical death pathway. Gene expression profiling revealed induction of RIPK1, PARP2, CYLD, JUN, TNF, LC3B in cells transfected with siMLL-AF4 and treated with zVAD-FMK, but not in siMLL-AF4/DMSO cells or appropriate controls. These genes have been recently described as key players in a novel programmed cell death pathway termed necroptosis. Necroptosis is phenotypically matched to necrosis, but opposed to the conventional view of the necrosis pathway, underlies tight regulation, sharing key modulators of the TNFR (tumour necrosis factor receptor) and TLR (Toll-like receptor) signalling pathways and involving autophagy. Our data suggests that in combination of caspase inhibition, MLL-AF4 knock-down results in activation of the necroptosis pathway. These findings illustrate the oncogene addiction of t(4;11)-positive cells to MLL-AF4, as RNAi-mediated oncogene ablation has the inevitable consequence of cell death.

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0022

INDUCTION OF APOPTOSIS BY SHORT-TERM, HIGH-DOSE KINASE-INHIBITOR EXPOSURE IS A COMMON MECHANISM IN CELLS TRANSFORMED BY ONCOGENIC KINASES

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Small-molecule tyrosine kinase inhibtors (TKI) have become a valuable tool in the treatment of a number of malignant diseases. Chronic myeloid leukemia (CML) has pioneered the way for targeted therapy with TKIs. It is widely accepted that continuous target inhibition of oncogenic kinases *in vivo* is a prerequisite for successful TKI treatment. The clinical experience with Dasatinib, a TKI approved for the treatment of BCR-ABL positive CML questions this paradigm: Dasatinib has a serum half-life of about 4h. Thus, administration of Dasatinib once daily results in transient target inhibition only. Nevertheless, excellent clin-

ical responses are observed when using this schedule. Recently, published data suggested, that this results from a more pronounced target inhibition upon a single dose of Dasatinib (100 mg) as compared to a single dose of Imatinib (400 mg). However, if equipotent doses of Dasatinib and Imatinib are applied to BCR-ABL positive cells *in vitro*, one can observe that Imatinib also induces apoptosis upon short-term exposure. Our current study addresses the question whether induction of apoptosis by short-term, high-dose kinase-inhibitor exposure is a common mechanism in cells transformed by oncogenic kinases. BCR-ABL, JAK2V617F, and FLT3-ITD transformed Ba/F3-cells were treated with different doses of specific TKIs (IC_{50}, IC80, 10xIC_{50}) for various periods of time and the percentage of cells in sub-G1-phase was measured at 24, 48, and 72h. To determine the relation between inhibition of specific oncogene-dependent intracellular signaling pathways (pSTAT5, pERK) and induction of apoptosis (cleaved caspase 3), we measured these molecules simultaneously at the single-cell level by flow-cytometry. To confirm flow-cytometry results, Western blot experiments were performed. In all three cell-models, significant induction of apoptosis after 24, 48, and 72h with all tested dose levels was observed when cells were treated with the respective inhibitor continuously. When the inhibitor was washed out upon a short incubation time, no significant induction of apoptosis was observed when IC_{50} and IC80 were used. However, interestingly, the 10xIC_{50} dose-level nevertheless lead to significant induction of apoptosis in all three cell-models. Flow-cytometry analysis revealed a rapid decrease of pERK and pSTAT5 after 2h, followed by a pronounced increase of cleaved caspase 3 after 8-10h. Western Blot experiments revealed early caspase 3 cleavage even with standard dose inhibitor treatment: As soon as 4h after initiation of inhibitor treatment cleaved caspase 3 was detectable, reaching a maximum level after 10h of treatment. When looking at total caspase 3, the earliest time point one could recognize a decrease in total caspase 3 was after 10h, with almost no detectable total caspase 3 after 16h. The aim of our study was to provide data for the hypothesis, that induction of apoptosis by short-term, high-dose kinase-inhibitor exposure is a common mechanism in cells transformed by oncogenic kinases. Our current derived data from three clinically relevant tyrosine-kinase-mutant driven cell-models, strongly confirm this hypothesis. The exact mechanism is currently investigated in our laboratory but unknown at this point of time. However, the data presented provide a strong rationale to test modifications in dose and schedule of currently used TKIs in clinical trials.

0023

CHANGES OF NUCLEOLIN EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Nucleolin (NL) is a nucleolar phosphoprotein involved in many essential biological processes which are often impaired in neoplastic cells. NL is associated with cell proliferation, cell cycle control, apoptosis, and it also regulates gene expression. Experimental data suggest that during apoptosis NL is involved in nucleolar-cytoplasmatic distribution of cleaved p89 fragment of poly(ADP-ribose) polymerase-1 (p89 PARP-1), this leads to NL accumulation in apoptotic bodies and its removal. The cytoplasmatic occurrence of p89 PARP is early and sure molecular evidence of apoptosis. According to literature data NL interacts with glucocorticoid receptor, is overexpressed in cancer cells and correlates with their proliferation activity. Cellular NL overexpression is associated with chemotherapy resistance. Steroids response is the one of the most significant prognostic factor in children with acute lymphoblastic leukemia (ALL). This study aims to evaluate changes of NL expression in peripheral blood mononuclear cells collected prior to and after 12 hours of prednisone administration, and their correlation with early treatment response in children with newly diagnosed ALL. *Patients.* This study comprised of 38 children (aged 15-206 months, median 51months) with ALL. All children were treated with prednisone in a dose 60 mg/m^2/day according to ALL IC 2002 protocol. The follow up time was 7-66 months (median 38 months). *Methods.* Peripheral blood mononuclear cells were collected prior to and after 12 hours of prednisone administration. Cytospin preparations of these cells were stained with Mouse Monoclonal IgG1 anti-Human Nucleolin (Santa Cruz Biotechnology) followed by Allophycocyanin, Crosslinked, Goat anti-Mouse IgG (Molecular Probes). In order to assess the rates of apoptotic cells respective slides were stained with Polyclonal Rabbit anti-PARP p85 fragment (Promega Corporation) followed by FITC conjugated Polyclonal Swine anti-rabbit antibody (DakoCytomation). Nuclear DNA was stained with propidium iodide (PI). Red fluorescence of DNA - bound PI was a contouring parameter. NL-associated long red fluorescence and p85 PARP green fluorescence were measured by laser scanning cytometer (LSC, CompuCyte, USA). Based on early treatment response patients were divided into 2 subgroups. Patients with absolute leukemic cell counts of less than 1000/µL of peripheral blood after 7 days of prednisone treatment and with bone marrow leukemic cells below 5% on day 15 were classified as early good responders (n=26). All remaining patients were classified as early poor responders (n=12). *Results.* After 12 hours from prednisone administration the percentage of apoptotic cells as well as p85 PARP expression were significantly higher in the group of early good responders (p=0.002 and p=0.04).Those alterations were not observed in the remaining group of patients. The mean pretreatment values of NL expression and after 12 hours from prednisone administration were significantly higher in the early poor responders group comparing to the the early good responders (p=0.003 and p=0.04). *Conclusions.* These data seems to indicate that NL overexpression may contribute to poor early treatment response, influencing the outcome for these patients.

0024

THE EFFECT OF P16 ON GLUCOCORTICOID RESPONSES IN A B-CELL LYMPHOBLAST CELL LINE

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Background. Glucocorticoid (GC) has been used as a major anticancer agent for the lymphoid malignancies and their effect appears via glucocorticoid receptor (GR). p16INK4 (p16) is a one of the cyclin-dependent kinase inhibitors acts as a negative cell cycle regulator. It has been suggested that p16 has some roles in GC related apoptosis in lymphoblsts, and the inactivation of p16 in B-cell acute lymphoblastic leukemia (ALL) may induce the lymphoblsts more resistant to GC. *Aims and Methods*. To evaluate the relationship between GC responses and p16 expression, we generated the p16-inactivated cells using a B-cell lymphoblast cell line, NC-37 through p16 siRNA transfection method. We evaluated the GR expression, apoptosis and cell viability between control cells (the p16+ cells) and the p16 siRNA transfected cells (the p16- cells) after a single dose of dexamethasone (DX) treatment. *Results.* There was a significant increase of the cytoplasmic GR expression after 6, 12 and 18 hours with proportional pattern, and followed by a sharp decrease at 24 hour in both cell groups. The GR expressions tended to be higher in the p16+ cells than in the p16- cells through 24 hours, and those at 18 hour was statistically significant(p<0.05). In apoptosis test assessed by flow cytometry using 2 monoclonal antibodies, we could observe a two-phase process of apoptosis in both cell groups; early apoptosis appeared along with GR changes through 24 hours, and late apoptosis appeared higher levels at 18 and 24 hours when the GR was already down-regulated. The latter phenomenon also tended to be more prominent in the p16+ cells than that of the p16- cells, and the result at 18 hour was statistically significant (p<0.05). In cell viability test assessed by Alamar blue staining, viable cells were decreased in both cell groups after DX treatment, but the degree of reduction was more prominent in the p16+ cells after 18 hours (p<0.05). *Conclusions*. These results suggest a relationship between GR expression and cell cycle inhibition, whereby, inactivation of p16 leads to reduced sensitivity to DX in lymphoblst cell line. This observation might have important implications for cancer therapy.

0025

PERIPHERAL BLOOD LYMPHOCYTE SUBSETS, TREG CELLS AND FOXP3 EXPRESSION IN β-THALASSEMIA MAJOR AND β-THALASSEMIA TRAIT PATIENTS

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Background. Infectious complications constitute an important part of the clinical spectrum of β-thalassemia, being associated with significant morbidity and mortality. Other than the high risks of blood-born infections associated with multiple transfusions, coexistent immune abnormalities have also been held responsible for the increased susceptibility of these patients to infectious diseases. A wide spectrum of immune abnormalities including both quantitative and functional defects has been reported by numerous studies relevant to β-thalassemic patients with multiple transfusions. These defects have been attributed both to the β-thalassemia itself and/or the applied therapeutic interventions. Naturally occurring regulatory T cells (Tregs) play a crucial role in the maintenance of immunological self-tolerance and negative control of various immune responses to non-self antigens. Forkhead transcription factor, FoxP3, is specifically expressed on Tregs and is a master control gene for the development and function of natural Treg cells. Increased antigenic stimuli due to repeated blood transfusions might change the Tregs ratio and FoxP3 expression on T lymphocytes in β-thalassemia patients. *Methods.* Thirty patients with β-thalassemia major who are regularly transfused, 30 patients with β-thalassemia trait who had never been transfused and 20 healthy age-matched children for control group were included in the study. Total lymphocyte count, total neutrophil count, IgG, IgA, IgM, peripheral blood lymphocyte subsets including naive and memory T cell, Treg cells and FoxP3 expression on Tcells were evaluated in these patients and control group. *Results.* Moderate abnormalities were observed in peripheral blood subsets, whereas a signifiant difference was noted in Treg cells and FoxP3 expression on these T cells in β-thalassemia when compared wih β-Thalassemia trait patients and control group. *Conclusions.* Our findings suggest that ß-thalassemia itself, either major or trait, may directly lead to permanent immune stimulation. In β-thalassemia major, immune system seems to be balanced in spite of immune stimulation as a consequence of increased Tregs ratio whereas in thalassemia trait, immune system seems to be continuously activated, associated with high frequency of infectious antigens alone. This is the first study, which evaluated effect of Tregs in β-thalassemia. Additional studies are required to establish alterations of Tregs and immunosupression more clearly in other transfusion dependent diseases.

Genomics and proteomics & cytogenetics and molecular diagnostics

0026

PERSONALISED MEDICINE - THE USE OF PROTEOMICS BIOMARKERS TO PREDICT RESPONSE TO THALIDOMIDE IN MULTIPLE MYELOMA

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Background. Multiple Myeloma (MM) is a disease characterized by a proliferation of malignant plasma cells. The understandings of haematological malignancies at the protein level are important for the prediction of therapy response. *Proteomics* describes the analysis of the entire proteome of a cell or tissue and incorporates multiple technologies including 2D-DIGE. There are a limited number of studies to date in MM; those already performed highlight the potential impact of these technologies in the discovery of potential novel proteins biomarkers associated with the drug resistance. As such, this approach aims to minimize that one-size-fits-all approach by matching the patient to a specific treatment based on the proteomic characteristics of that person's malignancy. Thalidomide alone or in combination represents an effective treatment strategy for newly diagnosed, relapsed and refractory MM patients. The identification of novel biomarkers could lead to more effective, individualized therapeutic strategies with improved patient outcomes. *Patients, Method and Material.* Serum samples of 36 newly diagnosed and relapse MM patients, who had had initial treatment with thalidomide based regimens were analysed. Based on D100 re-staging, 20 responders and 16 non responders to thalidomide were identified. Samples were analysed using 2D-DIGE, a technique based on pre-electrophoretic labelling of samples with one of three spectrally resolvable Fluorescent CyDyes (Cy2, Cy3, and Cy5) allowing multiplexing of samples into the same gel. Initially serum samples were immunodepleted, this step is designed to specifically remove 14 high- abundant Proteins representing approximately 94% of total protein mass. This allowed for easier analysis of low abundance proteins, which are more likely to be a source of potential biomarkers. All 2D-DIGE images were scanned and collected on a Typhoon Fluorescent Imager. Pooled samples were used as an internal standard to quantify expression changes with statistical significance. Statistical analysis and quantitation of protein expression were carried using DeCyder Extended Data Analysis (EDA) software. This allowed us through multivariate analysis to uncover and cluster the protein expression pattern. (Data derived from 2-D DIGE experiments). *Results.* 12 proteins have been identified to be differentially expressed in non-responders compared to responders: 7 were up-regulated and 5 were down-regulated (t-test ≤0.02). All 12 proteins were >1.25-fold differentially expressed, with changes up to 6.62-fold. For example Figure 1 shows statistical analysis of protein 1 using DeCyder BVA software. These proteins were increased 2.45 fold respectively in the immuno-depleted serum from non-responders compared to responders, (t-test 0.0046).

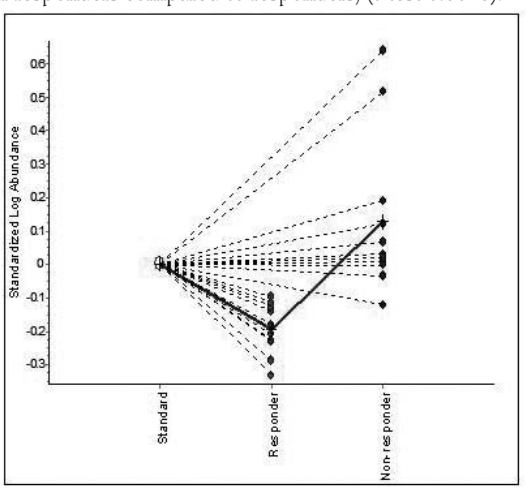

Figure 1.

Once the 12 panel proteins were established, further statistical analysis was performed using DeCyder EDA software. Principal Component Analysis (PCA) was used to separate the responders from the non-responders based on the panel of 12 statistically significant differentially expressed proteins (Figure 2). Each dot on this plot represents a clinical sample; clinical samples from the same experimental groups are located in the same distinct areas, i.e. contained in one half of the plot, confirming consistency of results. *Conclusions.* Accurate prediction of an individual patient's drug response is an important prerequisite of personalized medicine. Using a panel of proteomic biomarkers, we have demonstrated the feasibility of predicting sensitivity and response to thalidomide in previously untreated myeloma. We have so far identified 6 of these proteins and in the process of identification of remaining proteins and plan to confirm their predictive value in a larger group of patients.

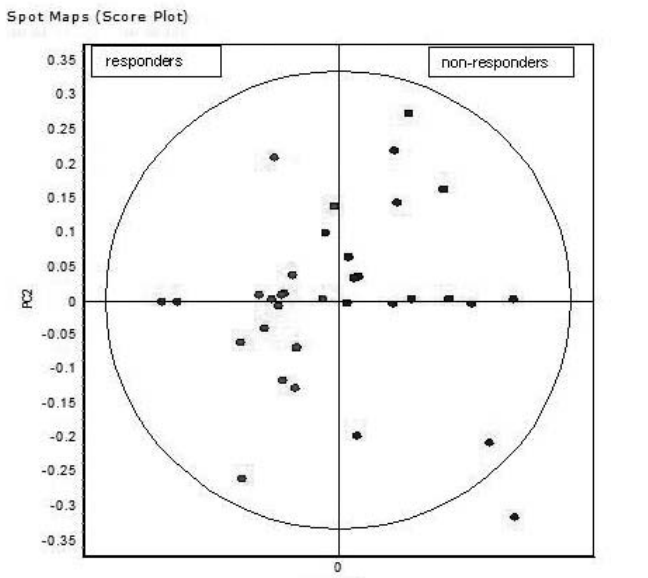

Figure 2.

0027

DIFFERENT RESPONSES TO ADRIAMYCIN AND RADIATION ON RAJI CELLS: A COMPARATIVE MITOCHONDRIAL PROTEOMIC STUDY

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Chemotherapy including adriamycin (ADR) and radiotherapy are both popular used in the treatment of non-Hodgkin's lymphoma (NHL) alone or combined together, but many side effects induced by ADR or radiation (e.g. dose-related cardiotoxicity, bone marrow depression, and *second tumor*) represent most challenging problems in the successful treatment of NHL. Furthermore, chemoresistance and radioresistance are both obstacles to achieve a satisfied therapeutic outcome. Former studies confirmed that the anti-tumor mechanism of both ADR and radiation were thought to contribute to the induction of apoptosis and inefficiency of DNA repair, which were closely regulated by mitochondria. Mitochondrial proteome, the main executor of diverse functions in this important subcellular organelle, has received considerable attention with the development of proteomics. To our most knowledge, there have been no simultaneous, quantitative proteomic studies on comparative mitochondrial proteome of lymphoma and their cell lines. *Aims.* The purpose of this study was to investigate different responses of mitochondrial proteome of Raji cells exposed to ADR and radiation respectively, and also to shed new lights on identify mitochondrial associated biomarkers as promising targets for NHL treatment. *Methods.* Two-dimensional differential in-gel electrophoresis (2D-DIGE) in combination with linear ion trap quadrupole-electrospray ionization tandem mass spectrometry (LTQ-ESI-MS/MS) were conducted as a non-biased approach to assess changes in the mitochondrial proteome of Raji cells following exposure to ADR and radiation respectively. *Results.* Total 1485 protein spots were located, of which 41 were chosen to be analyzed further (differently expressed ≥1.5-fold) and resulted in 55 unique proteins or peptides in three groups of Raji cells (control, ADR-treated, and radiation-treated) as showed in Table 1. Differentially expressed mitochondrial proteins distributed their functions in OXPHOS, redox, protein synthesis and degradation, DNA repair, transporters and channels, etc (Figure 1A). Compared to the control and radiation-treated Raji, ADR-treated group were accompanied by down-regulated proteins involved in OXPHOS, DNA repair, cells cycle, and protein synthesis. Intriguingly, proteins involved in above four kinds of functions in radiation-treated Raji were even higher than the control (Figure 1B). Total 7 proteins were common in three conditions, distributing their functions in OXPHOS, DNA repair, transporters and channels, proteins synthesis and degradation (Figure 1C).

Table 1.

Function Group	*Potein Name (Gene Name)*	*Control/ADR Trend (Folds)*	*Control/ Radiation Trend (Folds)*	*ADR/ Radiation Trend (Folds)*
OXPHOS	ATP SYNTHASE D CHAIN (ATP5H)	±1.93		
	ATP synthase subunit O, mitochondrial precursor (ATP5O)			±3.52
	LOC644189 Similar to Acyl-coenzyme A thioesterase 2 (ACOT2)	±3.52		
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	±3.52	±2.72	±2.57
	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial [Precursor] (HADH)	±3.52		
Energy metabolism	Adipophilin (ADFP)		±2.72	
	Carnitine O-palmitoyltransferase 2, mitochondrial precursor (CPT2)		±1.94	
Cell cycle	Prohibitin (PHB)	±2.53		±1.96
	HRAS-like suppressor 2 (HRASLS2)	±2.26		±2.57
	Kinetochore protein Hec1 (NDC80)	±3.52		
	Isoform Long of Antigen KI-67 (MKI67)		±2.72	
	Isoform 1 of Neuron navigator 3 (NAV3)		±2.72	
	Isoform 1 of Forkhead box protein M1 (FOXM1)		±2.14	±2.69
	ATPase family AAA domain-containing protein 3B (ATAD3B)		±1.94	
Transporters and channel	Heat shock 70kDa protein 9 precursor (HSPA9)	±2.29		
	Isoform 4 of Mitochondrial ATP-binding cassette sub-family B member 6 (ABCB6)	±2.26		±2.57
	Geranylgeranyl transferase type-1 subunit beta (PGGT1B)	±3.05		±2.74
	Isoform 1 of Bifunctional heparan sulfate N-deacetylase/ N-sulfotransferase 2 (NDST2)	±3.05		±2.74
	Hemoglobin subunit epsilon (HBE1)	±3.52	±1.94	±2.74
	HBA1 Hemoglobin subunit alpha (HBA2)	±3.52	±1.94	
DNA repair	DNA repair protein RAD52 homolog (RAD52)	±3.05	±2.14	±2.74
	DNA mismatch repair protein Msh3 (MSH3)	±2.26		±2.57
	Similar to Fanconi anemia group F protein (FANCF)	±2.45		
	ATP-dependent DNA helicase Q4 (RECQL4)		±2.14	±2.69
Protein synthesis	Elongation factor Tu, mitochondrial [Precursor] (TUFM)	±3.05	±2.14	±2.74
	KIAA0415 gene product (KIAA0415)	±3.05	±2.14	±2.74
	Isoform 2 of Uncharacterized protein C19orf60 (C19orf60)	±3.05		±2.74
	Something about silencing protein 10 (UTP3)	±3.05		±2.74
	Isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC)	±3.52		
	LOC344382 similar to Serine-threonine kinase receptor-associated protein?STRAP?	±3.52		
	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3 (CHCHD3)	±4.71		
	Mediator complex subunit 29 (MED29)		±2.14	±2.69
	Elongation factor G 1, mitochondrial (mEF-G 1)		±1.61	
	Isoform 1 of NTPase KAP family P-loop domain-containing protein 1 (NKPD1)		±1.94	
REDOX	Superoxide dismutase [Mn], mitochondrial precursor (SOD2)	±3.12		±3.52
	Peroxiredoxin 3 (PRDX3)			±1.56
Cell structure and motility	Vimentin (VIM)	±3.53		±3.44
	Poliovirus receptor-related protein 1 [Precursor] (PVRL1)	±2.26		±2.57
	Bullous pemphigoid antigen 1, isoforms 6/9/10 (DST)	±3.05		±2.74
	Centrosomal protein of 170 kDa (CEP170)	±3.05		±2.74
	MYH9	±2.67		
	MYH11	±3.52		
	ACTB Actin	±4.44	±2.14	
	ACTA2 Actin	±3.52	±2.72	
Signaling	Probable G-protein coupled receptor 81 (GPR81)	±3.52		
	Receptor expression-enhancing protein 2 (REEP2)		±2.14	±2.69
	CDNA FLJ12595 fis, clone NT2RM4001344 (FLJ12595)			±3.52
Other	Embigin precursor (EMB)	±3.05	±2.14	±2.74
	B2M Beta-2-microglobulin (β2M)	±3.05	±2.14	±2.74
Unknown	Noelin-2 precursor (OLFM2)	±3.28		±3.44
	CDNA FLJ41134 fis, clone BRACE2 (Q6ZWG4)	±3.52		
	CDNA FLJ25713 fis, clone TST05089		±2.14	
	Homo sapiens cDNA clone IMAGE:4663196 5', mRNA sequence		±2.49	
	Eukaryotic translation initiation factor 4E family member 1B (EIF4E1B)			±3.52
	CDNA FLJ25713 fis, clone TST05089			±2.69

Additionally, several proteins which had been reported to be associated with chemo- or radio-resistance such as HSPA9, PHB, ABCB6, and ATAD3B have also showed significant alterations in their abundance. *Conclusions.* This study examined different responses of mitochondrial proteome exposed to ADR and radiation respectively. Raji cells treated by ADR seemed to be undergoing more intensive stress than radiation-treated group, which indicating more effective anti-tumor outcome. Our finding suggested that proteins such as HSPA9, PHB, ABCB6, and ATAD3B should be investigated in more details to reverse the chemo- or radio-resistance as promising biomarkers in NHL treatment.

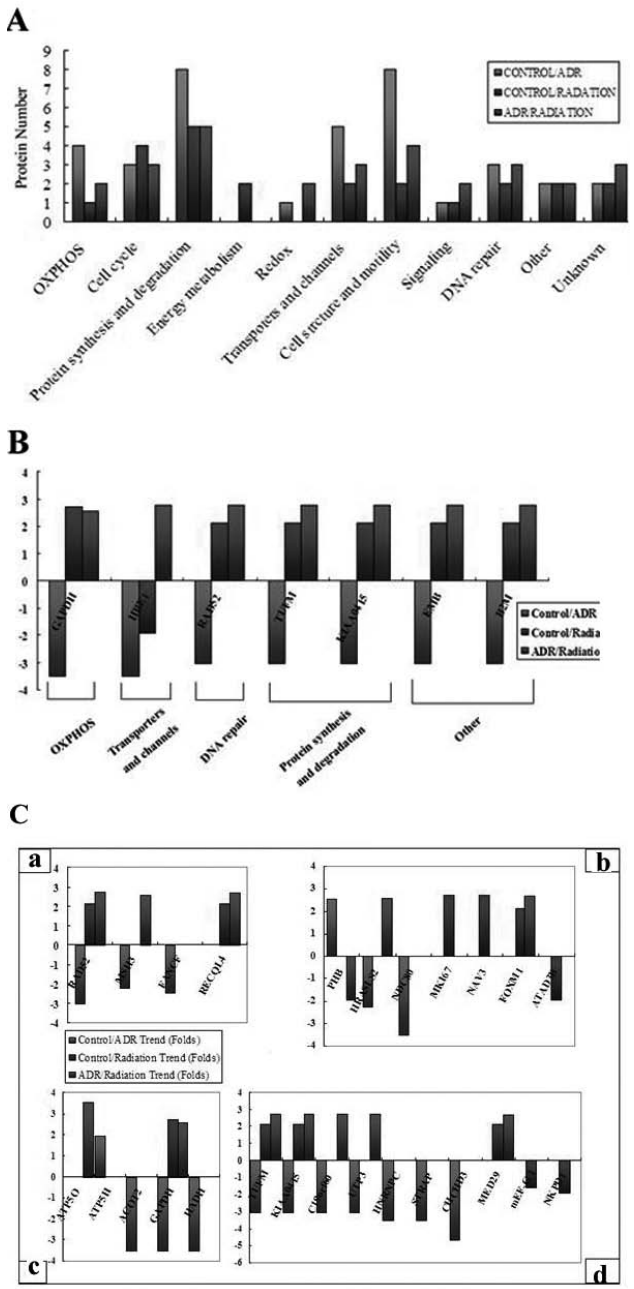

Figure 1. A. Identified proteins from three groups of Raji cells (control, ADR-treated, and radiation-treated) were categorized according to their functions. Differentially expressed mitochondrial proteins distributed their functions in OXPHOS, cell cycle, redox, protein synthesis and degradation, DNA repair, transporters and channels, ect. B. Seven proteins were common in three conditions, distributing their functions in OXPHOS, DNA repair, transporters and channels, proteins synthesis and degradation, etc. C. Mitochondrial proteins identified which involved in functions associated with apoptosis and DNA repair. a: proteins involved in DNA repair; b: proteins involved in cell cycle; c: proteins involved in OXPHOS; d: proteins involved in proteins synthesis and degradation.

0028

COMPARATIVE PROTEOMIC PROFILING OF SUBTYPE M1 AND M2 OF ACUTE MYELOID LEUKEMIA BY TWO-DIMENSIONAL ELECTROPHORESIS AND MASS SPECTROMETRY

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Background. Proteomic analysis of blastic cells of acute myeloid leukemia (AML) may help to distinguish different morphological/cytogenetic/molecular forms of the disease. The results of this analysis may also give a valuable information concerning prognosis and potential therapeutic targets in AML patients. This problem has not been evaluated thoroughly so far. Therefore, in order to identify new biomarkers associated with AML, a method for comparative proteomic analysis of mononuclear cells was elaborated. *Aims*. The aim of the study was to create proteomic maps of bone marrow and peripheral blood mononuclear cells of M1 and M2 subtype of AML. *Methods.* Twelve patients with AML-M1 and eleven with M2 diagnosed according to FAB classification, after obtaining the informed consent, were included into this study. Mononuclear cells were obtained from bone marrow and peripheral blood in three time points: at diagnosis, after induction polychemotherapy (daunorubicin 3, Ara-C 7 days) when the complete remission was achieved or not, and at the time of possible relapse. A traditional proteomic approach based on two-dimensional electrophoresis (2DE) and MALDI-TOF mass spectrometry was used. *Results.* Until now, we have identified 185 proteins among around 653 protein spots on 2DE gels. All the identified proteins have been classified (based on their function or sub-cellular localization) into the following groups: enzymes, structural proteins, gene regulation proteins, signaling proteins, cellular defense and stress related proteins, extracellular proteins, heat-shock proteins and chaperones, protein involved in protein processing, membrane proteins, nuclear proteins and miscellaneous proteins. Among the identified proteins, 12 displayed qualitative or quantitative differences in the accumulation pattern between particular patients with AML. Only protein spots that showed at least 1,5-fold increase or reduction in abundance were selected. We found that the best markers are: annexin III, 6-phosphogluconate dehydrogenase and vesicle amine transport protein 1 (Vat1 protein), which were almost exclusively present in M2 samples. Concentration of these proteins was below 2DE detection level in almost all M1 patients. Annexin III was found in all M2 and in two M1 samples (but in the latter relative concentration of this protein was very low). L-plastin was detected in all M1 and M2 samples, but in seven M2 patients we also detected its second isoform. Peroxiredoxin 6 and catalase were also increased in the M2 in comparison to M1 samples but differences were not so obvious. Several other proteins, e.g. purine nucleoside phosphorylase, peroxiredoxin 2 and vimentin also exhibited various accumulation in particular samples, but the observed changes did not correlate with FAB AML subtypes. Independently form FAB subtype, a visibly higher accumulation of annexin I was noted in remission state in comparison to patients resistant to the treatment applied. Interestingly, a higher accumulation of actin-χ 1 in patients who relapsed was also observed. *Summary and conclusions.* The traditional proteomic approach allowed to find marker proteins which distinguish between AML-M1 and M2 subtype. In our opinion some of them also seem to be useful in prediction of polychemotherapy resistance.

0029

THE ROLE OF TNF MICROSATELLITE POLYMORPHISMS IN SURVIVAL OF PATIENTS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The conditioning regimens performed prior to the hematopoietic stem cell transplantation (HSCT) are responsible for the release of proinflammatory cytokines such as TNFα. A correlation between TNFα serum levels and occurrence of post-transplantation complications such as acute GvHD has been reported. Previous studies have found an association between TNFα production and TNF microsatellite polymorphisms. *Aims.* The purpose of the present study was to investigate a possible influence of TNFα, TNFβ and TNFδ microsatellites on the outcome of HSCT. *Methods.* One hundred patients who underwent HSCT were included in the analysis of TNFα, TNFβ and TNFδ microsatellites. All of them received transplant from a sibling identical for HLA-A, -B, and -DRB1 loci. The microsatellite analysis was performed using PCR amplification with fluorescently labeled primers followed by electrophoresis on a 6% polyacrylamide gel in an automated sequencer (ALFexpress, Amersham Pharmacia, Uppsala, Sweden). *Results.* The distribution of alleles at the tested TNF microsatellites among the patients did not differ from the results obtained for healthy individuals previously tested, with the exception of an increased frequency of TNFα8 and TNFδ3 alleles among patients. This difference was statistically significant only before correction. Patients were then analysed with respect to their survival and presence of an individual allele. The only significant difference in the survival rates was found for TNFα microsatelite alleles. The presence of TNFα8 allele was associated with worse survival (p<0.01) while TNFa10 allele showed a correlation with better prognosis (p=0.03). However, the difference observed for TNFα10 allele was significant only before correction. *Summary*. The results suggest an association of TNFα8 allele with a worse outcome of HSCT. However, since this is an allele which is found rarely both among healthy individuals and patients, the value of this finding should be confirmed with a study including a larger number of subjects.

0030

SET-NUP214 EXPRESSION REQUIRES ALTERNATIVE SPLICING

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Background. The SET-NUP214 (TAF-1/CAN) gene fusion has been described as result of a chromosomal translocation t(9;9)(q34;q34) in a case of acute undifferentiated leukemia and more recently as result of recurrent cryptic deletion del(9)(q34.11q34.13) in T-acute lymphoblastic leukemia (T-ALL). The fusion protein appears to be involved in high-level expression of HOXA cluster genes in T-ALL and may contribute to the pathogenesis of the disease. *Aims.* Immortalized cell lines have often been used to elucidate the function of aberrantly expressed or mutated genes. We screened a panel of 141 leukemia/lymphoma cell lines to find cellular systems for SET-NUP214 research. *Methods.* RT-PCR analysis was performed to find SET-NUP214 - positive cell lines. Deletion del(9)(q23.11q34.13) was confirmed by fluorescence in situ hybridization (FISH) analysis with tilepath BAC and fosmid clones, array-based copy number analyses and quantitative genomic PCR. Genomic PCR plus sequencing analysis allowed detecting genomic SET and NUP214 breakpoints. Western blot analyses were performed to verify SET-NUP214 protein expression. *Results.* The T-ALL cell line LOUCY and the megakaryocytic leukemia cell line MEGAL expressed the SET(TAF-I β)-NUP214 fusion gene transcript and the TAF-I α -NUP214 splice variant. Results of FISH, array-based copy number analysis and quantitative genomic PCR were consistent with del(9)(q34.11q34.13). Genomic sequencing localized the breakpoints of the SET gene to regions downstream of the stop codon and to NUP214 intron 17/18 in both LOUCY and MEGAL cells. Expression of a SET exon 7 / NUP214 exon 18 fusion mRNA and the 140 kDa SET-NUP214 fusion protein was found in both cell lines. *Summary and Conclusions.* Cell lines LOUCY and MEGAL express the recently described SET-NUP214 fusion gene. Formation of the SET exon 7 / NUP214 exon 18 gene transcript requires alternative splicing as the SET breakpoint is located downstream of the stop codon in exon 8.

0031

THE UNUSUAL MLL REARRANGEMENT IN ACUTE MYELOID LEUKEMIA

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Background. Aberrations of the MLL gene (myeloid/lymphoid leukemia) located on chromosome band 11q23 are of great interest because of their high variability and association with unfavorable prognosis. One mechanism of interrupting the MLL gene is a partial tandem duplication (PTD). The MLL PTD has been described in about 6 - 10 % of adult patients with acute myeloid leukemia (AML) with normal karyotype and in most cases with trisomy 11 as a sole cytogenetic abnormality. In rare cases, the duplication is nontandem (PNTD) due to insertion of DNA from another chromosome. The MLL PNTD is generated as a consequence of multiple recombination events and can simulate a simple translocation. *Aim.* Presentation of an unusual and rare MLL rearrangement identified by cytogenetic and molecular methods. *Methods.* Bone marrow cells of all patients with diagnosis of AML were examined by conventional cytogenetics and FISH with a commercially available MLL Break Apart Rearrangement probe. Further investigations of the karyotype changes were performed by FISH with commercially available probes and/or by multicolor FISH and/or mBAND. At the molecular level, the MLL PNTD was analysed by RT-PCR and DNA sequencing. *Results.* During the years 2003-2008, we examined 171 patients at the time of diagnosis of AML and MLL PNTD was found in four cases (2,3%). In patient 1, the translocation t(10;11)(p12;q23) and the MLL PNTD including exons 2 through 9 with the insertion of the MLLT10 exon 10 was proved. In patient 2, the reciprocal complex translocation t(9;12;11)(p22;p13;q23) and the MLL duplication of exons 2 through 8 with the insertion of the MLLT3 exon 9 and 10 was identified. In patient 3, the translocation t(11;19)(q23;p13) and the MLL PNTD including exons 2 through 8 with cryptic insertion of MLLT1 exons 2 and 3 was detected. Complex aberrations 46,XY,der(11)del(11)(q13q23.3) ins(10;11)(p12;q23.3q23.1) were identified in patient 4. The MLL PNTD included exons 2 through 9 and the insertion of the exon 10 of the MLLT10 gene. *Conclusions.* The formation of new fusion genes is an important event in leukemogenesis. Many different processes leading to a MLL fusion have been described, however mechanisms triggering the formation of MLL PNTD remain unclear. The systematic and careful detection of the MLL gene aberrations in patients with hematological malignancies are extremely important due to its correlation with poor prognosis and aggressive progression of the disease.

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0032

COMPLEX CHROMOSOMAL ABERRATIONS IN BONE MARROW CELLS OF 86 PATIENTS WITH MYELODYSPLASTIC SYNDROMES (MDS)

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Karyotype of bone marrow cells represents one of the most valuable prognostic marker for MDS patients with profound impact on diagnosis and therapeutic decisions. Specific and recurrent aberations are delineated for diagnostic subgroups. In 10-20% of MDS patients complex chromosomal aberrations (CCA) can be found and precise identifications of chromosomal regions involved in CCA could yield detection of cryptic, recurrent, prognostically relevant aberrations and identification of candidate genes involved specially in progression of the disease. The aim of this study was to evaluate significance of specific chromosomes and/or chromosomal regions involved in CCA and to establish the exact localization and frequency of chromosomal breakpoints. Various modifications of molecular cytogenetic techniques were used for detailed analysis of CCA: FISH, mFISH/mBAND, CGH and/or array CGH. During 2002-2008 we examined bone marrow samples of 680 adults with MDS and in 96 of them (14.1%) CCA were found (in 86 patients at dg and in 10 cases during the disease progression). Out of 86 patients with

CCA at dg primary MDS was diagnosed in 74 (86.0%) of them, in 12 cases therapy related MDS was ascertained. Dg according to the WHO classifications: MDS NS 6x, RA 2x, RA/RCMD 6x, RCMD 7x, RAEB I 22x, RAEB II 23x, MDS-AML 19x and RARS 1x. Deletion of 5q31 region was proved in 75 patients (87.2%). In 35 cases mFISH analyses showed that parts of deleted No.5 were translocated into other chromosomes. The most recurrent partners of deleted No.5 in unbalanced translocations were chromosomes 17 (11x), 3 (6x), 7 (5x) and 12 (5x). Monosomy of No.5 was confirmed in one case only, thus proving that monosomy 5 is not separated diagnostic entity in MDS patients with CCA. The most frequent breakpoints on chromosome 5 were 5q33 (31x), 5q13 (25x), 5q14 (7x) and 5q12 (5x). Except No. 5 the most frequently involved in CCA were chromosomes 7 (37x), 3 (33x), 12 (31x), 17 (31x) and 11 (24x), the most recurrent breakpoints were at regions 7p11 (6x), 7q11 (6x) and 12p11 (7x). Presence of CCA at diagnosis were connected with poor response to the therapy and short overall survival. For survival analysis patients were classified into three groups according to the molecular cytogenetic findings: (1) del(5)(q) and additional CCA - 39 cases, (2) deleted chromosome 5 involved in CCA - 35 cases, (3) CCA without deletion of 5q - 12 cases. The shortest overall survival was found in group 2 (median 3 months), followed by group 1 (median 5 months) and 3 (median 9 months). Patients with MDS and CCA should be considered as a unique entity with extremely poor prognosis. Exact molecular cytogenetic analysis of all chromosomal aberrations which are involved in CCA will lead to redistribution of the patients into new prognostic groups, could improve the therapeutic outcome and contribute to the development of a new targeted treatment strategy.

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0033

INTER-LABORATORY PERFORMANCE EVALUATION OF A NEW RQ-PCR ASSAY AIMED AT MEASURING RESPONSE TO TREATMENT IN CHRONIC PHASE CML PATIENTS

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Background. Real-time quantitative PCR (RQ-PCR) measurement of BCR-ABL transcripts in blood has been proven to be a reliable alternative to bone marrow cytogenetics and is now used as in daily practice for monitoring of patients with Chronic Myeloid Leukemia (CML). Analysis of BCR-ABL transcript levels by RQ-PCR reflects the level of leukemic inhibition induced by therapy and provides a highly sensitive method to determine minimal residual disease (MRD). However, differences in RQ-PCR techniques (including choice of the control gene, calibration method and primers and probe design) lead to significant variability in BCR-ABL reported values (Zhang *et al.* JMD 2007, Branford *et al.* Leukemia 2006). Considering the influence BCR-ABL quantification can have on treatment decisions, a higher degree of standardization is required to allow consistent measures of drug response. *Aim.* This study aimed at evaluating inter-laboratory analytical performance of a new assay named *BCR-ABL Mbcr FusionQuant® Ez*. This assay was developed as an aid to evaluate major and complete cytogenetic responses in CML patients. The kit incorporates all reagents and enzymes required to run the RQ-PCR test, in an attempt to improve robustness of BCR-ABL quantification. *Methods.* Three study sites contributed to this evaluation, and analyzed the same 50 anonymous and blinded blood samples, containing different spiked levels of BCR-ABL positive cells. RNA extractions and BCR-ABL RQ-PCR measurements were performed according to the kit package insert on a Roche Light Cycler 1.2. BCR-ABL results were reported as Normalized Copy Number ratios (NCN=CNBCR-ABL/CNABLx100). *Results.* One hundred and thirty eight samples gave interpretable NCN values from the 150 samples (i.e. 92%). No statistically significant impact of the testing laboratory was found. Agreement between Cleveland and Ipsogen was calculated on 44 common interpretable samples, on 42 between Richmond and Ipsogen, and on 40 between Richmond and Cleveland. Taking Ipsogen NCN values as reference, 95.5% of Cleveland and 88.1% of Richmond NCN values were comprised in a 2-fold interval, and 100% within 5-fold. For Major Cytogenetic response (MCyR, threshold=8.89 NCN) all samples were found to be concordant between the 3 testing sites, corresponding to a Kappa coefficient of 1. Only one sample was found to be discordant between Ipsogen and Cleveland regarding Complete Cytogenetic response (CCyR, threshold=0.87 NCN). The same sample was also discordant between Ipsogen and Richmond. As a result, for CCyR, overall percent agreement between Cleveland and Ipsogen was found to be 97.7%, 97.6% between Richmond and Ipsogen and 100% between Richmond and Cleveland. Inter-laboratory agreement was equal to 0.962 (Kappa). *Summary and conclusions.* Overall, inter-laboratory agreement was perfect for MCyR (Kappa=1), and almost perfect for CCyR (Kappa=0.962). No discordant sample was identified between the 2 external laboratories. Therefore, our assumption that a significant proportion of inter-laboratory variability in BCR-ABL NCN reporting is related to the lack of standardization of test procedures is supported by this study using a kit including all reagents needed to run the experiment from patient's RNA. The increased robustness obtained with this kit should ultimately benefit the patients and allow for optimized treatment decisions.

0034

HIGH RESOLUTION MELTING ANALYSIS FOR THE RAPID DETECTION OF C-KIT MUTATIONS IN CORE BINDING FACTOR-ACUTE MYELOID LEUKEMIA

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Background. c-KIT gene encodes a transmembrane glycoprotein receptor that is member of the type III receptor tyrosine kinases family implicated in cell proliferation, differentiation and survival. Activation of c-KIT receptor may be achieved by gain-of-function mutations predominantly located within exon 8 and 17 and have been reported to occur preferentially in acute myeloid leukemias (AML) with CBF rearrangements [t(8;21) or inv(16)/t(16;16)]. *Aims.* We report the results obtained in a group of CBF AML patients by using a high resolution melting (HRM) method for simultaneous screening of exon 8 and 17 c-KIT mutations. *Methods.* The study included 69 CBF AML patients, 55 adults [30 with t(8;21) and 25 with inv(16)] and 14 children [8 with t(8;21) and 6 with inv(16)]. c-KIT mutations study was performed on genomic DNA extracted from bone marrow samples collected during active disease (diagnosis and relapse). The HRM assays were performed in duplicate using the LightCycler® 480 Instrument (Roche). Two DNA normal controls and one positive mutated DNA sample for each exon were included in each run. Thirty ng of DNA were amplified in 10 µL final volume containing 1x LightCycler®480 High Resolution Melting Master (Roche), 0.5 µmol/L of each primer-pair and 2.0 mM MgCl2 for exon 8 or 0.2 µmol/L of each primer-pair and 3 mM MgCl2 for exon 17. Results were analyzed as fluorescence versus temperature graphs using the Gene Scanning Software Version 1.5.0 (Roche). Differences in melting curve shape showed by the difference plot helps clustering the samples into groups. *Results.* Among 69 samples screened for the presence of c-KIT mutations, four samples in exon 8 and eight samples in exon 17 showed a clearly distinctive shape of the difference curve plot compared with those of the wild-type DNA. Direct sequencing confirmed presence of c-KIT mutations in 11 out of 69 (15.9%) AML patients (one infant and 10 adults). Mutations corresponded to insertion/deletions in exon 8 in three patients, point mutations in exon 17 in seven patients, and one who presented mutations in both exons (Table 1).

Table 1. Karyotype *c-KIT* mutations found exons 8 and 17.

Exon	Patient	Cytogenetics	Nucleotide change	Mutation description
8	19295	inv(16)	del ctacgac ins gggg	p.T417_D419delinsRG
	4474	inv(16)	del acttacgacaggctcg ins tcgaggatct	p.T417_V422delinsSRIL
	15702	inv(16)	del tac ins gtc	p.T417TfsX33
	18826	inv(16)	del ttacga ins cgg	p.T417TfsX33
17	4248	t(8;21), inv(16)	gac→cac	p.D816H
	15097	t(8;21), inv(16)	gac→cac	p.D816H
	34824	inv(16)	gac→gtc	p.D816V
	18826	inv(16)	gac→gtc	p.D816V
	4923	inv(16)	gac→tac	p.D816Y
	9153	inv(16)	gac→tac	p.D816Y
	34259	t(8;21)	gat→ggt	p.D820G
	23530	t(8;21)	aat→aag	p.N822K

No false positive or negative results were found and the assay sensi-

tivity achieved for both exons detected mutations up to dilution 1:100 of a positive DNA sample for exon 8 and exon 17 in wild-type DNA. c-KIT mutations were statistically associated with high white blood cell count (WBC) (p=0.012) and prognostic value analyzed in adult patients showed a significantly reduced relapse free survival (p=0.04) for patients with c-KIT exon 17 mutations. *Conclusions.* HRM is a rapid, sensitive, specific and low cost method for the screening of exons 8 and 17 c-KIT mutations.The incidence of c-KIT mutations was 15.9% in CBF- AML patients (one infant and 10 adults). Exon 17 mutations seems to confer an unfavourable prognosis to these patients.

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0035

ANALYSIS OF ACTIVATION INDUCED CYTIDINE DEAMINASE MRNA AS PREDICTOR OF DISEASE PROGRESSION AND RESPONSE TO THERAPY IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease. Some patients exhibit a stable, indolent disease and survive for many years without requiring any therapy, while other show rapidly progressive disease and die within months. Recently, some new prognostic factors, such as IgVH mutational status, 2AP-70 and Activation Induced Cytidine Deaminase (AID) mRNA expression were introduced to identify indolent versus progressive types of CLL bearing the potential to facilitate risk-adapted treatment strategies. *Aims.*The study was performed to evaluate the clinical value of AID mRNA expression as predictor of disease progression in early stages and response to therapy in later stages of CLL. *Methods.* The study included 42 B-CLL patients. They were divided into 2 main groups; group 1 patients (N=15) did not exhibit an indication to start antileukemic therapy at presentation, while group 11 patients (N=27) were indicated to receive antileukemic therapy. Group 11 patient were further subdivided into 2 subgroups according to the treatment protocol they received. Group 11 A patients (N=13) received fludarabine and cyclophosphamide (FC protocol), while group 11B patients received chlorambucil with or without prednisolone.An informed consent was obtained from the patients. Investigations were done to all patients including complete blood count, bone marrow examination, ESR, LDH, B2M, liver and kidney function tests. Immunophenotyping of leukemic cells and assessment of AID mRNA expression by quantitative real time PCR (QRT-PCR). *Results.* Higher expression of AID was associated with need for treatment. A cut-off value of AID expression of 5 units was introduced using youden's index for prediction of need for treatment with 83% sensitivity and 67% specificity. A shorter treatment free survival was associated with high AID expression. In groups IIA and IIB patients, AID expression showed an association with the type of response to antileukemic treatment, being higher in those not achieving complete remission or non-responding to treatment. Moreover, a cut-off value of AID expression of 4000 units was introduced for prediction of treatment refractoriness in patients who received chlorambucil with 80% sensitivity and 89% specificity. *Summary and Conclusions.* AID could be used to predict early disease progression and its ability to predict responsiveness to anti-leukemic treatment in CLL cases. Real time-PCR is a rapid and sensitive method for evaluating AID expression.

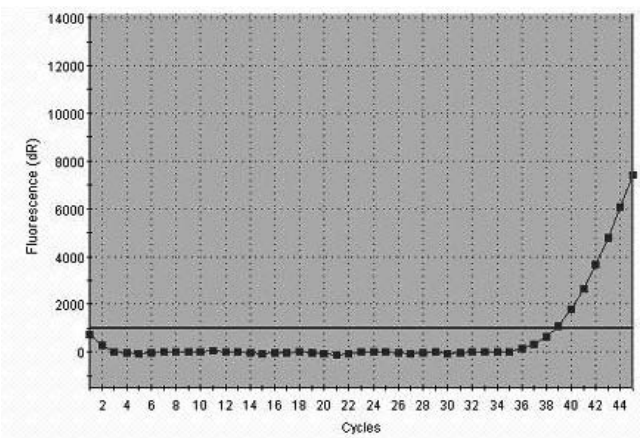

Figure 1. Amplification plot of AID.

0036

CONSTITUTIONAL RUNX1 DELETION IN NON-SYNDROMIC THROMBOCYTOPENIA; 21Q22 ITSN1 AS A CANDICATE GENE IN MENTAL RETARDATION

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We present a developmentally normal patient with isolated thrombocytopenia and myelodysplastic features associated with a constitutional submicroscopic 21q22 deletion (including RUNX1). In contrast, four cases were recently published of syndromic thrombocytopenia with 21q22 deletions and variable degrees of dysmorphic features and mental retardation (MR), suggesting a dosage-sensitive causative role of gene(s) on 21q22 in MR. One patient had developed myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) at the age of six years. A 5-year-old boy was referred with a history of frequent haematoma caused by a relatively mild thrombocytopenia. Growth and psychomotor development was normal as well as dysmorphologic evaluation. Bone marrow (BM) revealed an active, atypical megakaryopoiesis with slight dysplastic features. G-banding on BM showed a 46,XY karyotype. FISH analyses demonstrated two constitutional microdeletions of one chromosome 21; a 21q22 interstitial deletion with loss of RUNX1 and a terminal 21q deletion. High-resolution oligo array-CGH (105K Agilent) showed a 1.60 Mb interstitional deletion 21q22.11-12, including RUNX1, and a 2.22 Mb terminal 21q22.3 deletion. Several genes at 21q22.12 have been proposed as candidate genes to contribute to MR in syndromic thrombocytopenia. We compared the genotypes and phenotypes of the published cases with our case. This approach pointed to an association with MR of a minimally deleted region of 0.4 Mb containing the ITSN1. ITSN1 is a scaffold protein and has a role in endocytic vesicle trafficking in neurons. It has been implicated in Alzheimer's disease and Down syndrome, and could play a role in MR in these patients as a dosage-sensitive gene. We hypothesize that haploinsuffiency of ITSN1 is implicated in MR in syndromic thrombocytopenia with 21q22 deletions. Our case emphasizes the necessity of cytogenetic and molecular analyses of 21q22 for RUNX1 status in diagnosing patients with isolated or syndromic thrombocytopenia and supports RUNX1 haploinsufficiency in FPD/AML.

0037

DETECTION OF NUMERICAL CHROMOSOME ABERRATIONS IN RARE ACUTE MYELOID LEUKEMIA (AML) CELLS

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Background. Chromosomal aberrations, detected by karyotyping and fluorescent in situ hybridization (FISH), have high clinical significance for the diagnosis and treatment of leukemias, but the detection limits of the cytogenetic techniques are strongly affected by the abundance of the abnormal cells in a sample. Structural aberrations can alternatively be detected with high sensitivity by PCR but this technique lacks the component of visual verification (specificity) and in rare event cases of numerical aberrations, it is not applicable. The ImageStream system is a flow cytometry-based image analysis platform that produces high resolution images of cells prepared in suspension at rates that can exceed 100 cells per second. In order to adapt this technology for chromosome analysis a method was established to perform FISH in suspension (FISH-IS) and an extended depth of focus (EDF) modification was designed to enable detection and enumeration of FISH signals that lie anywhere within a 16 µm range of depth within the cell. *Aims.*The present study aims to demonstrate applicability of FISH-IS for detecting numerical chromosome aberrations and to establish specificity and sensitivity of detection. Additionally, its clinical application is demonstrated in the analysis of samples of patients with +8 AML. *Methods.* Healthy donor peripheral blood samples hybridized with chromosome enumeration probes (CEP) served as model systems of disomy (♀, CEP-X), monosomy (♂, CEP-X) and trisomy (♂, CEP-8 + CEP-Y). FISH-IS analysis was also performed on cryopreserved samples procured at diagnosis and remission from 12 patients previously diagnosed by conventional cytogenetics with +8 AML. *Results.* By titrating healthy donor male cells into female cell populations before hybridization, the sensitivity of detection in these controlled models of monosomies and trisomies was determined to be at least 1% with 100% specificity (analyzing 10.000 cells/sample). For the diagnostic AML samples, +8 was detected in abun-

dance by both conventional FISH (c-FISH) and FISH-IS but the % trisomies detected by c-FISH was generally higher than detected by FISH-IS. For the AML remission samples, the opposite was true. Whereas the % +8 in all remission samples was below the detection limit of c-FISH, the % +8 detected by FISH-IS was generally higher. Of note, for 8/12 patients, relapse samples were available for which 2 were determined to have relapsed with +8 by c-FISH which correlated with the 2 highest +8 scores determined by FISH-IS in the corresponding remission samples. *Summary and Conclusions.* It is important to consider that some of the discrepancies between the c-FISH and FISH-IS results may be due to the analysis of samples obtained in parallel rather than analysis of the exact same sample. The pre-clinical data demonstrate that FISH-IS detects numerical chromosome aberrations with a higher sensitivity and specificity level than conventional cytogenetic analyses. The preliminary clinical data suggest that FISH-IS can be used to detect numerical chromosome aberration in minimal residual disease in AML. Further studies are underway to verify the suggested observed correlation between +8 detected at remission and relapse.

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0038

A RAPID, SENSITIVE AND SPECIFIC REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR QUANTIFICATION OF THE JAK2V617F ALLELE BURDEN

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Background. The JAK2V617F mutation has emerged as an essential molecular determinant of chronic myeloproliferative disorders (CMPDs), occurring in >95% of polycythemia vera, and >50% of essential thrombocytosis or idiopathic myelofibrosis patients. A correlation between the proportion of mutant JAK2 allele and the propensity to a more symptomatic disease has been described. Moreover, JAK2V617F targeted therapy is expected to become available in the near future. Therefore, sensitive and specific quantification of the mutant allele is needed. However, most of the current assays are hampered by false positive results due to aspecific amplification. *Aim.* To evaluate the analytical and clinical performances of a real-time polymerase chain reaction (qPCR) assay using a combination of hydrolysis probes and a wild-type blocking oligonucleotide, all containing locked nucleic acids (LNA) for sensitive and specific detection of the JAK2V617F mutation. Moreover, we validated a procedure for precise quantification of the JAK2V617F allele burden. *Materials.* We used 116 DNA samples from patients suspected to suffer from a CMPD and dilutions of HEL cells known to carry the mutation to compare the LNA-based qPCR to those obtained by two previously published and well established methods: a qPCR[1] and an allele specific PCR.[2] In order to validate the accuracy of the LNA-based qPCR, 77 randomly selected samples were genotyped for the JAK2V617F mutation using conventional sequencing. In addition, 20 normal donor peripheral blood samples were analysed. *Results.* All assays detected the same 36 JAK2V617F positive patients out of 116 suspected CMPD diagnostic samples. In contrast to the other qPCR test[1], no amplification in normal donor DNA was observed in LNA-based qPCR, proving the specificity of the assay. Moreover, the latter was the sole assay able to detect as few as 0.1% of JAK2V617F allele drown into wild-type alleles. Low intra- and inter-assay variabilities with high efficiency of amplification ensure a good reproducibility of the LNA-based qPCR assay. Finally, quantification of the JAK2V617F allele burden showed similar levels among each of the different CMPD entities as described by other groups. *Conclusions.* The LNA-based qPCR is a rapid, robust, sensitive and highly specific assay for quantitative JAK2V617F determination. It can be easily implemented in clinical molecular diagnostic laboratories. Moreover, precise quantification allows determination of JAK2V617F burden at diagnosis as well as the evaluation of the response to novel JAK2 inhibitors.

References

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0039

MYELODYSPLASTIC SYNDROMES: USEFULNESS OF GENE EXPRESSION PROFILING FOR PROGNOSIS AND CELL MORPHOLOGY CLASSIFICATION

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Background. The myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic malignancies, characterized by cytopenias in at least one lineage of peripheral blood cells, ineffective hematopoiesis and hypercellular bone marrow. Gene expression profiling in MDS using microarray technology has demonstrated that several genes are useful for subtype discrimination, assessment of prognosis and distinguish *de novo* AML with myelodysplasia from MDS-associated leukemia. *Objective*. Reproduce these results using low density arrays. *Patients and Methods*. Sixty-nine patients with MDS were studied (18 RCMD, 2 RA, 16 RAEB, 3 RARS, 3 MDS with hipoplasia, 14 AML with myelodysplasia, 8 CMML and 4 MDS associated with isolated del(5q)). Bone marrow samples were obtained by aspiration at the time of initial diagnosis. Cells were obtained after hypotonic lysis of erythrocytes, washed in PBS, labeled using MACS $CD34^+$ MicroBeads and selected with miniMACS magnetic cell separation columns according to the manufacturer's instructions. Purity of the selected $CD34^+$ cells was evaluated by flow citometry. Total RNA was extracted from these samples and one microgram was converted to cDNA by reverse-transcription using random primers. Sixty-one genes among those proposed in the different microarray studies and 3 endogenous genes were selected and a quantitative low density array (Micro fluidic card, MFC) was performed. Lineal models were used to detect differences in the expression profiles of genes in logarithmic scale from MFC (cytological and cytogenetic groups as categorical variables and prognosis score as an ordinal one). Observed p-values were then compared to their expected distribution assuming lack of association in a QQ-plot. Genes with a high deviation from the expected distribution were considered as significant. *Results.* Several genes were found to be significantly expressed according to: 1) Cytological subgroups: in MDS associated with isolated del(5q) PF4, DCK, MAP3K12, DYNLL1 and RB1CC1 genes were overexpressed. In AML with dysplasia PF4 was underexpressed and DCK was overexpressed. In RAEB RAC1 was underexpressed; 2) cytogenetic abnormalities: In patients with del(5q) PF4, MAP3K12 and DYNLL1 genes were overexpressed; 3) prognostic score (IPSS): PF4 expression decreased from better to poor prognosis. ITPR1 expression increased from better to poor prognosis; 4) any gene allowed us to differentiate MDS-associated leukaemia from *de novo* AML with myelodysplasia. *Conclusions.* In MDS a significant correlation between the expression of some genes and cytological subgroups, cytogenetics abnormalities and prognosis score was found. We could not reproduce exactly the gene expression profiles described in the literature due to the low number of patients in some MDS subtypes. Granted: PI-05-1409, FIJC-P-EF-08 and RD06/0020/1056 from RETICS.

0040

MOLECULAR CHARACTERISATION OF TRANSLOCATION (7;8) IN A CASE OF T-CELL LYMPHOBLASTIC LYMPHOMA

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Background. The number of cytogenetically identified chromosomal translocations increases rapidly. The consecutively deregulated expression of genes or expression of neogenes is important for pathogenesis and development of malignant tumours in many instances. Unravelling these translocations precisely down to the molecular level may give therefore new insights in tumorigenesis. *Aims.* Precise molecular characterisation of chromosomal breakpoints in tumor samples and characterisation of affected genes is our goal. *Methods.* We here describe clarification of a leukaemia sample derived from a patient with T-cell-lym-

phoblastic lymphoma. Cytogenetically a split in FISH analysis within TRB was found. To localize the precise breakpoints of the chromosomal alteration in this case, FT-CGH (fine tiling comparative genomic hybridisation) was used. Observed imbalances were further examined using LM-nested-PCR (ligation-mediated nested PCR). In brief: sample and reference DNA is separately digested with six restriction enzymes (DraI, PvuII, EcoRV, StuI, SmaI, HindII) and adaptors are ligated. A pair of gene specific primers is designed at the border of deletion spots observed upon FT-CGH analysis. After first round PCR a nested PCR is performed using a gene specific primer and an adaptor primer. The expected germline product can be predicted using database and restriction sites. PCR-product with aberrant size upon gel analysis can be distinguished from germline PCR-products, are gel extracted and sequenced. Sequencing results reveals presence of translocations comparing the data with genomic databases. Presence of these alterations is confirmed with gene specific primers analyzing undigested DNA. *Results.* The FT-CGH analysis showed small deletion (about 2 kb) in J1 region of TRB (TRBJ1). Using libraries of restricted and ligated DNA nested PCR was performed with pair of primers gene specific and pair of adaptor primers. Upon gel electrophoresis of PCR products of DraI digested sample two bands were observed: an expected germline band about 4 kb and an atypical band of 2.6 kb size. The atypical band was cut out, extracted and sequenced. Sequencing revealed a translocation (7;8) with the breakpoints: 142,196,004 on chr.7 (TRBJ1-4 region) and 128,824,471 on chr.8 (telomeric of the c-Myc gene). The second break was localized at position 142,194,034 on chr.7 and 128,824,487 on chr.8. This translocation was confirmed on genomic DNA with gene specific primers. *Summary and Conclusions.* Using combination of both methods (FT-CGH and LM-PCR) in the case of lymphoblastic-T-cell-lymphoma we characterized for the first time exact breakpoints of translocation (7;8). Here known protooncogene c-Myc is transposed upstream of TRBJ1 region. The c-Myc protooncogene is known to be a partner gene for a translocation in Burkitt lymphoma with the varying immunoglobuline sites (as a V(D)J recombination mediated translocation). In Burkitt lymphoma this type of translocation causes overexpression of c-Myc. In our work we showed for the first time, that in T-cell-lymphoblastic lymphoma c-Myc is rearranged to the TRB gene, resembling the c-Myc-Ig rearrangement observed in B cell lymphomas.

0041

A BRANCHED DNA-BASED TECHNIQUE FOR DETECTION OF BCR-ABL GENE PRODUCTS

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Background. Genetic abnormalities especially translocations are probably the critical pathogenetic events in acute leukemias which lead to development and progression of disease. The specific genetic abnormalities can be used not only for diagnosis but also to determine the prognosis, therapy protocols and therapy response. BCR/ABL test for CML is one of the mostly used tests in molecular hematology laboratories in recent years. There are different techniques to display translocations but, both FISH and Q-RT-PCR have been widely used. Both of them are expensive, need special equipments and skilled technicians. Other than higher contamination risk, false positive and negative results are important defects of these tests. Therefore it has been necessary to develop new technique which is easy to standardize, cheaper, easier and does not need skilled technicians. *Aim*. Herein we used branched DNA (bDNA) technology for detection and quantification of BCR-ABL gene product with specific probes which has been used for diagnosis and quantification of viral nucleic acids. *Methods.* K562 (b3-a2) and MEG-01 (b2-a2) cell lines were used as positive controls, HepG2 (hepatic carcinoma cell line) was used as a negative control in order to determine the test conditions and sensitivity assessment. We also evaluated the presence of BCR-ABL gene product in blood samples of 22 CML patients by bDNA assay. The sensitivity and specificity of this approach were compared to Q-RT-PCR (lightCycler- Roche diagnostic corporation) results. The cell lines and 10 µL of whole blood from CML patients were lysed with lysing mixture, the cell samples were incubated with capture probes which can hybridize with BCR-ABL gene products and recognize two different fusion gene mRNA (b3-a2 and b2-a2). Unbound probes were removed by washing and again hybridized with label probes. Two additional washes used to remove unbounded material and substrate added to make the signal visible using a luminometer. Spesific probes for β-glucuronidase (GUSB) gene were used as a control probe to detect GUSB mRNA expression in three cell lines. *Results.* GUSB gene was expressed in all three cell lines and the expression level correlated well with cell count. BCR-ABL gene mRNA was also expressed in K562 and MEG-01 cell lines and comparable with cell count but no expression was found in Hep G2 cell line even cell count increase. Detection of the BCR/ABL signal by bDNA assay was observed in 12 patients. These twelve patients were also showing a positive PCR signal. All patient samples giving a negative signal using bDNA assay were also negative by QRT-PCR. BCR/ABL signal values obtained with bDNA-based assay were comparable with those obtained with Q-RT-PCR. The bDNA-based assay was also able to detect transcript variants (b3-a2 and b2-a2). *Conclusions.* Our results suggest that branched DNA assay is a quantitative and reliable assay that can be employed in hematological malignancies, characterized by translocations leading to the formation of hybrid genes.

0042

GENOMIC IMBALANCES IN HCV-RELATED CRYOGLOBULINEMIAS

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Background. Hepatitis C virus (HCV) is estimated to infect 130-170 million people worldwide. A cluster of endemic HCV is found in Southern Italy, with infection rates ranging from 4.6-10.4% and prevalence increasing to 34% in the elderly. HCV causes viral hepatitis, liver cirrhosis and hepatocellular carcinoma and is associated with B-cell lymphoproliferative disorders, including the cryoglobulinemic syndrome. Usually benign, mixed cryoglobulinemia (MC) evolves to overt lymphomas in 5-10 % of cases. Previous claims of IgH/BCL2 rearrangement in MC were not confirmed (Matteucci C *et al.*, Leukemia 2008;22:219-222; Sansonno D *et al.*, Hepatology 2006;43:1166-7). FISH screening detected a 3q trisomy in 1/19 MC cases, suggesting genomic imbalances may arise in this disease (Matteucci C *et al.*, Leukemia 2008;22:219-222). *Aim.* To trace the genomic profile of HCV-related MC in a new series of patients using Metaphase Comparative Genomic Hybridization (M-CGH). *Methods.* 27 patients with HCV-positive MC (21 females, 6 males, median age 61 years, range 44-80; type II MC in 25; type III MC in 2) were selected from patients referred to Internal Medicine and Clinical Oncology, University of Bari Medical School. HCV genotype was 1b in 15 cases and 2a2c in 12. Liver histology indicated chronic active hepatitis in 26 and cirrhosis in 1. Clinical symptoms in all matched a typical cryoglobulinemic syndrome, with 2 showing peripheral lymphoadenopathy, splenomegaly, and a oligo/monoclonal circulating B-cell population. DNA was extracted from peripheral blood mononuclear cells in 25 cases and from bone marrow cells in 2. M-CGH experiments were performed as previously described (Matteucci *et al.,* CGC 2002;135:28-34). Analysis was performed with a CytoVision system (Applied Imaging, Genetix Limited, New Milton, Hampshire, UK). Genomic gains and losses were defined by comparing the green-to-red ratio profiles with the 99.5% dynamic standard reference interval.

Table. Clinico-immunological data in 27 HCV-related MC.

N.	AGE/SEX	LIVER HISTOLOGY	GENOTYPE	ALT (U/L)	RF (U/L)	IgM (mg/dL)	CRYOCRIT (%)	IC	HCV VIRAL LOAD (U/mL)	BCR	FOLLOW UP (months)
1	75/F	CIRRHOSIS	2a/2c	82	89	143	5	IgM k - IgG	2,700,000	OLIGOCLONAL	44+
2	70/F	CAH	1b	44	3	1200	4	IgM k - IgG	850,000	POLYCLONAL	80+
3	58/F	CAH	1b	96	20000	1175	10	IgM k - IgG	1,938,000	nd	58+
4	56/F	CAH	2a/2c	39	96	126	1	IgM k - IgG	14,109	nd	46+
5	46/F	CAH	2a/2c	17	477	826	1	IgM k - IgG	181,000	POLYCLONAL	87+
6	80/F	CAH	1b	75	69	66	2	IgM-IgG (III)	47,750	nd	53+
7	70/M	CAH	2a/2c	38	300	139	2	IgM k - IgG	35,000	nd	21+
8	73/F	CAH	1b	143	2080	569	16	IgM k - IgG	1,927,000	nd	31+
9	80/F	CAH	1b	101	304	163	9	IgM k - IgG	133,000	POLYCLONAL	57+
10	47/F	CAH	1b	36	69	195	3	IgM k - IgG	760,000	nd	25+
11	64/F	CAH	2a/2c	21	43	255	1.4	IgM k - IgG	769,000	OLIGOCLONAL	87+
12	53/M	CAH	2a/2c	35	121	119	1	IgM k - IgG	612,000	nd	20+
13	44/M	CAH	2a/2c	119	171	139	2	IgM k - IgG	2,312,000	nd	20+
14	52/F	CAH	2a/2c	34	12	73	8	IgM k - IgG	319,000	POLYCLONAL	73+
15	80/F	CAH	2a/2c	33	1520	215	10	IgM k - IgG	1,366,000	nd	46+
16	71/M	CAH	1b	24	2	330	2	IgM k - IgG	173,000	MONOCLONAL	94+
17	67/F	CAH	1b	134	4800	295	9	IgM k - IgG	400,000	nd	65+
18	61/F	CAH	1b	27	756	264	10	IgM k - IgG	2,530,000	MONOCLONAL	95+
19	58/M	CAH	2a/2c	83	2950	538	18	IgM k - IgG	869,000	MONOCLONAL	53+
20	61/F	CAH	1b	124	463	268	4	IgM k - IgG	993,000	nd	45+
21	55/M	CAH	1b	47	101	294	5	IgM-IgG (III)	378,000	nd	83+
22	80/F	CAH	2a/2c	32	35	136	9	IgM k - IgG	741,000	nd	43+
23	78/F	CAH	1b	25	1140	743	10	IgM k - IgG	398,000	nd	97+
24	75/F	CAH	1b	74	189	154	3	IgM k - IgG	29,700	nd	82+
25	70/F	CAH	1b	77	1270	377	12	IgM k - IgG	47,200	nd	52+
26	62/F	CAH	2a/2c	88	22	83	1	IgM k - IgG	43,000	nd	36+
27	55/F	CAH	1b	43	32	131	6	IgM-IgG (III)	210,000	nd	26+

CAH: chronic active hepatitis; RF: rheumatoid factor; IC: immunocomplexes; BCR: B-cell receptor

Results. Genomic imbalances were found in 2/27 cases (7.4%). Case n.1 had a chromosome 8 long arm gain and short arm loss. Case n. 16 had a 8q21-qter region gain and a 6q16-qter loss. M-CGH profiles were normal in 25 cases. *Conclusions.* For the first time we showed genomic imbalances occur in around 7% of MC in absence of NHL and found 8q21-qter duplication was a recurrent event underlying oligo/monoclon-

al B lymphocyte benign expansion. 8q duplication was previously found in 12% of HCV-related NHL (Matteucci C *et al.*, Leukemia 2008;22:219-222). Unlike all the other MC cases in this series, both cases with 8q duplication had clinical features mimicking B-cell NHL i.e. peripheral lymphoadenopathy and splenomegaly. Both remained stable over 44 and 94 months of follow up. These data suggest genetic analysis should be included in the work-up of HCV-related cryoglobulinemic syndromes in order to elucidate pathogenetic mechanisms of lymphomagenesis and to select candidates for antiviral and/or anti B-cell expansion treatment.

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0043

FRAGMENT LENGTH ANALYSIS SCREENING FOR CEBPA MUTATIONS DETECTION IN ACUTE MYELOID LEUKEMIA

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Background. CEBPa encodes a protein member of the basic region lucine zipper (bZIP) transcription family that is essential for myeloid differentiation. CEBPa gene consists of two N-terminal transactivating domains (TAD1 and TAD2) and a C-terminal basic and leucine zipper region (bZIP). Inactivating CEBPa mutations have been reported predominantly in normal karyotype acute myeloid leukaemia (AML) and have been related with a favourable outcome. *Aims.* Our objective was to set up a rapid fragment analysis method for the screening of CEBPa mutations and to validate this method in a group of AML patients. *Methods.* The study included 70 AML patients with normal or intermediate-risk karyotype. Twenty-nine patients (41%) showed FLT3-ITD, NPM1 mutations or both. CEBPa mutations study was performed on genomic DNA extracted from bone marrow samples collected at diagnosis. Three PCR were performed using three primer pairs to amplify TAD1, TAD2 (including region between the two domains) and bZIP domains with forward primers fluorescently labelled with FAM. Thirty nanograms of DNA were amplified in a 25 µL reaction containing 7% DMSO and 0.3U of Taq Expand High Fidelity for TAD1 and bZIP fragments and 10% DMSO, 1x Pfx Amp Buffer, 1x PCR Enhancer Solution and 2U Platinum Taq DNA Polimerase for TAD2 fragment. Initial denaturation at 95°C for 3 min was followed by 35 cycles of denaturation at 95°C 1 min, annealing at 55°C for 40 sec and extension at 60°C for 1 min. A final extension of 94°C 30 sec and 60°C 45 min was performed. The mixtures were electrophoresed on an ABI PRISM 3130 Genetic Analyzer and results were analysed using the GENEMAPPER software (Applied Biosystems). Results were confirmed by sequence analysis using ABI PRISM terminator cycle sequencing kit v1.1 (Applied Biosystems) on the ABI PRISM 3130 Genetic Analyzer. *Results.* Eleven out of 70 (16%) patients showed altered electropherograms. Sequence analysis confirmed nucleotide sequence variation in all the cases. These variations were the polymorphism P194_H195dup in two patients and CEBPa mutations in the remaining nine patients (13%) (Table 1).

Table 1. Characteristics of *CEBPa* mutations detected in AML patients.

	PATIENT	MUTATION	PROTEIN	COMMENTS
ONE MUTATION	8861	bZIP:c.1499_1518dup	A303_V308dup	In-frame duplication in BR
	14011	bZIP: c.1525_1527dup	Q312dup	In-frame duplication in LZ
	17210	bZIP: c.1532dup	L315fsX320	Frameshift duplication in LZ and STOP
TWO MUTATIONS	3889	TAD1:c.852_853dup and bZIP:c.1525_1527del	S21fsX160 and Q312fsX358	N-terminal stop and frameshift deletion in LZ
	7100	TAD1: c.779_782dup and bZIP:c.1520_1521ins	I68fsX108 and T310_Q311insL	N-terminal stop and in-frame insertion in LZ
	9147	TAD1:c.838del and bZIP:c.1518_1553dup	Q83fsX159 and E309_D320dup	N-terminal stop and in-frame insertion in LZ
	12816	TAD1:c.790_791dup and bZIP:c.1497_1538dup and c.1538_1539ins	I68fsX160 and A303_L315dup and L315_E316insDR	N-terminal stop
	13356	TAD1: c.858del and bZIP: c.1500_1501ins	K90fsX159 and A303_K304insF	N-terminal stop and in-frame insertion in BR
	15635	TAD1: c.899del and bZIP:c.1507_1530dup	G36fsX159 and R306_K313duo	N-terminal stop and in-frame duplication in LZ and BR

BR: basic region in bZIP domain, LZ: leucine zipper in bZIP domain

Six out of nine patients had two mutations and three patients had a single mutation. All patients were sequenced in parallel and no additional mutations were detected. When we consider FLT3 and NPM1 status we observed that 41 patients without FLT3 or NPM1 mutations showed 9 (22%) CEBPa mutations. By contrast no CEBPa mutations were detected in any of the 29 patients with FLT3 and/or NPM1 mutations. The subgroup of patients FLT3 and NPM1 negatives with CEBPa mutated showed a trend to a better relapse free survival than wild-type patients (75% vs. 23%, p=0.09) at 2 years. *Conclusions.* Fragment analysis is a rapid, specific and sensitive method for CEBPa mutation screening. The incidence of CEBPa mutations was 22% in the group of AML with normal or intermediate karyotype and without FLT3 or NPM1 mutations. In this group without molecular markers CEBPa mutations may establish a subgroup of patients with better prognosis.

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0044

MUTATIONS IN THE IRF ASSOCIATION DOMAIN OF THE TRANSCRIPTION FACTOR GENE ICSBP MAY BE ASSOCIATED WITH LEUKEMOGENESIS IN SHWACHMAN-DIAMOND SYNDROM

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Background. Patients with congenital bone marrow failure syndromes (CMBF) have an increased risk for developing MDS or AML. Yet it is not known which gene defects may induce the malignant transformation. ICSBP (interferon-consensus site binding protein), coding for a transcription factor of the IRF family controlling myeloid cell differentiation, is one putative candidate gene, since ICSBP has been identified as one of the genes most dramatically down-regulated in G-CSF-treated patients diagnosed as CN compared to G-CSF-treated healthy controls. *Aim.* The aim of our study was to investigate patients with congenital bone marrow failure syndromes for ICSBP mutations, deletions or methylation defects leading to a down-regulation of ICSBP. *Methods.* DNA was extracted from peripheral blood or bone marrow of 50 CBMF including Shwachman-Diamond syndrome (SDS, 21), Fanconi anemia (FA, 6), Diamond-Blackfan anemia (DBA, 10), and congenital neutropenia (CN, 13) patients. Screening for mutations in the DNA binding domain (DBD) and in the IRF association domain (IAD) of ICSBP was done by direct sequencing. Furthermore, the promoter region of ICSBP was analyzed for *de novo* methylation and for deletion in the chromosomal region 16q24.1 by fluorescence in situ hybridisation (FISH) using BAC. *Results.* The substitution c:827 G>A leading to Arg276His was found in 3 of 21 SDS patients. These point mutation was constitutional, since they were also present in the oral mucosa, but was not present in a control group (>150 patients). Another substitution c:602 C>T; p:Ala201Val was observed in one of ten DBA patients investigated. The known SNP (rs61995933) was found in one of six FA patients. No alterations were found in 13 CN patients. By means of methylation specific PCR we found no evidence for promoter methylation in the patients diagnosed with CBMF. Furthermore, no significant changes in this chromosomal region were observed by means of FISH analysis. *Conclusions.* Inactivation of the transcription factor ICSBP may disturb normal hematopoietic differentiation and may also play a role in triggering leukemogenesis in patients with congenital bone marrow failure syndromes. Interestingly, one SDS patient carrying an ICSBP point mutation developed leukemia with a complex karyotype supporting our hypothesis. Functional consequences of the mutation remain to be determined.

0045

A NEW RELIABLE FISH METHOD FOR IDENTIFYING MULTIPLE SPECIFIC CYTOGENETIC ABNORMALITIES IN ACUTE MYELOID LEUKEMIA

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Background. Cytogenetics is considered the most important prognostic factors in acute myeloid leukaemia (AML). However, often it fails to completely resolve complex karyotypes and cryptic translocations. *Aim.* To analyze the capability of the Chromoprobe Multiprobe AML Panel for the detection of the most important chromosomal abnormalities in AML and cryptic rearrangements using eight non liquid probes attached to a glass device. *Methods.* We analyze 80 patients diagnosed with AML in parallel by chromosome banding analysis and interphase FISH using the panel which includes: -5/del(5q), -7/del(7q), TP53, del(20q), PML/RARα, AML1(RUNX1)/ETO, MLL, and CBFβ/MYH11 probes. Briefly, slides were prepared pipetting 4 µL of cell suspension onto all eight areas of the template slide. Before the hybridization, the probes were dissolved back into solution adding 2 µL of hybridization solution to each of the eight areas on the device. Then, the template slide was aligned with the probe device and both were replaced in a humid chamber in a 37 °C water bath overnight (Figure 1A). Fluorescent signals were analyzed using Metacyte v3.5.1 image analysis software (Metasystems Inc., Germany) (Figure 1B). *Results.* Using both cytogenetics and multiprobe FISH, we identified 7 patients (9%) with good risk cytogenetics: four patients with inv(16), two patients with t(8;21), and one t(15;17). Five rearrangements were detected by both methods, whereas one inv(16) and one t(15;17) were just detected by FISH. Regarding patients with intermediate risk cytogenetics, we found complete concordance between both methods in all the 33 patients (41%) with normal karyotype, except for the aforementioned cryptic t(15;17). Nineteen additional patients (24%) of the intermediate category had other miscellaneous structural or numerical defects. The multiprobe AML panel was useful in the characterization of chromosomal aberrations in 15 patients (19%) with adverse risk cytogenetics. It showed a good correlation with conventional karyotype in all cases except one, who had a cryptic deletion of the CBFβ allele and was detected only by FISH. However, FISH was ineffective to detect aberrations in 6 cases (7.5%) that involved chromosome regions not represented in the multiprobe panel. However, the panel helped to identify aberrations in 9 patients (11%) without metaphases (7 cases) or with non evaluable chromosomes (2 cases): 3 MLL rearrangements, 2 monosomy 7, one of them also with del(5q), and one inv(16). No alterations were found in the remaining 3 cases. *Conclusions.* We showed the panel is a step forward in the right direction to make FISH easier to handle. The panel identified several chromosomal abnormalities within all cytogenetic risk groups and helped us to clarify complex karyotypes. It also provided a valuable tool for cases without or not assessable metaphases. In conclusion, the panel can be a complementary technique for the identification of most common rearrangements in AML, particularly in cases with poor quality or not available metaphases. Given the importance of chromosome aberrations for prognostication in AML, this method helps in the diagnosis and treatment decision making.

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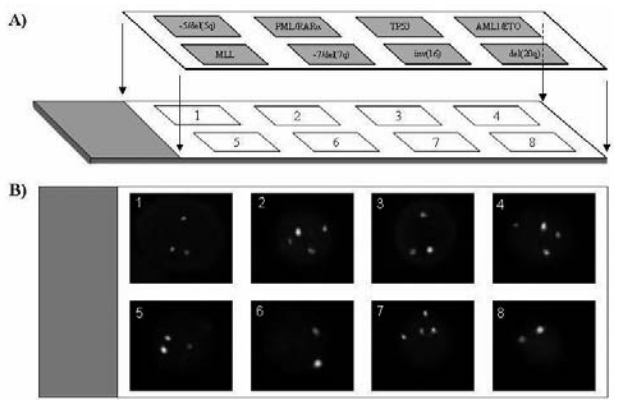

Figure 1.

Cytogenetics and molecular diagnostics

0046

ACUTE MYELOID LEUKEMIA WITH BIALLELIC CEBPA GENE MUTATIONS AND NORMAL KARYOTYPE REPRESENTS A DISTINCT GENETIC ENTITY ASSOCIATED WITH A FAVORABLE CLINICAL OUTCOME

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Background. Acute myeloid leukemia (AML) without chromosome abnormalities (cytogenetically normal AML, CN-AML) represents a molecularly heterogeneous disease in which mutations in a small number of genes have shown to define subgroups with prognostic significance. Point mutations in the CEBPA gene occur at about 8-12% of CN-AML patients and confer a favorable prognosis with an increased overall survival. CEBPA mutations are found as either biallelic (biCEBPA) or monoallelic (moCEBPA). *Aims.*We set out to explore in a large number of CN-AML patients whether the number of mutated alleles in CEBPA is of prognostic relevance. *Methods.* We screened bone-marrow samples of 467 CN-AML patients within the AMLCG study for the presence of CEBPA gene mutations using a multiplex PCR-based fragment length analysis. We identified 58 CEBPA gene mutations in 38 patients of which 20 patients had biCEBPA mutations, 18 patients had moCEBPA mutations and 423 patients had wildtype (wt) CEBPA. These three subgroups were analyzed for clinical parameters and for additional mutations in the NPM1, FLT3 and MLL genes. Furthermore, we obtained gene expression profiles (GEP) using oligonucleotide microarrays. *Results.* Only biCEBPA patients had an improved median overall survival when compared to patients with wtCEBPA (not reached vs. 20.4 months; p=0.018) whereas moCEBPA and wtCEBPA patients had a similar intermediate outcome. Interestingly, biCEBPA patients were never associated with mutated NPM1 (0 vs. 43%; p=0.000) and rarely associated with FLT3-ITD (5 vs. 23%; p=0.059), whereas moCEBPA patients had a similar frequency of mutated NPM1 and a significantly higher association with FLT3-ITD (44 vs. 23%; p=0.037) compared to wtCEBPA. Furthermore, biCEBPA but not moCEBPA patients showed a high concordance to previously identified GEP signatures of CEBPA mutant AML patients. *Conclusions.* Biallelic disruption of the N and the C-terminus of CEBPA is required for the favorable clinical outcome of CEBPA mutated patients and represents a distinct molecular subtype with a different frequency of associated gene mutations and a distinct GEP. These findings have major prognostic impact for risk-adapted therapeutic strategies in AML.

0047

MENINGIOMA GENE 1 (MN1) EXPRESSION IS A SENSITIVE MARKER FOR MINIMAL RESIDUAL DISEASE DETECTION IN ACUTE MYELOID LEUKAEMIA

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Background. The assessment of minimal residual disease (MRD) has currently become a necessary strategy to better address treatment intensity in human leukemias. However, so far the applicability of this strategy has been limited to those leukaemia subsets characterized by genetic markers amenable to sensitive detection by PCR Meningioma 1 (MN1) gene overexpression has been reported in acute myeloid leukaemia (AML) patients. The aim of the study was to characterize the patients presenting MN1 overexpression and to establish the role of MN1 as a marker for MRD detection. *Methods.* 158 AML patients, 30 CML patients and 50 healthy donors were analyzed for MN1 expression by RQ-PCR. In 20 patients bearing a fusion gene transcript (FG), we analyzed MN1, WT1 and FG during follow-up and in 7 patients also NPM1 mutations has been quantified by RQ-PCR. *Results.* The MN1 levels were extremely low in normal samples: median value of 136 MN1/104ABL copies in PB (range 9-300) and 254 in BM (range 80-500) and 12,9 (range 11-19) in

CD34+ cells. By contrast, about 50% of the AML samples with normal karyotype (NK) showed high MN1expression with a median of 5136 copies/104 ABL copies (range 852-90230) and 6780, (range 1367-15900) in PB. All samples carrying the CBFβ-MYH11 FG expressed a significantly higher amount of MN1 transcript as compared to controls (p<0.0001 in both BM and PB): median 46950, (range 2149-98000) in BM and 34500, (range 1400-67999) in PB. About 50% of the samples with AML1-ETO FG abnormally expressed MN1: median 16950, (range 3500-34000) in BM and 3475, (range 1260-56000) in PB. Finally, the APL samples expressed MN1 values comparable to those of healthy subjects in both BM and PB (p=0.05 and 0.08). No association between FLT3 mutations and MN1 was demonstrated. In contrast, MN1 is typically overexpressed in patients with NPM mutations. 36 out of 47 NPMc+ patients were characterized by abnormal MN1expression. We were unable to confirm the correlation between EVI1 and MN1 expression (r= 0.06) reported in literature. Finally, MN1 is not expressed in CP CML (10 cases) but is overexpressed during AP (n=10) and BC (n=10) (median 49100 and 62741 respectively). Finally MRD has been detected by measuring MN1, FG, WT1 and in 7 cases also NPM. MN1 expression always paralleled that of the FG. Furthermore, MN1 strictly parallels WT1. In addition, MN1 is more sensitive than NPM1 in predicting relapse. In all the cases MN1 rose at least two months before relapse. *Conclusions.* These data show that 47% of patients with NK are characterized by abnormal MN1 expression. The overexpression is typical of AML with NPMc+ and inv(16) and CML in AP and BC. MN1 could therefore represent a marker for MRD in patients with normal karyotype and it seems to be more sensitive than NPM1. Increased MN1 expression in BM during follow up was always found to be predictive of an impending hematological relapse.

0048

MEASUREMENT OF MICRORNA EXPRESSION LEVELS BY QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION IS A USEFUL TOOL TO PREDICT OUTCOME IN PATIENTS WITH B CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. B-cell chronic lymphocytic leukaemia (B-CLL) is the most common form of adult leukaemia in the Western World. FISH abnormalities, differential expression of cell surface markers and IgH mutation status have been validated as useful prognostic markers. Besides, differences in microRNA (miR) expression patterns have been proposed as novel prognostic markers in B-CLL largely on the basis of miR expression arrays. *Aim.* However, arrays are not practical for routine diagnosis and therefore we tested these data by quantitative real-time polymerase chain reaction (qRT-PCR) and correlated it with clinical and biological outcome measures. *Methods.* We performed fluorescent in situ hybridization (FISH) and IgH mutation analysis on enriched B-CLL peripheral blood samples (>80% CD19+;CD5+) from 79 clinically annotated patients collected at our institution. FISH results were confirmed by comparative genomic hybridization (aCGH). Next, samples and normal CD19 positive B-cell controls were subjected to qRT-PCR for miRs-155, 223, 15a, 16, 29c, 150, 34a and 21. Results were correlated to clinical and biological features. *Results.* To date, the data show that miR-155, miR-150 and miR-29c are significantly over-expressed in samples from patients with B-CLL compared to normal controls. In contrast, miR-223 expression is down-regulated. Interestingly, miR-15a and 16 expression levels are not significantly different in del13q14.1 vs non-del 13q14.1 subsets. We are currently comparing miR-15a and 16 expression levels to CGH analysis of the 13q14.1 locus. In accordance to previous studies, MiR-223 under-expression is associated with unmutated IgH locus whereas miR-155 over-expression is predictive of advanced stage disease. *Conclusions.* Taken together, we demonstrate that miR expression levels measured by qRT-PCR correlate with biological and clinical features of the disease and warrant further validation as potetially useful prognostic markers within large clinical trials.

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0049

STANDARDIZATION OF MINIMAL RESIDUAL DISEASE FOR MINOR-BCR-ABL TRANSCRIPTS IN PH+ALL: A EUROPEAN APPROACH OF THE EWALL AND ESG-MRD-ALL CONSORTIA

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Background. The level of mininmal residual disease is increasingly being recognized as an important prognostic parameter in a number of hematologic diseases and is used to guide therapy in adult and childhood acute lymphoblastic leukaemia. Reverse-transcription real-time quantitative PCR (rtPCR) for quantification of BCR-ABL mRNA transcripts in peripheral blood and bone marrow is employed routinely during treatment of patients with Philadelphia chromosome positive chronic myeloid leukaemia (CML) or acute lymphoblastic leukaemia (Ph+ALL). However, laboratories may differ substantially in their methodology and analysis strategy, making interpretation and comparison of results difficult or impossible. As a consequence, recommendations for the harmonization of RQ-PCR for BCR-ABL have been established for the p210bcr-abl transcript characteristic of CML. In case of Ph+ ALL and the p190bcr-abl transcript, neither the interlaboratory variability nor the optimal approach for standardisation and/or harmonisation of RQ-PCR for m-BCR-ABL have been established. Therefore, it was the aim of this collaborative European study to assess the variability of m-BCR-ABL detection between numerous well established laboratories involved in diagnosis and MRD monitoring of adult and pediatric patients with Ph+ ALL. We here report the results of the first m-BCR-ABL laboratory control rounds involving 21 participating laboratories from the EWALL and the ESG-MRD-ALL. *Aims.*As a first step towards standardization of m-BCR-ABL detection, the first two laboratory control rounds focused on a comparative analysis of the variability of RNA-extraction and of real-time PCR, the sensitivity of real-time PCR, the proportion of false positive/negative results. *Methods.* Serial dilutions of the BCR-ABL positive cell line Sup B15 in the BCR-ABL negative cell line Nalm 6 were prepared to yield dilutions of 10%, 2 %, 1 %, 0.1 %, 0.01%, 0.001%. Nalm 6 cells alone served as a negative control. The total amount of cell preparation was 5×10E+06 cells stabilized in 2 mL of TRIZOL and frozen at -20°C until shipment. For lab round one, participants were asked to perform RNA-extraction, cDNA-synthesis, and BCR-ABL/ABL measurement in three different experiments. In lab round 2, cDNA was sent out centrally and only the quantitative PCR were to be performed. *Results.* The median measured RNA-amount ranged from 0,07-2,65 (median 0,5 µg/µL), resulting in abl copy numbers ranging from 1.03E+03 to 1.93E+06 (median 1.05 E+05). 20 of 21 participating laboratories detected BCR-ABL in the low-level sample 0.01%. 12 of 20 evaluable participating laboratories failed to detect the 0.001%. For lab round 2, only 4 of 14 evaluable laboratories to date failed to detect the 0.001%. 3 laboratories detected the negative control as positive in lab round 1, none of the 14 evaluable laboratories detected the negative control as positive in lab round 2. *Summary.* Initiation of laboratory control rounds and beginning implementation of standardized methodology resulted in improved variability and lower frequency of false-positive results in the second quality control round. Despite these improvements, further standardisation of methodology is needed to improve results of m-BCR-ABL quantification to ensure comparability of MRD analysis between laboratories and clinical trials at the European level.

0050

JAK2 V617 ALLELE BURDEN AND THROMBOSIS IN PATIENTS WITH POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA

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The V617F mutation of the JAK2 gene is frequently detected in patients with Myeloproliferative Neoplasms (MPN) and it seems to be directly implicated in the pathogenesis of these diseases. It is found in virtually all the patients with Polycythemia Vera (PV) and about half the patients with Essential Thrombocythemia (ET) or Idiopathic Myelofibrosis (IM). Given the increased incidence of thrombotic episodes in these patients, research has been made on the possible correlation of the V617F mutation to the development of thrombosis with contradicting results. In the present study we examined the possible correlation of the JAK2 V617F allele burden with the development of thrombosis in patients with PV and ET. Furthermore, patients were tested for the presence of the most common hereditary risk factors for thrombosis (HRFT): the G1691A mutation of Factor V (FV Leiden), the G20210A mutation of Factor II (FII) and the homozygosity C677T of the MTHFR gene, with the intention of investigating possible synergic action between the presence of V617F mutation and the HRFT in developing thrombosis in patients with MPN. We studied 58 patients with ET and 41 patients with PV. Diagnosis was based on the WHO criteria. We identified the JAK2 V617F mutation by using DNA PCR followed by digestion of PCR product with BsaXI restriction enzyme and quantified the JAK2 V617F mutational load with quantitative Real time DNA PCR. The result was expressed as a percentage of mutated to mutated plus non-mutated JAK2 copies. The patients that carried more than 50% copies were considered of being homozygous for the mutation. The mutations of FV, FII and MTHFR were determined using allele-specific PCR. Seventy patients were found positive for JAK2 V617F mutation [39/41 (95.1%) with PV and 31/58 (53.4%) with ET]. Quantitative real time PCR was performed in 67/70 JAK2 V617F$^+$ patients. Overall, 28/67 patients carried homozygous clones (26/38 with PV and 2/29 with ET). Three of 87 patients with available data carried the G1691A mutation of FV, 4/87 the G20210A mutation of FII and 11/87 were homozygous for the C677T mutation of the MTHFR gene. In 14/87 patients we observed coexistence of JAK2 V617F mutation with one HRFT. 24/99 patients developed a thrombotic episode. Correlation of the frequency of thrombotic episodes with presence of: (i) JAK2 V617F mutation (ii) homozygous JAK2V617F clones and (iii) HRFT, showed an important increase in thrombotic episodes only in the concomitant presence of JAK2 positivity and one HRFT (5/14, 36%). Adversely, the presence of V617F on its own does not seem to increase the chance of thrombotic episodes. Conclusively, this study indicates the synergic role of the V617F mutation of the JAK2 gene with the HRFT on the development of thrombosis, bringing up the need of determining HRFTs in JAK2$^+$ patients with MPNs. However, the increased frequency of thrombosis in patients with MPNs independent of the presence of JAK2 mutation, imposes the quest for additional predisposing factors that lead to the high frequency of thrombosis in these patients.

0051

MONOCLONALITY TESTING USING PYROSEQUENCING

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Background. In medicine, and in particular in hematology, clonality studies have been used to establish the single origin of tumors and to differentiate nonmalignant from malignant states. X-chromosome inactivation based assays exploit the fact that during female embryonic development most of the genes of one X-chromosme are inactivated in each cell. The subsequent progeny of each cell maintains the same inactivated genes, resulting in a mosaic of cells expressing genes from one X-chromosome or the other. On the contrary, in a malignant clonal population genes from the same X-chromosome are inactivated. Current assays are technically laborious and demanding, and not suited for high throughput analysis. *Aims.* In this study we describe a phenotypic clonality test based on pyrosequencing of single nucleotide polymorphisms (SNPs), present in mRNAs transcribed from the active X-chromosome. *Methods.* Eight single nucleotide polymorphisms (SNPs) from eight different X-chromosomal genes (MPP1, GK, CFP, EBP, PIGA, SPRY3, SUV39H1, CHST7) were chosen, localized in regions of the X-chromosome with a stable inactivation pattern and with detectable expression in hematopoietic cells. DNA and RNA were extracted from peripheral blood of 69 normal female blood donors (19 to 75 years old). Previous to RNA extraction blood cells were separated into mononuclear (lymphocytes and monocytes) and polynuclear (granulocytes) fractions, using dextran and Ficoll gradients. Purity of cell fractions was confirmed by flow cytometry. Pyrosequencing reactions for each SNP were set up in triplicates and analysed on a Pyrosequencer 96MA machine (Qiagen). For each blood donor, analyses were performed for DNA and the two RNA fractions, with primers specific for the eight chosen SNPs. Pathologic samples (peripheral blood or bone marrow) were also obtained from twelve patients with various suspected myeloproliferative and myelodysplastic syndromes, with histiocytosis, PNH or with AML. *Results.* Analysis of DNA samples allowed to establish for each SNP the frequency for informative heterozygosity, which varied from 34% (for SPRY3) to 60% (for PIGA), according to the SNP studied. For 67/69 blood donors ≥ 1 SNP gave informative results (mean: 3 to 4 SNPs). For MPP1, CFP, EBP, PIGA, SPRY3, and SUV39H1, levels of heterozygosity were distributed around 50 %, as expected for non-skewed X-inactivation. The SNPs GK and CHST7 showed a different distribution, with skewed as frequent as non-skewed samples. Interestingly, skewed expression of GK and CHST7 were found in 6 blood donors, in which the other SNPs showed a normal distribution. These results argue for extensive variability in X-linked gene expression or inactivation, depending on the gene studied. Using combinations of informative SNPs and comparing X-inactivation between different cell lineages, we were able to confirm clonality in some of the patients studied. Data on these patients is still under study and will be presented. *Conclusions.* Pyrosequencing of a combination of different SNPs is a highly precise method for the distinction between clonal and non-clonal cell populations. Comparisons between patterns of inactivation in different cell lineages of the same patient are needed to correctly interpret inactivation patterns in hematologic diseases.

0052

COOPERATING MUTATIONS OF RECEPTOR TYROSINE KINASES/JAK2/RAS SIGNALING PATHWAYS IN *DE NOVO* AML WITH MLL TRANSLOCATIONS AT DIAGNOSIS AND RELAPSE

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Background. MLL gene located at 11q23 is fused to a variety of partner genes through chromosomal translocations in acute leukemia. Two-hit model of leukemogenesis has been proposed for acute myeloid leukemia (AML). MLL translocation (MLL-T) is considered a class II mutation. The occurrence of cooperation of class I mutations in AML with MLL-T is not clear. Aims. We sought (1) to determine the frequencies of collaboration of class I mutations involving receptor tyrosine kinases (RTK)/JAK2/Ras signaling pathways in *de novo* AML patients with MLL-T at diagnosis and to assess their prognostic impact, (2) to analyze the mutation status and pattern changes at relapse to determine the roles of cooperating mutations in relapse. *Patients and methods.* Sixty-five patients had *de novo* AML with MLL-T which was detected by cytogenetic, Southern blot or FISH analyses at initial diagnosis. RT-PCR was used to detect common MLL fusion transcripts. cDNA panhandle PCR was used to identify the rare MLL partner genes. MLL fusion partners included 19 AF9, 12 AF10, 11 AF6, 10 ELL, 3 ENL, 2 AF4 and one each for CBL, LARG, LCX, MSF, and SEPT. Mutational analyses were performed on bone marrow samples at diagnosis, complete remission, and relapse by DNA/cDNA PCR with GeneScan analysis for FLT3/ITD, PCR-RFLP followed by direct sequencing for FLT3/TKD, DNA/cDNA-PCR with direct sequencing for c-KIT, c-FMS, N-Ras, K-Ras or PTPN11, and allele-specific PCR for JAK2V617F. Results. At diagnosis, FLT3-ITD mutations were detected in 3 and FLT3-TKD in 8; c-KIT mutation in 1, c-FMS in 3, N-Ras in 8, K-Ras in 12, and PTPN11 mutations in 4; none had JAK2V617F. All the mutations detected at diagnosis were not present in the complete remission samples, indicating these mutations were leukemia-specific. Together, cooperating mutations involving RTK/ JAK2/ Ras pathways occurred in 55% (36/65) of *de novo* AML patients with MLL-T. Three patients had two class I mutations (2 FLT3-TKD plus N-Ras, 1 FLT3-TKD plus K-Ras muta-

tions). Twenty of 23 patients who relapsed had relapse samples available for comparative analysis. Three patients retained the same N-Ras mutations at relapse whereas one with Gly12Asp changed to Gly12Cys at relapse. Two relapsed with identical K-Ras mutations, one gained and another one lost K-Ras mutation. All 3 patients who harbored FLT3-TKD mutations at diagnosis lost the mutations at relapse. Another one lost PTPN11 mutation at relapse. All the 5 treated patients carrying FLT3-ITD, c-FMS or c-KIT mutations at diagnosis had an overall survival and event-free survival of less than 6 months. Conclusions. Our results showed that more than half of AML patients with MLL-T carried mutations involving RTK/JAK2/RAS pathways at diagnosis and FLT3-TKD, K-Ras or PTPN11 mutations might lose in relapse. AML patients with MLL-T harboring RTK mutations had a poor outcome.

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0053

PROGNOSTIC SIGNIFICANCE OF TELOMERE LENGTH, MOLECULAR CYTOGENETIC FINDINGS AND IMMUNOPHENOTYPIC FEATURES IN PATIENTS WITH B-CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The clinical course of patients with B-chronic lymphocytic leukemia (B-CLL) is highly variable. Therefore, several indicators related to the genetics and biology of B-CLL are increasingly used for prognosis and treatment response prediction. Recently, some evidences suggest that short telomeres are associated with poor outcome and telomere restriction fragment length (TRF-L) might be of predictive significance. *Aims.*The aim of this study was to evaluate telomere restriction fragment length in a cohort of patients with B-CLL and to correlate these findings with interphase FISH (I-FISH) analyses of chromosomal aberrations, telomerase activity, IgVH mutational status and ZAP-70 and CD38 expression. *Methods.* During years 2007-2008, peripheral blood samples of 77 patients with B-CLL (40 male, 37 female, mean age 67) were analyzed. 21 patients were studied at time of diagnosis and 66 patients were examined at follow-up (45 untreated, 11 after treatment). I-FISH analyses were performed using DNA probes: 1) CLL Probe panel for regions 17p13.1 (gene p53), 11q22.3 (gene ATM), 13q14.3, 13q34 and 12p11.1-q11; 2) LSI IGH (14q32) Break probe for detection of 14q32 aberrations. Telomere length - TRF index in kilobases (kbp) was determined by Terminal Repeat Fragment (TRF) method using Telo TAGGG Telomere Length Assay kit. The cases with TRF shorter than 6.7 kbp (mean TRF from 18 age matched healthy individuals, median=61 years) were considered as cases with reduced telomeres. Telomerase activity was detected using TeloTAGGG Telomerase PCR ELISAPLUS kit. ZAP-70 expression analysis was performed by flow cytometry, PCR "touch down" methodology was used for mutational analyses of IgVH genes. *Results.* Patients were grouped according to molecular cytogenetic findings as follows: good prognosis (del 13q14 as a sole aberration) - 32 patients; standard prognosis (normal findings, +12 and/or combination of monoalelic and bialelic deletion 13q14) - 40 patients; poor prognosis (deletion of ATM and/or p53 genes) - 5 patients. IgVH mutational status was analyzed in 74 patients - in 48 mutated, in 20 unmutated IgVH status was proved, in 6 patients the germline IgVH genes were not identified. 28 of 72 patients were ZAP-70 positive and CD38 positivity was detected in 18 out of 76 examined patients. High heterogeneity in telomere length was observed in a range of 3.50 kbp - 15.5 kbp (median 6.1 kbp), reduced telomeres were proved in 48 patients. A correlation between TRF-L and IgVH mutational status was confirmed whereas IgVH mutated patients showed long and IgVH unmutated short telomeres (p=0.001). Detailed results of TRF-L analyses in comparison with other prognostic features will be presented. *Conclusions.* Finding new prognostic markers in early stage of B-CLL is a subject of intensive research. We confirmed that TRF-L analyses have predictive value and can contribute to prognostication in B-CLL patients.

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0054

B-CELL LYMPHOPROLIFERATIVE DISORDERS WITH T(11;14)(Q13;Q32) OR T(14;18)(Q32;Q21) SHOW VARIATIONS IN THE PATTERN OF ADDITIONAL CYTOGENETIC ABERRATIONS, GENE EXPRESSION PROFILE AND ANTIGEN EXPRESSION

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Background. The t(11;14)(q13;q32) is considered a marker of mantle cell lymphoma (MCL), while the t(14;18)(q32,q21) is associated with follicular lymphoma. Both translocations have also been reported in other B-cell malignancies, including chronic lymphocytic leukemia (CLL). *Aims and Methods.* We evaluated immunophenotypic as well as genetic characteristics occurring in association with t(11;14) and t(14;18) in order to define specific entities. 51 B-cell lymphoproliferative cases with t(11;14) and 26 with t(14;18) were studied by chromosome banding, FISH, immunophenotyping and gene expression analyses (HG-U133+ 2.0, Affymetrix). *Results.* Based on immunophenotyping 13 of 26 t(14;18)+ cases were classified as NHL and 13 cases as CLL (9 CLL, 4 CLL/PL). The mean number of cytogenetic aberrations in addition to t(14;18) was 1.1 in CLL cases and 4.2 in NHL cases (p=0.016). A complex karyotype (≥3 aberrations in addition to t(14;18)) was identified in 9 of 13 B-NHL cases (69.2%), however, in none of the 13 CLL cases (p=0.0002). In CLL the only additional recurring aberrations were +12 (n=7) and -13q (n=5). Additional aberrations in B-NHL cases were +1q (n=3), -4q (n=2), -6q (n=5), +7 (n=3), +12 (n=4), -15q (n=2), +der(18)t(14;18) (n=2), +21 (n=2), +22 (n=2), and +X (n=2). Notably, deletion 13q was not detectable in t(14;18)+ B-NHL. Two antigens were significantly higher expressed in t(14;18)+ B-NHL: CD10 (mean positivity (mp) 34.9% vs. 1.7%, p=0.006), and CD38 (60.9% vs. 29.8%, p=0.014), whereas 3 antigens were significantly lower: CD11c (38.6% vs. 14.5%, p=0.005), CD23 (66.6% vs. 24.0%, p=0.001), and CD5 (80.8% vs. 29.5%, p<0.001). Gene expression analysis identified 28 significantly differentially expressed genes. In cases with t(11;14)+ 39 of 51 cases were classified as NHL and 12 were classified as CLL (3 CLL, 9 CLL/PL). The mean number of aberrations in addition to t(11;14) were 2.1 in CLL and 5.8 in NHL cases (p=0.011). Whereas 26 of 39 NHL cases (67%) revealed a complex karyotype, only 3 of the 12 CLL cases (25%) did (p=0.011). In CLL the only additional recurring aberrations were +3q (n=3), +12p (n=2), -13q (n=2), +15q (n=2), -17p (n=4) and in NHL (observed in at least 5 cases): -1p (n=8), +3q (n=20), -6q (n=5), -8p (n=11), +8q (n=12), -9p (n=8), -9q (n=8), +11q (n=8), -11q (n=9), -13q (n=11), +13q (n=8), +15q (n=5), and -17p (n=13). CD23 was significantly higher in t(11;14)+ CLL as compared to t(11;14)+ B-NHL: (mp 48.8% vs. 20.8%, p=0.0001). CD22 was significantly higher expressed in t(11;14)+ B-NHL (mp 72.3% vs. 56.9%, p=0.036). However, gene expression profiling did not identify significant statistical differences in gene expression. *Summary.* t(14;18)+ CLL were characterized by a low number of chromosome aberrations and a higher expression of CD11c, CD23, and CD5. In contrast, t(14;18)+ NHL frequently demonstrated a complex karyotype and a higher expression of CD10 and CD38. Differences in gene expression were observed. Although, t(11;14)+ CLL were characterized by a lower number of additional chromosome aberrations as compared to t(11;14)+ NHL and a higher expression of CD23 and a lower expression of CD22, no global differences in the gene expression profile were identified.

0055

ASSOCIATION OF INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION RESPONSE CRITERIA WITH CONVENTIONAL METAPHASE CYTOGENETICS IN CML PATIENTS ON THERAPY

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Background. The Philadelphia (Ph) chromosome and its molecular equivalent, the BCR-ABL fusion gene, represent the pathogenetic cause and a useful target for detection and follow up monitoring of chronic myeloid leukemia (CML). Cytogenetic analysis of bone marrow metaphases (Cy) has been established as the standard method. In contrast, interphase fluorescence in situ hybridization (IP-FISH) has been increasingly applied in many studies due to recent optimization of the technique but is not represented in current treatment guidelines. The aim of our study was to define IP-FISH response criteria which correspond best with complete and major cytogenetic responses. *Methods.* In order to quantitatively compare results of both methods 1,750 bone marrow

samples from 748 CML patients at different stages of CML were analyzed in parallel with Cy and IP-FISH. 5 patients with known Ph negative/BCR-ABL positive CML were excluded and not considered for analysis. These patients can be monitored by BCR-ABL FISH on metaphases or interphase nuclei only as conventional cytogenetics is not informative. 643 patients in chronic phase were analyzed during treatment with an imatinib based therapy, ten patients received interferon α (IFN). 74 patients at different stages of the disease received a therapy with second generation tyrosine kinase inhibitors: nilotinib, n=18 (chronic phase n=13, accelerated phase n=2, blast crisis n=3); dasatinib, n=56 (chronic phase n=41, accelerated phase n=4, blast crisis n=11). 21 patients received no therapy or the therapy was not evaluable. The correlation between Ph positive metaphases and the proportion of FISH positive interphase cells was determined by using the Spearman´s rank correlation coefficient. The chi-square test was used to compare IP-FISH and Cy data. We calculated probability levels for different threshold values of IP-FISH response groups using the minimum p-value approach. The optimally separating threshold value was chosen as cut-off point. *Results.* Cy and IP-FISH data showed a good correlation (r=0.87; $p<0.0001$). The following cut-off values were defined: ≤30% interphase positive cells by FISH was found to correspond best with major cytogenetic response (MCyR; ≤35% Ph^+ metaphases by Cy); ≤6% interphase positive cells by FISH revealed to be concordant with complete cytogenetic response (CCyR; 0% Ph^+ metaphases; Table 1). 96.2% of samples with ≤30% IP-FISH positive cells had a major cytogenetic response. Of 1,163 samples in CCyR, 99.3% showed a percentage of ≤6% IP-FISH positive cells. 82 of 1,163 samples (7.0%) with 0% Ph^+ metaphases by Cy were IP-FISH positive (median 3%, range 1-21% positive interphases). IP-FISH showed false negative results in 10 of 1,090 samples (0.92%) with a median of 8% Ph^+ metaphases (range 4-40%). In conclusion, IP-FISH data are comparable with metaphase cytogenetics but the cut-off points differ. The prognostic value of IP-FISH data should be analyzed in prospective controlled studies.

Table 1.

	FISH positive interphases	
	0-30%	**>30%**
MCyR (0-35% Ph+ metaphases)	n=1,331	n=8
no MCyR (>35% Ph+ metaphases)	n=52	n=359
	FISH positive interphases	
	≤6%	**>6%**
CCyR (0% Ph+ metaphases)	n=1,155	n=8
no CCyR (>0% Ph+ metaphases)	n=88	n=499

0056

FREQUENT MONITORING OF WILMS' TUMOR GENE EXPRESSION LEVELS IN PERIPHERAL BLOOD FOR EARLY DIAGNOSIS OF ACUTE LEUKEMIA RELAPSE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Wilms' tumor gene WT1 expression levels have been extensively studied using bone marrow (BM) as the most common source for assessment of minimal residual disease (MRD) in acute leukemia. Aims. We examine whether peripheral blood (PB) is available as an alternative source for MRD assessment or not. *Methods.* Between April 1994 and December 2001, 44 transplants for 41 patients with acute leukemia were performed with BM or peripheral blood stem cells at Osaka University Hospital. We obtained PB samples once every one to three weeks from day 20-400 after transplant. WT1 expression levels in PB were measured by real-time reverse-transcription polymerase chain reaction methods The WT1 expression level in K562 cells was defined as 1.0 and serial dilutions of K562 cDNA were used for construction of standard curves. Results. As a background level in PB, we defined WT1 levels of less than 2×10^{-4}, based on the expression in samples of 24 healthy volunteers and leukemia-specific chimeric-gene-negative samples from 4 patients who had the minor bcr-abl or AML1-MTG8 chimeric gene. If WT1 levels increased to 2.0×10^{-4} or more, we would obtain PB samples once or twice per week until WT1 levels decreased to less than 2.0×10^{-4} or patients relapsed. Of 33 patients who are in CR, 18 are alive at a median follow-up of 2117 days (range, 909-3709 days), 15 died of transplant-related mortality at a median follow-up of 332 days (range, 52-551 days). In 324 of 352 PB samples (92.0%) obtained from 33 patients who are in CR, WT1 levels distributed less than 2.0×10^{-4}. In only 28 of 352 PB samples (8.0%), WT1 levels ranged from 2.0×10^{-4} to 2.0×10^{-3}, but returned ultimately within the background level ($<2.0\times10^{-4}$). On the other hand, hematologic relapse ultimately occurred on 11 transplants at a median time to relapse of 157 days (range, 43-535). In PB samples within 120 days before relapse of 9 transplants, WT1 levels increased over the background level at a median of day -41 (range, -106 to -14), and then increased to more than 2.0×10^{-3} at least once before relapse. In the remaining 2 transplants, WT1 levels of more than 2.0×10^{-3} were observed only at the time of relapse, although WT1 levels showed 1.4×10^{-3} and 7.6×10^{-4} over the background level, on day -7 and -21 before relapse, respectively. Furthermore, based on kinetics of WT1 levels prior to relapse, we found the following two relapse patterns: a rapid increase in a doubling time of less than 7 days, and a slow increase in that of 7 days or more. *Conclusions.* WT1 levels in PB of more than 2.0×10^{-4} suggested the presence of MRD after transplants. Patients with WT1 levels in PB of more than 2.0×10^{-3} ultimately relapsed, and thus we need frequent monitoring of WT1 levels in PB until they decrease to the background level. In particular, for early detection of impending relapse with a rapid increase in a doubling time of less than 7 days, we need blood sampling for WT1 assessment once or twice weekly.

0057

DNA METHYLATION PROFILES OF DIFFERENT LYMPHOID MALIGNANCIES

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Background. There is increasing evidence that, in addition to genetic aberrations, epigenetic processes play a major role in carcinogenesis. Particularly, aberrant methylation of promoter CpG islands is known to be a major inactivation mechanism of tumor-related genes. Studies in different tumor types suggest that specific tumors may have their own distinct patterns of methylation. *Aims.* To compare the methylation profile of tumor suppressor genes (TSG) in multiple myeloma (MM), chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) patients. *Methods.* We have evaluated the methylation status of p15INK4b, p16INK4a, p14ARF, SOCS-1, p27KIP1, RASSF1A and p73 (TAp73 isoform) TSG genes in newly diagnosis patients with CLL, MM and MCL. Forty-four CLL patients (23 males; median age: 65 years); 44 MM (21 males; mean age: 67 years) and 6 MCL (2 males; median age: 61 years) were analyzed. All patients gave informed consent and the study was approved by the local Ethics Committee. Peripheral blood (PB) samples from 10 normal individuals and CpGenome Universal Methylated DNA (Chemicon International) were used as negative and positive controls, respectively. DNA was extracted from bone marrow cells (BM) of MM patients, PB of LLC patients and controls and BM or lymph nodes of MCL using phenol/chloroform method. Methylation status was performed using Methylation Specific PCR technique and DNA sequencing. The methylation index (MI; ratio between the number of genes methylated and the number of genes analyzed) was also calculated. *Results.* All pathologies showed TP73 and p15INK4bgenes methylated. TP73 showed similar percentages in both CLL and MCL (70% and 67%, respectively) and a lower frequency in MM (45%). The frequencies of p15INK4b were: 67% for MCL, 32% for MM and 16% for CLL. SOCS-1 and p14ARFgenes methylation were more frequent in MCL (83% and 100%) than MM (52% and 29% of cases, respectively) and, both genes lacked methylation in CLL patients. Low frequencies were observed for p16INK4a (4%, 7% and 0%) and RASSF1A genes (0%, 2% and 0%) for CLL, MM and MCL, respectively. All patients lacked methylation at p27KIP1 gene. None of the target genes were methylated in normal samples. Our MSP results were confirmed by sequencing analysis. In LLC the MI ranged from 0 to 0.29, with a median de 0.14, corresponding to one gene/sample. In MM the MI ranged from 0 to 0.71, with a median de 0.28, corresponding to two gene/sample while in MCL the median MI was 0.43 (range: 0.29-0.57), corresponding to three genes/sample. *Conclusions.* Highest frequencies of methylation for MCL and lowest levels for CLL were observed. CLL had a strong association with TP73 methylation. The perturbation of cytokine signalling via silencing of SOCS-1 gene seems to be important in both MCL and MM. The comparison of the profiles of this three tumor types showed important similarities and

differences, suggesting individual patterns of methylation. These results indicate that epigenetics would have a different value in the pathogenesis of these entities.

0058

TET2 DELETION IS A COMMON DENOMINATOR IN HAEMATOLOGICAL PATHOLOGIES WITH 4Q24 REARRANGEMENTS

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Background. Chromosome deletions, the most frequent copy number changes in MDS and AML, produce diverse molecular events, such as gene fusion, loss of microRNA, and tumour suppressor gene inactivation or haploinsufficiency, most of which remain to be characterized. *Aims.* To characterise the 4q rearrangement in 9 patients with haematological diseases. *Methods.* G-banded metaphases were karyotyped according to ISCN (1995). To study del(4q), fluorescence in situ hybridization (FISH) was performed with 22 DNA clones mapping from 4q21 to 4q26. Patient materials included bone marrow, peripheral blood (PB) cultured with PHA, $CD3^+$ PB lymphocytes, and buccal mucosa cells at diagnosis and during disease evolution. FICTION studies (Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms) used clone RP11-16G16 labelled in green (Spectrum green, Vysis) combined with anti-CD34, -CD133, -CD13, -CD33, -CD14, -CD19, -CD20, -CD7, and-CD3 antibodies labelled in red (CY3, Jackson-Immunoresearch/ListarFISH, Milano, Italy). Exon 6 TET2 mutations were analysed by DHPLC and direct sequencing using the following primers: TET2_EX6FOR 5'- CTTATCTGCTGCAAGTACC-3' and TET2_EX6REV 5'- CACGCTGAACTCTCTTCCTT-3'. *Results.* We collected 2 cases of CMML and 1 of AML with interstitial 4q deletions and 6 with translocations (1 severe transient pancytopenia; 1 persistent therapy-related leucopenia without bone marrow dysplasia; 1 CMML, 3 AML). FISH analysis identified a 4q deletion at the translocation breakpoint in all 6 cases and identified a common deleted region of about 75kb at band 4q24, corresponding to clone RP11-16G16, despite of varying deletion sizes. The common deleted region contains only TET2, a putative tumour suppressor gene. In the patient with severe transient pancytopenia constitutional karyotype suggested mosaicism, confirmed by FISH which detected del(4)(q24)/TET2 on PB, $CD3^+$ lymphocytes and cells from buccal mucosa. In 1 patient with AML FICTION demonstrated $CD34^+$ cells as well as myeloid, T- and B-cell lymphoid lineages carried del(4)(q24). Wild-type TET2 exon 6 was found in 7/9 cases. Additional karyotype or cryptic changes were found in 5/9 cases, del(5q) was present in four. *Summary and Conclusion.* In benign and malignant haematological pathologies we demonstrated for the first time that: del(4)(q24) involves TET2, a member of a well-conserved protein family with unknown functions, often at the 4q24 translocation breakpoint; no concomitant second allele mutations at TET2 exon 6 were present; del(4)(q24)/TET2 targets $CD34^+$ haematopoietic cells in AML. As it occurred as a germline event in the patient with transient pancytopenia who has not developed haematological malignancies it remains to be determined whether and how it plays a causative role in disease pathogenesis.

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0059

COMBINATION OF FT-CGH AND LM-PCR ALLOWS MOLECULAR CHARACTERIZATION OF CHROMOSOMAL ALTERATIONS IN T-ALL CASES

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Background. Chromosomal abnormalities, like deletions, amplifications, inversions or translocations play a key role in malignant transformation. However, only a portion of these abnormalities has been clarified down to the molecular level so far. The precise clarification of such genomic alterations in leukaemia allows identification of genes involved in malignant transformation. Furthermore, this information can be useful for monitoring of the disease progression. *Aims.*In this study combination of fine tiling comparative genomic hybridization (FT-CGH) and ligation mediated PCR (LM-PCR) was used for cloning and molecular characterization of novel chromosomal breakpoint regions and gene rearrangements in T-cell acute lymphoblastic leukaemia (T-ALL). *Methods.* The TCRA/D split was shown previously by fluorescent in situ hybridization (FISH) in 68% of malignant cells suggesting an inv(14)(q11q32). Since chromosomal translocation involving the TCRA/D locus are usually accompanied by DNA losses due to V(D)J recombination, a custom fine-tiling oligonucleotide array of 385,000 oligonucleotides (NimbleGen) covering 24 Mb of different genomic areas, including the T-cell receptor α/δ locus (TCRA/D) on 14q11.2, was designed for comparative genomic hybridization (CGH). All DNA losses within TCRA/D were further analyzed by LM-PCR. *Results.* FT-CGH analysis revealed several mono- and biallelic deletions within the TCRA/D locus between positions 21,635 K, 21,976 K, 21,989 K and 22,093 K and were further analyzed by LM-PCR. Using a sets of nested primers located at the borders to the deleted regions amplification products differing from the germline control were obtained. Sequence analysis of these non-germline LM-PCR products revealed one physiological TCRD rearrangements between TRDV1 and TRDJ1 (position 21,635 K and 21,989 K). In addition sequencing of atypical fragments of the LM-PCR products obtained within the breakpoint at 21,976 K revealed the inversion of chromosome 14 with the breakpoint at sequence 21,977,838 of 14q11 together with the sequence of 105,948,661 of 14q32 in the IGH locus. LM-PCR analysis of the remaining breakpoint of the TCRA/D locus at position of 22,093 K with reverse gene specific primers revealed a translocation 14q11 at position 22,092,696 joined together with IGHV4-61 at 106,166,169 of 14q32.33. By this chromosomal aberration genomic regions of about 114,858 bp within the TCRA/D locus and 217,508 bp of the IGH locus (105,948,661-106,166,169) were deleted. The identification of this inv14(q11q32) confirmed the split observed with fluorescence in-situ hybridization (FISH) probes for the TCRA/D locus in 68% of cells. *Summary and Conclusions.* We show that the combination of FT-CGH and LM-PCR allows the amplification and molecular characterization of gene rearrangements and chromosomal translocation in patient samples with limited percentage of malignant cells. Furthermore, complex genomic alterations which are not visible using conventional cytogenetic procedures can be unraveled. This precise information can be used for minimal residual disease monitoring and most importantly may help to better understand the pathophysiology of T cell leukaemia.

0060

AN ACCURATE MLPA SCREENING METHOD FOR MLL-PTD DETECTS A LOW FREQUENCY OF MLL-PTD IN PEDIATRIC ACUTE MYELOID LEUKEMIA

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Background. Mixed-lineage leukemia (MLL)-partial tandem duplications (PTD) are found in 3-5% of adult acute myeloid leukemia (AML) samples, and are associated with a poor prognosis. In adult AML, MLL-PTD is either detected as sole abnormality or in conjunction with trisomy 11 or FLT3-ITD. Until now, studies in pediatric AML are scarce; reported large differences in frequency of MLL-PTD; and are often based on RT-PCR, which can give false-positive results. *Aim.* Retrospectively, a cohort of 286 children with AML was screened for MLL-PTD using multiplex ligation-dependent probe amplification (MLPA), which is a method to detect copy number differences of specific sequences. MLPA is less laborious as compared to genomic RT-PCR, and therefore suitable for screening a large number of samples. Moreover, this may resolve the problem of false positive cases when using RT-PCR. *Method.* We designed a reaction mix for MPLA-analysis containing probes for exon 2 to 13 of MLL for MLL-PTD detection and exon 17 of MLL as internal control. A probe in the serpinB2 gene was used as external control. If possible, screening was also performed for MLL-PTD transcripts with RT-PCR (n=226). The method is currently validated on an independent cohort of adult AML, for whom Southern Blot was performed to detect

MLL-PTD. *Results.* We detected MLL-PTD in 7/286 patients (2.4%), indicating a low frequency in pediatric AML. In these patients MLL-PTD transcripts were also present. Moreover, MLL-PTD transcripts were detected in 7 patients with an MLL-rearranged AML, but without evidence for MLL-PTD using MLPA, hence they were considered false-positive results. This was not encountered in the other 219 AML samples screened with RT-PCR without MLL-rearrangement. Three patients had normal cytogenetics; 1 patient had a trisomy 11, while for 3 patients no conventional cytogenetic data were available. Moreover, in 4 patients a FLT3-ITD was detected, in 1 other patient a FLT3 tyrosine kinase domain mutation, and in another patient a mutation in NRAS. MLL-PTD was not related to sex. The median age of patients with MLL-PTD was 7.5 (4.8-18) years and the median white blood cell count 95×10^9/L (44-170). Survival analysis was restricted to the subset of patients treated according to uniform DCOG and BFM treatment protocols (n=184). In this cohort, patients with MLL-PTD (n=5) had similar 3-years event-free survival rates (pEFS) compared to patients without MLL-PTD (40 vs. 44%, p=0.98). *Summary and Conclusions.* In conclusion, the frequency of MLL-PTD in pediatric AML has probably been overestimated in earlier studies. In this study we used MLPA as an accurate stand-alone screening method for MLL-PTD on the genomic level, which revealed a frequency of only 2.4% in pediatric AML.

0061

FLOW CYTOMETRIC DETECTION OF NG2 ANTIGEN FOR EARLY IDENTIFICATION OF CHILDHOOD ACUTE LEUKEMIAS WITH MLL GENE REARRANGEMENTS

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Background. Pediatric acute leukemias with MLL gene rearrangements form a distinct group characterized by specific biology, high clinical aggressiveness and frequently serious prognosis. Split-signal fluorescent in situ hybridization (FISH) is currently gold standard for the detection of all rearrangements to breakpoint cluster region of MLL gene on chromosome 11q23. However, this investigation requires special laboratory settings and the results are usually obtained within several days. Data from the literature indicate that surface expression of NG2 antigen on leukemic cells as detected by flow cytometry might predict the presence of MLL gene rearrangements. Such flow cytometric results are readily available immediately at diagnosis. *Aims.* Evaluation of flow cytometric detection of NG2 antigen expression as a surrogate marker of the MLL gene rearrangements in pediatric acute leukemias. *Patients and Methods.* The study group consisted of 247 consecutive children with acute leukemias treated at the centers of Polish Pediatric Leukemia and Lymphoma Study Group (PPLLSG), including 207 pactients with acute lymphoblastic leukemias (ALL) and 40 patients with acute myeloid leukemias (AML). In all patients, bone marrow or peripheral blood slides obtained at initial diagnosis were analyzed with MLL split-signal FISH. In parallel, expression of NG2 antigen on blast cells was determined with multicolor flow cytometry using monoclonal antibody 7.1. *Results.* MLL gene rearrangements were found with split-signal FISH in 24 patients with *de novo* acute leukemias (9.7%). MLL was rearranged in 17 of 207 patients with ALL (8.2%). This incidence is comparable to data obtained in European and American studies. Interestingly, MLL aberrations were more frequent in AML patients occurring in 7 of 40 cases (17.5%). Such frequency is higher than usually described in literature (approximately 10%). NG2 antigen expression was found in 25 acute leukemia patients (10.1%), including 18 patients with ALL (8.6%) and 7 AML patients (17.5%). The results obtained with FISH and flow cytometry were largely overlapping. In ALL patients, the sensitivity of MLL gene rearrangement detection via flow cytometric NG2 expression assessment reached 82%; specificity of the test was very high (98%), with positive predictive value of 77% and negative predictive value of 98%. In AML patients, the sensitivity of NG2 test reached only 43%, with specificity of 88%, positive predictive value of 43% and negative predictive value of 88%. *Conclusions.* The flow ctometric assessment of NG2 antigen expression is highly specific and useful screening test for MLL gene rearrangements in pediatric ALL.

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0062

DELETION OF 14Q INVOLVING IGH GENE IN PATIENTS WITH MULTIPLE MYELOMA

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Chromosomal translocations involving the IGH locus on 14q32 are one of the most frequent chromosomal abnormalities in patients with multiple myeloma (MM). Recent publications have shown that IGH in B-cell malignancies can be also involved in deletions affecting chromosome 14. Deletion involving IGH gene can be divided into 3 categories detected by FISH: whole IGH deletion, deletion of IGHV part (telomeric) of IGH and deletion of 3´IGH flanking (centromeric) sequences. Deletions including telomeric part of IGH gene are usually results of somatic VDJ recombination of IGH sequences. It has been suggested that deletions of 3´IGH flanking sequences can occur through deletions of whole der(14) soon after translocation affecting IGH gene, especially t(4;14) or as a results of interstitial del(14q). The aim of the present study was to determine frequency and type of IGH deletion using molecular cytogenetic methods and to correlate these findings with other cytogenetic abnormalities in our cohort of 200 MM patients. We detected deletions involving IGH gene in 42 (21%) patients using FICTION method with LSI IGH probe (Abbott-Vysis, Downers Grove, IL, USA). The deletions were grouped into 3 categories: 1. Deletion of whole IGH (15 cases); 2. IGHV deletion (14 cases); 3. Deletion of 3´IGH flanking sequence (13 cases). IGH deletions were found together with RB1 deletion in 29 out of 42 patients. Monosomy of chromosome 13 was confirmed in all patients with whole IGH deletion and RB1 deletion (12 patients). In 11 patients with whole IGH deletion the monosomy of chromosome 14 was proved by using 14qTEL specific Probe (Abbott-Vysis) and TCR A/D (14q11) (Dako, Stockholm, Sweden). Terminal deletion of (14q) not involving TCR A/D locus was confirmed in 4 patients. The translocation was proved in 7 out of 13 patients with 3´IGH deletion, in 5 patients t(4;14) and in another 2 patients t(11;14). Using CGH/arrayCGH deletion of 14q involving 3´IGH flanking sequence was proved in 2 patients. Finally, our data confirmed that deletions involving IGH were detected in 21 % patients in our cohort of 200 MM patients and should be considered as a marker of monosomy 14, translocation involving IGH gene or deletion of 14q. Deletions involving IGH most often coexist with monosomy 13 or RB1 deletion.

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0063

A NOVEL CHROMOSOMAL TRANSLOCATION T(11;14)(Q24.1;Q32) INVOLVING IGH IN CHILDHOOD B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKAEMIA

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Rearrangements involving IGH gene on chromosome 14q32.3 are well known in mature B-cell malignancies and have been more recently described in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL). IGH translocations are usually reciprocal and bring genes on other chromosomes into close apposition with the IGH locus, where their expression is deregulated due to the presence of potent B-cell-specific transcriptional enhancers. A two-year-old girl was diagnosed with an ALL common type and treated according to AIEOP-ALL-00 Protocol. Death occurred after four months due to haematological toxicity. Cytogenetic analysis of PB and BM blasts revealed a t(11;14)(q24-32;q32). FISH analy-

sis with IGH break-apart probe confirmed the rearrangement of the IGH locus between chromosomes 11 and 14. Cloning by LDI-PCR localized the breakpoint on chromosome 11q24.1 within the intronic region 1 of BC089451, a non coding gene. Quantitative real-time PCR showed over-expression of BLID mRNA, located 14Kb downstream the BC089451 gene. BLID codes for a protein containing a BH3-like domain essential for apoptosis. FISH studies performed with 11 close BACs to confirm the breakpoint junction identified a 585Kb deletion on der(11), with complete SORL1 loss. SORL1 controls intracellular trafficking of amyloid precursor protein and is causally linked to the pathogenesis of Alzheimer disease. Moreover SORL1 is down-regulated in high-grade astrocytoma. Deletion of SORL1 could play a role in leukemogenesis, like the IGH-ID4 association in the t(6;14)(p22;q32) with PAX5 and CDKN2A deletions. The functional consequence of BLID over-expression due to IGH enhancer juxtaposition is currently unknown. Mutational analysis of BLID BH-3 like domain is ongoing. The translocation to the Ig locus may result not only in deregulated expression of the incoming oncogene, but also in mutations due to the action of the Ig somatic hypermutation mechanism. In our case, a mutation of BLID in BH3 domain could result in a protein not inducing apoptosis.

0064

K-RAS MUTATIONS IN CYTOGENETICALLY NORMAL AML PATIENTS

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RAS genes encode a family of membrane-associated G-proteins which are important for the transduction of receptor signalling into cellular processes such as proliferation, differentiation and apoptosis. Transforming mutations in the three functional RAS genes - H-RAS, K-RAS and N-RAS - cause constitutive activation of the RAS proteins, and have been identified in many types of human cancers, including hematologic malignancies such as acute myeloid leukemia (AML). AML is a genetically heterogeneous disease and karyotype analysis allows classification of clinically distinct leukemia subtypes. Almost half of AML patients are classified as cytogenetically normal (CN-AML), and prognostically significant mutations identified in this group of leukemias allow further sub-classification, which is important for risk-directed therapeutic intervention. The aims of this study were to investigate the incidence and influence of activating K-RAS mutations on clinical outcome in CN-AML patients. Following institutional guidelines in accordance with the Declaration of Helsinki, patient samples were collected upon informed consent. Primary leukemia cells were obtained from peripheral blood or bone marrow aspirates from patients (<60 years old), mononuclear cells were purified by Ficoll-Hypaque gradient centrifugation, and samples were evaluated by cytomorphology, cytochemistry, multiparameter flow cytometry, cytogenetics, fluorescence in situ hybridization, and molecular genetics in parallel. Cytogenetic R-banding analysis was accomplished and only patients with a normal karyotype upon chromosome-banding analysis were included in this study. K-RAS sequencing was accomplished by K-RAS-specific amplification of cDNA prepared from total RNA and mutations were confirmed by directly sequencing patient chromosomal DNA. Activating K-RAS mutations were detected in 3 of 143 (2.1%) CN-AML samples and occurred concomitantly with nucleophosmin-1 (NPM1) exon 12 mutations but did not overlap with N-RAS mutations, FLT3 internal tandem duplications (FLT3-ITD), or MLL partial tandem duplications (MLL-PTD). All K-RAS mutations were observed in codons 12 or 13, and no non-canonical mutations were identified. Interestingly, the one K-RAS$^+$/NPM1$^+$ patient who underwent autologous bone marrow transplantation had no relapse and is still alive after more than 2400 days from diagnosis. The two K-RAS$^+$/NPM1$^+$ patients who did not receive bone marrow transplantations following induction therapy both relapsed after 330 and 391 days, respectively. In CN-AML patients with NPM1 mutations, the benefit of bone marrow transplantation has been shown to be restricted to the subgroup of patients with the prognostically adverse genotype FLT3-ITD. Our findings suggest that CN-AML patients with activating K-RAS mutations may compose an additional sub-group of NPM1$^+$/FLT3-ITD- CN-AML patients who might benefit from bone marrow transplantation.

Cellular immunotherapy and vaccination

0065

TARGETING LEWIS Y-EXPRESSING MULTIPLE MYELOMA AND ACUTE MYELOID LEUKEMIA WITH GENE-MODIFIED T CELLS DEMONSTRATING MEMORY PHENOTYPE

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Background. AML and MM are sensitive to immune control as evidenced by T cell mediated allogeneic graft versus leukemia/myeloma effect. Adoptive immunotherapy with gene-modified T cells has shown clinical activity in some solid tumors and B cell non Hodgkin lymphomas. The carbohydrate antigen Lewis Y (LeY) is a tumor associated antigen expressed on numerous epithelial cancers. *Aim*. Our aim was to generate gene-modified clinical-grade T cells directed against LeY-positive hematological malignancies. Moreover, we aimed to produce cells that possessed T cell memory, essential for *in vivo* T cell persistence and long-term control of tumor cell targets. *Methods and Results*. MM and AML cell lines were found to express differing levels of the LeY antigen ranging from negative (median fluorescence intensity (MFI) equal to mature lymphocytes (lymph) as internal control) to strongly positive (up to10xMFI lymph). Furthermore, 25/46 (54%) and 15/29 (52)% of primary MM and AML bone marrow samples were LeY-positive (≥5xMFI lymph), respectively. LeY-expression did not correlate with patient age, gender, clinical risk status, cytogenetic abnormalities, extent of previous chemotherapy, degree of bone marrow infiltrate, disease subtype, cytopenias in peripheral blood (MM and AML), LDH, WBC > or ″ 30×10^9/L (AML), β2 microglobulin, albumin or pretreatment with bortezomib, thalidomide or lenalidomide (MM). We developed a novel retroviral vector construct enabling efficient transduction of PBMC-derived T cells with resultant high expression (up to 65%) of a single-chain anti-LeY chimeric T cell receptor comprising T cell activation via CD3zeta and CD28 signaling domains. Under GMP conditions, we achieved >100-fold expansion of T cells using a 12 day culture protocol. Anti-LeY T cells lysed LeY-positive tumor cells *in vitro* while sparing LeY-negative control tumor cells and moderately LeY-positive neutrophils (>3xMFI lymph). Similar transduction rates were achieved in CD4 and CD8 T cell subsets. End-of-culture T cells showed low expression levels of CD45RA and CCR7, moderate levels of the co-stimulatory molecules CD27 and CD28, and active proliferation in response to IL-2 and IL-15, suggesting an effector memory phenotype. On re-exposure to LeY expressing tumor cells, anti-LeY T cells displayed active proliferation and IFN-gamma production. *In vivo* efficacy was demonstrated in three independent experiments of a MM xenograft mouse model showing improved disease free survival of mice receiving anti LeY T cells compared to control mice treated with non-transduced T cells. Transplantation of syngeneic murine anti LeY T cells into sublethally irradiated Balb/C mice subsequently monitored for up to two years was found to be safe without generation of lymphoproliferative disorders arising from the anti-LeY T cells and no impact on OS compared to irradiated control mice not receiving adoptive T cell transfer. *Conclusions.* Consequently, we are about to undertake a first-in-human phase I trial of autologous anti-LeY T cells for patients with LeY-expressing MM or AML. LeY is a promising and immunologically relevant target for T cell immunotherapy and our anti-LeY T cells are likely to demonstrate persistence in patients, an outcome which will be specifically addressed in our upcoming study.

0066

PEPTIDE VACCINATION ELICITS LEUKEMIA-ASSOCIATED ANTIGEN-SPECIFIC CYTOTOXIC CD8$^+$ T-CELL RESPONSES WITH POTENTIAL CLINICAL RELEVANCE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Results from bone-marrow transplantation, as well as remission phenomena after viral infection, suggest that CLL might be

targeted effectively by T-cell based immunotherapy. The receptor for hyaluronic acid mediated motility (RHAMM) is a tumor-associated antigen in chronic lymphocytic leukemia (CLL). $CD8^+$ T cells primed with the RHAMM-derived epitope R3, which is restricted by HLA-A2, effectively lyse $RHAMM^+$ CLL cells. *Aims.*We initiated a phase I clinical trial of R3 peptide vaccination and monitored immune responses against R3 epitope in patients with early stage CLL. *Methods.* Six HLA-A2+ CLL patients were vaccinated four times at biweekly intervals with the R3 peptide (ILSLELMKL; 300 μg/dose) emulsified in incomplete Freund's adjuvant; GM-CSF (100 μg/dose) was administered concomitantly. Detailed immunological analyses were conducted throughout the course of peptide vaccination including assessment of R3-specific T-cells by tetramer staining and ELISpot assays, evaluation of T-cell subsets which play a role in regulation of immune responses ($CD3^+CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ Tregs, Th17, $CD8^+CD137^+$, $CD8^+CD103^+$ and IL-17 producing $CD8^+$ T cells ($CD8^+IL\text{-}17^+$)) as well as assessment of TNF, TGFβ, IL-10 and IL-2 serum levels. *Results.* No severe adverse events greater than CTC I° skin toxicity were observed. Four patients exhibited reduced white blood cell counts during vaccination. In 5/6 patients, R3-specific $CD8^+$ T cells were detected with the corresponding peptide/ HLA-A2 tetrameric complex; these populations were verified functionally in 4/5 patients using ELISpot assays. In patients with clinical responses, we found increased frequencies of R3-specific $CD8^+$ T cells that expressed high levels of CD107a and produced both IFN-γ and granzyme B in response to antigen challenge. Interestingly, vaccination was also associated with the induction of regulatory T cells in four patients. We could find a correlation between the frequency of Tregs and activated $CD8^+CD69^+$ T cells. Interestingly, $CD8^+CD137^+$ cells correlated with $CD8^+IL\text{-}17^+$ T cells. Further, we noted a correlation between IL-2 concentrations in the serum and Treg frequencies. *Conclusions.* This study provides a proof of concept that peptide vaccination is safe, feasible and able to mount immunological responses even in the immunosuppressive environment of CLL. The manipulation of therapeutic schema as well as the definition of a group of patients who will mostly profit from this treatment could help to translate immunological responses to significant clinical benefit.

0067

EPIGENETIC MANIPULATION OF TUMOUR ANTIGEN EXPRESSION: A STRATEGY FOR SELECTIVELY UP-REGULATING THE GRAFT-VERSUS LEUKAEMIA RESPONSE IN PATIENTS ALLOGRAFTED FOR HAEMATOLOGICAL MALIGNANCIES

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Background. There is compelling evidence to suggest the curative effect of reduced intensity allografts in acute myeloid leukaemia (AML) and other haematologic malignancies is mediated by a graft-versus-tumour (GVT) immune reaction. Cancer testis antigens (CTA) represent a family of immunodominant proteins that are variably expressed in haematological malignancies and represent a potential target of a GVT response. Importantly a number of CTA genes demonstrate promoter hypermethylation in solid tumours which can be reversed using demethylating agents such as azacytidine. *Aims.*To determine whether donor T cell responses to CTAs are present in patients allografted for AML and multiple myeloma (MM) and whether epigenetic therapies can be used to manipulate T cell mediated killing of haemopoietic targets. *Methods.* We first screened 37 patients with AML and 8 with MM, who had not received demethylating agent treatment, for T cell responses to 25 peptides derived from 10 CTA genes, including BAGE, LAGE, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-C2, RAGE-1, by interferon-γ cytokine secretion assay (IFN-γ CSA). Subsequently, we studied the effect of epigenetic modifying reagents on the immunogenicity of tumour cell lines, primary tumours and normal primary fibroblast. We examined expression of 15 CTAs and costimulatory molecules important for T cell activation in AML, MM and Hodgkins' lymphoma cell lines before and after exposure to the demethylating agent azacytidine (Aza) and the histone deacetylase inhibitor sodium valproate (VPA), both as single agents and in combination. Finally we have used an interferon-γ ELISA assay to determine the effect of Aza and VPA on the recognition of target cells by CTA-specific T cells. *Results.* We found CTA specific T cells in 11.1% of patients (5 of 45) with frequencies ranging from 0.0005-0.2% (median 0.024%). We showed that expression of CTAs including HAGE, SACA3, SPANXB, PASD1 and SSX1 was induced in a dose dependent manner by Aza alone but not with VPA alone. We also observed that expression induced by Aza was further increased by combination treatment with VPA. Furthermore, we show increased expression of costimulatory molecules such as CD86 in AML and MM cell lines treated with either Aza or VPA. Induction of CTAs and costimulatory molecules was confirmed *in vitro* in primary AML cells and *in vivo* in AML patients on an Aza trial. The tumour-specific activity of Aza was confirmed in normal primary fibroblasts in which CTAs SPANXB, MAGEA4, HAGE and SSX1 were not induced. Finally we demonstrate that Aza-induced expression of the CTA MAGEA1 in MM cell lines was accompanied by increased recognition by MAGEA1-specific T cells. Consistent with the expression data, T cell recognition was increased further by combination treatment with VPA. *Conclusions.* These studies confirm the importance of members of the CTA family as targets of a T cell mediated immune response. Our data demonstrate that the expression of these putative immunodominant antigens in haematological malignancies in disease such as AML and myeloma, can be significantly and selectively up-regulated by epigenetic therapies with functional increases in target cell recognition. Our observations support the use of adjunctive epigenetic therapies after allogeneic transplantation with the aim of augmenting a GVL response.

0068

HIGH-DOSE RHAMM-R3 PEPTIDE VACCINATION FOR PATIENTS WITH ACUTE MYELOID LEUKEMIA, MYELODYSPLASTIC SYNDROME AND MULTIPLE MYELOMA

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Recently, we have demonstrated immunological responses and positive clinical effects of a peptide vaccination in ten patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and multiple myeloma (MM) overexpressing RHAMM using 300 μg RHAMM-R3 peptide. Here, we report the second cohort of nine patients with AML, MDS and MM vaccinated with a higher peptide dose of 1000 μg RHAMM-R3 peptide. The vaccine was given four times at a biweekly interval and GM-CSF was added for five days each vaccination. Similar to the patients vaccinated with 300 μg peptide only mild drug-related adverse events were observed such as erythema and induration of the skin. Immunological analysis were performed using ELISpot assays for Interferon gamma and Granzyme B, tetramer staining and chromium release assays. Moreover, regulatory T cells were quantified during vaccination. In this second cohort of patients treated with 1000 μg peptide we detected specific immune responses in a lower frequency (33%) in contrast to patients in the 300μg cohort (70%). In these patients with immune responses we found an increase of $CD8^+$/HLA-A2/RHAMM-R3 $tetramer^+/CD45RA^+/CCR7^-/CD27^-/CD28^-$ effector T cells in flow cytometry and an increase of R3-specific $CD8^+$ T cells in ELISpot assays. Two patients with positive immune responses showed a significant decrease of regulatory T cells. One patient without positive immune and clinical effects showed an impressive increase of the frequency of regulatory T cells (5.03-15.9%). Three patients treated with 1000 μg showed positive clinical effects. One patient with MDS RAEB 2 showed a reduction of leukemic blasts in bone morrow to lower than 5%, one MDS patient an improve of peripheral blood counts and one patient with multiple myeloma a reduction of light chain in serum. However, the patients in the 300 μg cohort showed a higher frequency of positive clinical effects. Taken together, RHAMM-R3 peptide vaccination induced both immunological and clinical responses. Therefore, RHAMM constitutes a promising structure for further targeted immunotherapies in patients with different hematological malignancies. However, higher doses of peptide do not improve the frequency and intensity of immune responses in this clinical trial.

0069

CLINICAL RESPONSES IN ALLOGRAFTED ACUTE LEUKEMIA PATIENTS WITH RESISTANT DISEASE USING A COMBINED CHEMO-IMMUNOTHERAPEUTIC TREATMENT STRATEGY

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Background. Chemotherapy manipulates the host milieu through different mechanisms including provision of lymphoid space, elimination of host anti-donor immune reactivity, suppression of regulatory T cells and induction of activating cytokines. Animal models have demonstrated that the serum peak concentration of activating cytokines is reached 48 hours after a chemotherapeutic treatment. We hypothesized that acute leukemia patients with resistant disease who have already undergone an allogeneic stem cells transplant (SCT) may benefit from donor lymphocyte infusions (DLI) performed closely after chemotherapy. *Aims.* Aim of this study was to analyze the immunologic and clinical effects of a chemo-immunotherapeutic treatment strategy consisting of chemotherapy followed 48 hours later by DLI in allografted acute leukemia patients. *Methods.* Four patients with acute myeloid leukemia evolved from a myelodisplastic syndrome and 1 with acute lymphoid leukemia, who had presented evidence of disease recurrence after an allogeneic SCT (1 from an haplo-identical donor, 2 from a matched unrelated donor [MUD] and 2 from a sibling), underwent 2 cycles of DLI (CD3=1×10^7/Kg of recipient body weight for haplo and MUD, and 1×10^8/Kg of recipient body weight for siblings) 2 days after a lymphodepleting chemotherapeutic treatment. These patients underwent cytofluorimetric analysis of the peripheral blood for CD3, CD3/CD4, CD3/CD8, CD3/CD4/CD25, CD16/CD56, CD20, CD7, CD38, HLA-DR and intracytoplasmic staining for γ IFN, α TNF, IL-2, IL-10 at days 0, 2, 5, 10, 20, 30 from DLI; two patients who underwent 2 cycles of standard DLI served as controls. *Results.* Analyses of the results demonstrated that from day 2 throughout the entire study period, patients who received DLI after chemotherapy showed a significant increase of activated elements, as documented by lymphocytes expressing the CD7/CD38/HLA-DR antigens, compared to controls (p=0.003); moreover, when considering the intracytoplasmic staining for specific cytokines, gamma IFN production was also superior for patients belonging to the study group (p=0.09). Four of the 5 patients who received DLI after chemotherapy presented for the first time a clinical picture of graft-versus-host disease (GVHD), although they had previously undergone many cycles of standard DLI; in these 4 patients, the onset of GVHD was associated to an hematologic complete remission (CR). The first three patients are now in persistent CR with a follow-up of 21, 7 and 2 months, respectively. The fourth patient obtained a CR, but died of pneumonia 2 months after the treatment. The fifth patient, who did not show any sign of GVHD, relapsed 4 weeks later. *Summary and Conclusions.* These observations suggest that donor lymphocytes may undergo a process of activation when infused into allografted patients who have received a chemotherapeutic protocol in the preceding 2 days; this seems to be associated with the onset of GVHD and with evidence of a clinical response. These results advocate a possible new chemo-immonotherapeutic strategy that can be taken into consideration for patients who have undergone an allogeneic SCT and have evidence of resistant/residual disease.

0070

RNA-MODIFIED DENDRITIC CELLS AS THERAPEUTIC VACCINES

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Background. Messenger RNA (mRNA)-based gene transfer has gained an important interest over the last decade, especially in the field of immuno-gene therapy of cancer. *Aims.* The aim is to prepare and to use immunostimulatory autologous dendritic cells (DC), the most professional antigen-presenting cells, as an adjuvant treatment for malignant hematological disease and for chronic infectious disease in an antigen-specific manner. *Methods.* In this area, most researchers have exploited low voltage electrical pulses (electroporation) as a means to introduce coding RNA into the cells. mRNA electroporation has become a method of choice for transfecting DC, given its superior cytoplasmic expression efficiency, its simplicity over viral transduction protocols and its clinical safety profile because of a strictly transient expression profile and the inability to integrate into the host genome. Furthermore, it allows the simultaneous introduction of antigens and immunostimulatory proteins into DC through co-electroporation of multiple mRNA sequences. Recently, optimized strategies to produce highly translatable mRNA and more insights into the immunostimulatory properties of RNA structures further advocates the use of RNA for vaccination purposes. *Results.* We are currently performing several phase I/II clinical trials using antigen RNA-electroporated DC in several disease models such as acute myeloid leukemia (AML), human immunodeficiency virus (HIV) infection and cytomegalovirus (CMV) reactivation or infection following allogeneic stem cell transplantation (allo-SCT). Feasibility, safety, immunogenicity and clinical effects were investigated. We have shown successful GMP-grade DC generation and vaccine production in AML patients in remission, in stable HAART-treated HIV patients and in allo-SCT patients. No serious adverse events or toxicity were observed upon vaccination. For AML patients in remission, we observed a molecular anti-leukemic response by a reproducible decrease in tumor marker by molecular minimal residual disease monitoring as well as a significant increase in tumor antigen-specific $CD8^+$ T-cell responses following DC vaccination. Feasibility and immunomonitoring data will be presented for the HIV and CMV trials. *Conclusions.* In conclusion, we provide evidence that RNA loading by electroporation provides a versatile gene therapy tool for the design of DC-based therapeutic vaccines in cancer and infectious diseases.

0071

IN VITRO GENERATION OF BOTH CONVENTIONAL AND UNCONVENTIONAL MATURE TCRAB CELLS FROM HUMAN HEMATOPOIETIC STEM AND PROGENITOR CELLS

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Background. The *in vitro* generation of mature T cells from human hematopoietic stem and progenitor cells (HSPC) could fulfill two existing needs. First, it could enhance and quicken T cell immune reconstitution after stem cell transplantation (SCT). Second, by generation of tumour antigen specific T cells it could provide an efficient therapy for numerous malignancies and could enhance GVT effect in the context of allogeneic SCT, without aggravating GVHD. When human HSPC are cultured on the murine stromal cell line OP9-transduced with the Notch ligand Delta-like-1 (OP9-DL1), they differentiate to T cells which can proliferate and produce interferon-γ upon polyclonal stimulation. The nature of the mature cells generated in these cultures, however, has not been well studied so far. *Aims.* As thymic epithelial cells (TEC) are not present is this co-culture system, and as they are thought to be the main cell type mediating positive selection of conventional TCRαβ cells, we investigated whether the T cell populations generated in OP9-DL1 cultures contain mature conventional TCRαβ cells, illustrating their positive selection. *Methods.* $CD34^+$ HSPC from postnatal thymus (PNT) or cord blood were cultured on OP9-DL1, in the presence of the cytokines Flt-3L (5 ng/mL), SCF (2.5 ng/mL) and IL-7 (5 ng/mL), and in some experiments IL-15. At repetitive timepoints, an aliquot of the cells was analysed phenotypically. Mature T cells were transferred to feeder cells, consisting of irradiated JY cell line and PBMC, in the presence of PHA (1 mg/mL). After 7 days, IL-2 (50 IU/mL) was added to the culture. Every 14 days, cells were restimulated. Before functionality assays, cells were stimulated overnight with PMA and ionomycin. Results Phenotypically mature $CD27^+CD1^-$ TCRγδ as well as TCRαβ cells were generated in OP9-DL1 cultures. Few mature $CD4^+$ single positive (SP) TCRαβ cells were observed. Mature $CD8^+$ SP cells co-expressed variable ratios of CD8αβ and CD8αα dimers, suggesting that the $CD8^+$ SP T cells consisted of two different cell populations. TCRαβ CD8αα and TCRγδ cells both expressed the IL-2Rβ receptor consitutively and both proliferated on IL-15 without prior TCR stimulation, a characteristic of unconventional T cells. These latter cells produced IFN-γ but virtually no IL-2 upon activation. In contrast, CD8αβ and CD4 TCRαβ cells were unresponsive to IL-15, but could be expanded upon TCR stimulation. These T cells had the characteristics of conventional $CD4^+$ (ThPOK, CD40L, high levels of IL-2 and IL-4 production upon stimulation) and $CD8^+$ (granzyme, perforin and IFN-γ production upon stimulation) T cells. *Conclusions.* We can conclude from these data that OP9-DL1 supports the development of both unconventional T cells and T cells with the characteristics and functionality of post-selection T cells. This suggests that a process similar to positive selection on TEC may be operative, although less efficient than in the thymus. We are currently investigating the *in vitro* anti-tumor capacities of both populations.

0072

IMMUNOSTIMULATORY LEUKEMIC DENDRITIC CELLS CAN EFFICIENTLY EXPAND LEUKAEMIA-REACTIVE T CELLS FROM HSCT DONORS

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Allogeneic hematopoietic transplantation (allo-HSCT) is the only curative option for patients affected by high-risk acute myeloid leukemia (AML). This is possibly due to the ability of allogeneic immune system to eradicate leukemic stem cells. In order to improve the efficacy of the allogeneic immune system against leukemia, we exploited the unique ability of myeloid blasts to differentiate into leukemic dendritic cells (LDC). We observed that a short (48h) exposure to calcium ionophore A23187 and IL-4 is able to induce LDC differentiation in a large proportion (86%) of *de novo* and secondary high-risk AML. Compared to original blasts, LDC significantly up-regulate molecules of the immunological synapse (CD86, CD80, HLA-DR, CD54 and CD58) while maintaining an intact leukemic antigenic (c-kit, CD34, WT1) expression profile. This possibly suggested a direct access of leukemic stem cells to the process. This favourable phenotype correlates with a high T-cell stimulatory capacity, similar to that of healthy mature DC. Most importantly, LDC proved to be superior to the original blasts in promoting the expansion of leukemia-reactive T-lymphocytes from related and unrelated HLA-identical and haploidentical donors. The extent of T-cell expansion directly correlated with the sensitivity of AML blasts to LDC differentiation. T lymphocytes stimulated with LDC in the presence of IL-7 and IL-15 were of central memory phenotype and reacted against the original leukaemia *in vitro* and *in vivo*. When infused in NOD/Scid mice, transplanted with the original leukaemia, LDC-stimulated T lymphocytes induced long-term (>18 weeks) complete remissions in the majority of mice, suggesting that this approach may be active against leukemic stem cells. These results show that functionally competent LDC can be generated from the majority of high-risk AML and may be used for an integrated allogeneic immunotherapeutic approach.

0073

IN VITRO ACTIVATION OF MYELOMA-SPECIFIC CYTOTOXIC T CELLS BY MUC1 PULSED DENDRITIC CELLS

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Background. Multiple myeloma is a hematological malignancy with weak immunogenicity and defective function of the immune system. An important issue for immunotherapy of myeloma is the identification of appropriate tumor-associated antigens. Recently, MUC1 was detected on a majority of myeloma cell lines. *Aim.* We studied antigen-specific and HLA-A2-restricted cytotoxic activity against MUC1-positive ARH77 myeloma cell line *in vitro*. *Methods.* A HLA-A2 specific MUC1-derived nonapetide (TSAPDTRPA) was used as a tumor-associated antigen. Dendritic cells (DCs) were generated from HLA-A2 positive PBMCs using GM-CSF and IL-4 and were matured with TNFα. Myeloma-specific cytotoxic activity of MUC1-reactive CTL was established by repeated stimulation of CTL via dendritic cells loaded with MUC1-derived nonapeptide. MUC1-reactive IFN-γ$^+$ T cells were sorted with immunomagnetic beads (MACS) and further expanded *in vitro*. Specific cytotoxicity of MUC1-reactive T cells against ARH77 myeloma cell line was evaluated. *Results.* The IFN-γ subset of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells after repeated stimulation via DCs reached 1.31±0.15% and 1.05±0.12%, respectively and after MACS enrichment reached 66.94±7.19% of IFN-g$^+$ CD3$^+$CD4$^+$ and 64.59±6.03% IFN-γ$^+$ CD3$^+$CD8$^+$ T cells. At a ratio 20:1 (effector:target cells) cytotoxicity of IFN-γ$^+$ T lymphocytes vs. ARH77 reached 17.16±5.13% compared to non-specific killing of allogeneic PBMC (negative control) which was 0.79±0.39%. At a ratio 40:1 IFN-γ$^+$ T cells were able to kill 36.14±6.51% of ARH77 MUC1-positive target cells while the cytotoxicity against allogenic PBMC remained negligible 0.77±0.38%. The differences between experimental and control groups were significant (p<0.01). *Conclusions.* We were able to demonstrate that MUC1-reactive T cells can be identified and expanded using a relatively simple *in vitro* techniques consisting of antigen-specific stimulation, immunomagnetic sorting and rapid expansion. In summary, MUC1-pulsed DCs could be a good candidate for future clinical vaccination trials.

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0074

CYTOKINE INDUCED KILLER CELLS: *IN VITRO* DIFFERENTIATED T CELLS WITH ANTI-TUMORAL ACTIVITY

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Background. Cytokine-Induced Killer (CIK) cells are CD3$^+$/CD56$^+$ positive cells obtained *in vitro* by stimulation of mononuclear cells with IFN-γ, monoclonal antibody anti-CD3 and IL2.They show considerable cytotoxic activity against leukemic cell lines and fresh samples and potent anti-tumoral activity *in vivo* in mice with little GVHD. CIK cells represent then a promising tool for immunotherapy of haematological malignancies. *Aims.*We aim to obtain a full ontogenetic and functional characterization of *in vitro* generated CIK cells to better understand their therapeutical potentiality in cancer patients. *Methods.* CIK cells were generated *in vitro* by stimulation of mononuclear cells or sorted T cell subsets with INF-gamma, anti-CD3 and IL-2. They were fully characterized in terms of phenotype, cytotoxic activity and gene expression in comparison with CD56$^-$ T cells and NK cells also present during the culture and with circulating CD3$^+$/CD56$^+$ cells. *Results.* By culturing sorted subpopulations we demonstrate that CIK derive only from proliferating CD3$^+$/CD56$^-$/CD8$^+$ T cell subset and not from the few CD3$^+$/CD56$^+$ cells present in the peripheral blood of normal o seem to be terminally differentiated and don't divide further. Purified CIK cells express high donors. CFSE proliferation assay show that the same CD3$^+$/CD56$^+$ cells generated in vitr level of NK markers CD56 and NKG2D, but lack expression of other NK specific activating (NKp30, NKp44, NKp46, CD16) and inhibitory (KIR2DL1, KIR2DL2, KIR3DL1, NKG2A, CD94) receptors. Similarly to NK cells, they present a large granular lymphocyte morphology and can kill K562 target in a 4 hours calcein-release assay. CIK cells, similarly to T cells present in culture, are mostly CD8$^+$, TCR α-β and show a polyclonal usage of Vβ chains. They are CD45RA$^+$, CCR7$^-$, CD62L weakly positive, CD11a$^+$, CD27$^+$, CD28$^-$ and then they can be classified as effector memory T cells. Also gene expression analysis confirme that CIK are more related to CD56$^-$ T cells compared to NK cells. In fact, among 1076 immune-related genes analysed, only 50 are deregulated in CIK cells compared to T cells instead 115 compared to NK cells. A lot of genes up-regulated in CIK cells compared to CD56- T cells confirmed also by immunophenotyping (MIP1α, Perforin, FasL, TNFα) are consistent with a inflammatory phenotype. Circulating CD3$^+$/CD56$^+$, contrary to *in vitro* expanded CIK cells, are oligoclonal, poorly cytotoxic for K562 also upon IL-2 stimulation and express lower levels of CD56 and NKG2D. *Conclusions.* CD3$^+$/CD56$^+$ CIK cells derive from proliferating CD3$^+$/CD56$^-$/CD8$^+$ T cells present throughout the culture. Gene expression and phenotypic analysis demonstrate that CIK cells are more related to T cells than NK cells. Differentely for T cells, CIK cells show cytotoxic activity against the target K562.CIK cells, except for NKG2D and CD56, which are not specific NK cell markers, do not express to a significant extent the other activating or inhibitory NK receptors. By virtue of the CD45RA, CCR7, CD27 and CD28 expression, in addition to Perforin, FasL and MIP1α CIK cells can be considerated as fully differentiated memory CD8 T cells. They may represent an even more advanced stage of *in vitro* differentiation of CD56$^-$ T cells.

Acute lymphoblastic leukemia - Clinical

0075

A NOVEL DASATINIB-SENSITIVE RCSD1-ABL1 FUSION TRANSCRIPT IN CHEMOTHERAPY-REFRACTORY ADULT PRE-B LYMPHOBLASTIC LEUKEMIA WITH T(1;9)(Q24;Q34)

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Background. Dasatinib is an oral broad-spectrum kinase inhibitor and predominantly targets BCR-ABL, SRC, C-KIT and PDGFR tyrosine kinases. It has been approved for clinical use for chronic myeloid leukemia (CML) and Philadelphia chromosome positive (Ph^+) acute lymphoblastic leukemia (ALL) patients carrying the 9;22 translocation and the resulting BCR-ABL1 fusion gene. In other subtypes of ALL, the clinical efficacy and putative molecular targets of dasatinib are unknown. *Aims.* The aim of this study was to characterize the molecular background of a clinical B-ALL entity characterized by a t(1;9) translocation and dasatinib sensitivity. *Methods.* Cytogenetic characterization of blood and bone marrow (BM) samples was done by G-banding and with FISH assay using 1p36 and 1q25 -specific probes, chromosome 9 painting and a BCR/ABL1 ES dual color extra signal translocation probe. Molecular characterization of the translocation was done with specific primers for RCSD1 and ABL1 using qualitative PCR. The PCR products were subsequently gel purified and sequenced in both directions. *Results.* The index patient is a 40-year old male patient with a chemotherapy-resistant pre-B ALL, who failed two consecutive induction therapies. G-banding indicated a balanced 1;9 translocation in leukemic blasts. FISH analysis suggested that the translocation occurs between ABL1 and an unknown gene located in chromosome 1q24-25. Based on the involvement of the ABL1 gene, the patient was started with dasatinib therapy (140 mg QD) and hematological remission was achieved 2 weeks later. After 4 weeks, patient was in complete cytogenetic remission which was maintained with dasatinib monotherapy until allogeneic BM transplantation was performed. A relapse occurred 1 year after transplantation, but was successfully re-treated with dasatinib. Molecular characterization of the translocation with specific primers covering the kinase domain of ABL1 gene (located in chromosome 9) and most of the exons of RCSD1 (located in the long arm of chromosome 1) yielded 2 positive PCR products with different molecular size. Sequencing revealed that the longer PCR product consisted of the 3 first exons of RCSD1 gene fused to ABL1 gene starting from exon 4. The shorter PCR product consisted of the first 2 exons of RCSD1 gene fused similarly to exon 4 of ABL1 gene. The predicted oncogenic product of the novel RCSD1-ABL1 fusion gene is in-frame and encodes the entire tyrosine kinase domain of ABL. *Conclusions.* In Ph^+ALL dasatinib is a novel promising treatment option. Results presented here suggest that dasatinib (and other tyrosine kinase inhibitors) may have clinical efficacy in other types of B-ALL as well. Although t(1;9)(q24;q34) translocation with RCSD1-ABL1 fusion gene is an uncommon subtype of acute leukemia, our results warrant the search for this novel fusion gene. It could be included in multiplex PCR panels commonly used in the routine diagnostic workup of acute leukemia enabling a rational and effective selection of optimal treatment modalities for each patient.

0076

LONG-TERM FOLLOW-UP OF HIV-INFECTED ADULT PATIENTS WITH BURKITT'S LYMPHOMA OR LEUKEMIA TREATED WITH INTENSIVE SPECIFIC CHEMOTHERAPY AND RITUXIMAB

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Background. A GMALL-derived PETHEMA trial (BURKIMAB) proved that rituximab could be associated with specific intensive chemotherapy to treat HIV-positive patients with Burkitt's lymphoma or leukemia (BL), with a survival similar to HIV-negative patients but significantly greater short-term toxicity (Cancer 2008;113:117-25). *Aims.* We aimed to study the impact of such immunochemotherapy on the clinical and immunologic status of HIV-positive long-term survivors. *Patients and Methods.* Twenty-four HIV-infected patients were included in the trial between July 2003 and December 2008. Treatment included a cyclophosphamide (Cy) and prednisone (PDN) five-day prephase, followed by six intensive 7-day cycles at 3-4 week intervals including rituximab in each cycle (plus two additional doses at the end of therapy), and several of the following: Cy, iphosphamide, vincristine, vindesine, dexamethasone, HD-MTX, HD-ARAC, teniposide, etoposide, and doxorubicin, together with intrathecal CNS prophylaxis with MTX+ARA-C+DXM. 14 patients (58%) achieved complete response and completed the scheduled treatment. All patients received HAART concomitantly to and after immunochemotherapy. *Results.* 14 patients, followed for more than one year (median 37 months, range 13-43), were selected for long-term evaluation of clinical and immunological status. 13 (93%) were males, with a median age of 37.5 (range 31-54). 3 (21%) were in immunologic and virologic response to HAART treatment at the time of diagnosis and all of them remained in the same situation after completing immunochemotherapy and during later follow-up. Four patients in immunologic response and with detectable viral load achieved and maintained a virological response after resuming treatment. Finally, 5/7 (71%) patients with uncontrolled HIV infection at diagnosis achieved and maintained both an immunologic and viral response after treatment. After a follow-up of 496 patient-years, no durable response losses have been reported, 4 HIV-associated infections were diagnosed including atypical mycobacterial infections (2), blastocystis enteritis and syphilis (one each) and one patient developed Kaposi's sarcoma. Up to date, no fatalities have occurred. *Conclusions.* Our results prove that specific immunochemotherapy for HIV-related BL/ALL3 has no significant long-term impact on the clinical and immunologic status, and is associated with a low incidence of HIV-associated events.

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0077

THE LONG-TERM OUTCOME OF ELDERLY PATIENTS WITH PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (PH+ALL) IN THE IMATINIB ERA

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Background. The use of imatinib combined with chemotherapy has improved the short-term outcome of elderly patients with Ph^+ ALL. However, few data are available on the long-term impact of tyrosine kinase inhibitors on survival. Aims. In this presentation, we update the survival data of a cohort of 30 patients treated from January 2003 to November 2004, with a median follow-up of 64 months. *Methods.* Patients with Ph^+ ALL, aged 55 years or older were treated according to the AFR09 protocol developed by the GRAALL (Leukemia 20;1526, 2006) which included a pre-phase with steroids, an induction treatment with chemotherapy, a consolidation phase with imatinib and steroids, and 10 maintenance blocks, including two 2-month blocks of imatinib. Overall, imatinib was given for 6 months during this 2-year regimen. A group of 21 patients treated during the pre-imatinib era with a similar chemotherapy regimen is used as a control. Results. Out of 30 patients included in the study, 27 achieved a complete response. At last follow-up (November 2008), 7 patients were still alive (23%, 95% C.I.: 10-42%), including 4 patients who had experienced a molecular (n=3) or a hematological (n=1) relapse. Patients surviving after a molecular relapse had been salvaged with various regimens, including imatinib or dasatinib given alone or combined with chemotherapy. The single patient offered an allogeneic stem cell transplantation relapsed and died. No clinical or biological characteristics (age, blood counts, and steroid sensitivity) at diagnosis allowed for prediction of long-term survival. Compared with patients treated during the pre-imatinig era, the survival advantage con-

ferred by the use of imatinib could be confirmed with a longer follow-up (Figure). Summary/conclusions. In the imatinib era, approximately 20% of elderly patients are alive 3 years after a diagnosis of Ph+ ALL was made. In this study characterized by a relatively short exposure of patients to imatinib, molecular relapses could be successfully managed by additional treatment with tyrosine kinase inhibitors with or without chemotherapy.

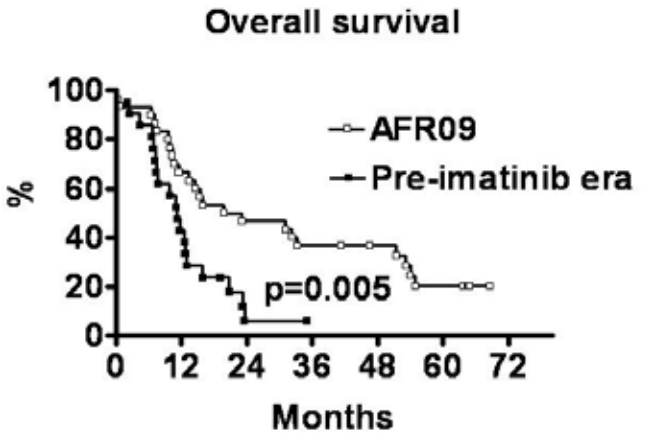

Figure.

0078

THE HUMORAL IMMUNE RESPONSE DURING THE L-ASPARAGINASE THERAPY - THREE CLASSES OF ANTIBODIES

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The use of L-asparaginase - a crucial agent in the treatment of malignant blood disorders - is often associated with the appearance of hypersensitivity reactions. During the treatment anti-asparaginase antibodies may cause the clinical manifested allergy and/or decrease enzyme activity. The aim of the study was to assess the presence of anti-asparaginase antibodies during two consecutive therapy courses, containing L-asparaginase in three classes of immunoglobulin: IgG, IgM and IgE and to analyze a putative clinical importance of the anti-asparaginase antibodies. The study group includes 80 children and adolescents with newly diagnosed ALL, treated according BFM protocols. The real serum asparaginase activity during the treatment was established before a next administration of the drug, during the phase of induction and reinduction therapy. On the last day of the treatment with asparaginase, the blood samples were collected for the examination of anti-asparaginase antibodies in classes IgG, IgM and IgE. Both assays were based on ELISA method. At the end of induction phase, anti-asparaginase antibodies were found in 28% of children in class IgG, in 25% of children in class IgM and in 18% of children in class IgE. In 30% of children the antibodies in only one class were found, however in 5% the presence of all three of antibodies was noted. The median concentration of the antibodies amounted 39.56 µg/dL (33.0; 57.4) for IgG, 7.49 µg/dL (5.88; 8.87) for IgM and 1.47 (1.14; 2.33) for IgE. In the reinduction phase of treatment anti-asparaginase IgG antibodies were found in 45%, IgM antibodies in 51% and IgE antibodies in 18% of patients. The median concentration of anti-asparaginase antibodies amounted 79.3 µg/dL (41.4; 213.0) for IgG, 9.7 µg/dL (6.78; 12.4) for IgM and 2.0 µg/dL (1.3; 3.3) for IgE. Antibodies in class IgG and IgM, especially in the reinduction phase of treatment, were associated with the clinical manifested hypersensitivity/allergy against asparaginase (IgG p=0.0059, IgM p=0,009, IgE p=0.07) and asparaginase activity (IgG 55 vs. 297.6 AU, p<10-5, IgM 63.3 vs. 281 AU, p<10-5). The IgE antibodies did not show such an association (hipersensitivity reaction p=0.07, asparaginase activity 113 U/L vs. 161 U/l, p=0.39). *Summary.* The use of asparaginase leads to the development of several classes of anti-L-ASP antibodies. The frequency and the concentration of anti-asparaginase antibodies are significant higher in the second course of treatment. The most important of the clinical point of view seem to be IgG and IgM antibodies but not IgE antibodies.

0079

LIPOSOMAL CYTARABINE FOR THE PROPHYLAXIS OF CENTRAL NERVOUS SYSTEM RELAPSE IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA. INTERIM ANALYSIS OF THE PALG 5-2007 ALL STUDY

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Background. Prophylaxis of central nervous system (CNS) involvement is one of the principles of the treatment of adults with acute lymphoblastic leukemia (ALL) and includes cytostatics administered intrathecally by lumbar puncture. However, results of the preceding study by the Polish Adult Leukemia Group (PALG 4-2002) revealed poor compliance to the regimen with only 55% of patients receiving the planned 6 i.t. injections during induction-consolidation phase. We hypothesized that the introduction of the depot liposomal cytarabine formulation (DepoCyte, Mundipharma, Cambridge, UK) characterized by prolonged activity in the cerebro-spinal fluid may increase compliance, by reduction of the total number of i.t. injections. *Aim.* The goal of this prospective, multicentre PALG 5-2007 ALL study was to evaluate the feasibility and safety of intrathecal prophylaxis based on the use of liposomal AraC, instead of native cytostatics (AraC + Mtx + Dexamethasone). *Methods.* According to the protocol, during pre-treatment phase, all patients received the first 'triple' i.t. injection. However, those with symptoms of post puncture syndrome, previously identified as a risk factor for non-compliance, continued with liposomal AraC injections (3x during induction-consolidation), while the remaining patients were further treated with native cytostatics (6x). I.t. liposomal AraC (50 mg) was administered on strictly defined days in order to avoid toxicity when combined with i.v. cytostatics. In particular, during consolidation, the i.t. therapy was given 48h hours after high doses of i.v. AraC or Mtx and 14 days before the next i.v. AraC or Mtx. *Results.* Among 70 patients registered between Jan and Dec 2008, liposomal AraC was introduced in 12 cases (8 male, 4 female) with B-ALL (n=10) or T-ALL (n=2). One patient was Ph-positive. The median age equaled 30 (19-57) years. Among 11 patients who completed induction-consolidation treatment in CR, all but one received planned 3 i.t. liposomal AraC injections. The rate of compliance was 10/11 (91%). For the total number of 31 injections, adverse events were reported in 3 cases: 1 patients developed brain edema (during Mtx-containing consolidation), 2 patients experienced post puncture syndrome. With the median follow-up of 7 (5-12) months, none of the patients developed CNS relapse. *Conclusions.* For the first time we report results of a prospective study on the prophylactic use of liposomal AraC, within a uniform induction-consolidation protocol for adult ALL. This early evaluation suggests that the procedure is feasible and safe. Reduction of the total number of i.t. injections probably facilitates compliance to the protocol.

0080

QUALITATIVE AND QUANTITATIVE CONCORDANCE IN MRD DATA, ASSESSED BY FLOW CYTOMETRY AND RT-PCR OF FUSION GENE TRANSCRIPTS IN INFANTS WITH MLL-REARRANGED ALL

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Background. Minimal residual disease (MRD) monitoring by flow cytometry (FC) or real-time quantitative polymerase chain reaction (RQ-PCR) is a strong tool for risk-adapted treatment in childhood acute lymphoblastic leukemia (ALL). ALL in infants is known to be very specific leukemia subset with frequent MLL-rearrangements and poor outcome. Prolonged MRD monitoring is essential during infants' ALL treatment. IgH/TCR rearrangements monitoring by RQ-PCR is well-standardized but very laborious and costly approach. Hence other methods - FC and RQ-PCR of fusion gene transcripts (FGt) - can be used for long-term MRD monitoring. *Aim.* To evaluate qualitative and quantitative concordance between MRD assessed by FC and FGt copy number (CN) measured by RQ-PCR in infants with primary and relapsed MLL-rearranged ALL during treatment. *Methods.* Tandem application of multicolor FC for MRD detection and RQ-PCR for FGt CN has been performed in 72

follow-up bone marrow samples from 15 infants with MLL-rearranged B-lineage ALL. 20 samples were obtained during remission-induction, 36 - during post-induction and 16 - during relapse treatment respectively. FGt CN was measured by RQ-PCR according to «Europe against cancer» recommendations. ABL has been used for normalization. MRD value was calculated as previously described. *Results and discussion*. Sensitivity of FC MRD detection varied from 10-4 to 10-5. RQ-PCR sensitivity ranged from 5-10-5 to 10-5. 7 of 72 samples (9.72%) were MRD-negative by both methods, 2 (2.78%) - were negative by FC but positive by RQ-PCR. Remaining 63 samples (87.50%) were MRD-positive by both methods. High qualitative concordance (97.22%) between FC and RQ-PCR was obtained. Samples with and without normal lymphoid regeneration were analyzed separately, because presence of normal B-cell precursors (BCP) in follow-up samples is known to be an obstacle for FC data analysis. Qualitative concordance in BCP-negative and BCP-positive samples was similar (96.43% and 97.73% respectively). In contrast, low quantitative concordance was found between FC and RQ-PCR (r=0.54). Low quantitative concordance was also observed in BCP-positive and BCP-negative samples (r=0.64 and r=0.59 respectively). Significant quantitative difference in FC and RQ-PCR data could be associated with variability of FG expression during treatment that does not correspond to the cell number. Moreover, percentage of tumor blasts among all nucleated cells is calculated during FC MRD detection, while MRD value in RQ-PCR of FGt is corresponded to the initial FGt and control gene levels. FC appears to be better for the quantitative MRD assessment; however FGt detection in RQ-PCR is more appropriate for MRD qualitative analysis because of its higher sensitivity. Hence, FC is more applicable for early treatment stratification and RQ-PCR of FGt - for later time-points. *Conclusion.* Tandem application of FC at early time-points and FGt detection by RQ-PCR at later time points seems to be a useful tool for MRD monitoring in infants with MLL-rearranged ALL.

0081

OUTCOME FOR ADOLESCENT AND YOUNG ADULTS UP TO AGE 35 YEARS (AYA) WITH ACUTE LYMPHOBLASTIC LEUKEMIA TREATED ON THE PEDIATRIC PROTOCOLS OF MOSCOW-BERLIN 91 AND 2002

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Background. Over the past decades, several research groups have demonstrated improvement in survival for adolescents and young adults (AYA) up to age 35 years with acute lymphoblastic leukemia (ALL) using pediatric regimens compared to adult treatment regimens. Original national pediatric protocols ALL-MB 91 and 2002 have shown high efficiency of treatment of children in Russia. *Aims.* The purpose of the study was to assess the efficacy and toxicity pediatric protocols ALL-MB 91 and 2002 for adolescents and AYA with ALL. *Methods.* Enrollment on the study began in December 1997. Inclusion of patients (pts) in protocol ALL-BFM 90 (n=43) was completed in September 2005 and ALL-MB 91/2002 - March 2008 (n=34). In protocols ALL-MB 91/2002 the pts receive four drug induction with dexametasone 6 mg/m^2 daily for 36 days, daunorubicin 45 mg/m^2 for 2 doses, vincristine 2 mg weekly for 5 doses and and intrathecal (IT) cytarabine and IT methotrexate and IT prednisolone weekly for 6 doses. Consolidation therapy included L-asparaginase in a constant dose of 10000 ME/m^2 weekly for 18 doses and 6-merkaptopurine 50 mg/m^2 (100%) daily and methotrexate 30 mg/m^2 (100%) weekly with weekly doses adjusted according to white blood cell count. Central nervous system (CNS) irradiation is performed for pts with CNS involvement at diagnosis and for patients with T-cell ALL and a high presenting white blood cell count. Traditional maintenance was carried out up to 24 months. The protocol ALL-BFM 90 called for the purpose of comparison as an effective standard therapy. *Results.* 78 (m - 8, f - 30) pts have been enrolled. 77 pts are valuable (1 withdrew on day 1 of therapy). The median age is 19.3 years (range 15-35). 37 (86%) pts are in complete remission (CR) on the protocol ALL-BFM 90 vs. 29 (88%) pts - ALL-MB 91/2002. Respectively 3 (7%) and 3 (9%) pts died in the induction. 3 (7%) and 1 (3%) pts is refractory to therapy. 5 (12%) and 1 (3%) pts died in CR from significant toxicities. Respectively 9 (21%) and 3 (9%) pts relapsed. 4 (33%) pts have CNS relapse, and 6 (50%) have bone marrow relapse. 6-years event free survival (6y-EFS) has 54 vs.77% (median of observation 5.7 years, p>0.05), and 6-years overall survival (6y-OS) has 65 vs. 82% (p>0.05) respectively. Myelosuppression toxicities of ALL-MB 91/2002 protocols have less significant compared with the ALL-BFM 90. In postremission period the most frequent significant toxicities are neutropenia Grade 4 (21 vs. 66%, p<0.05), and thrombocytopenia Grade 4 (0 vs. 62%, p<0.05), and infectious Grade 3-4 (32 vs. 55%, p>0.05). *Conclusions.* Protocols ALL-MB 91/2002 is effective therapeutic regimes for ALL. Further studies with higher power are needed to determine if this treatment regimen offers an advantage to AYA patients with ALL.

0082

HUMORAL IMMUNITY TO DIPHTHERIA, TETANUS, MEASLES, AND HAEMOPHILUS INFLUENZAE TYPE B IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA AND RE-VACCINATION RESPONSES

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Background. Loss of immunity to previous vaccinations and timing of re-vaccinations in children receiving chemotherapy remains controversial. *Aims.* To investigate the immunity to vaccine preventable diseases in children with acute lymphoblastic leukemia (ALL) before and after chemotherapy and to evaluate re-vaccination responses. *Methods.* Sixty-one patients with acute lymphoblastic leukemia and 13 healthy siblings were enrolled. Relapsed patients or patients undergoing stem cell transplantation were excluded. The study was approved by the local ethical committee and written informed consents of patients, parents and controls were obtained. Türk ALL-2000 protocol (TRALL-2000), a modified BFM-95 protocol was administered. Newly diagnosed patients (group 1), patients on maintenance chemotherapy (group 2) and patients that completed chemotherapy (group 3) were three study groups. Serum samples were stored at -80°C until analysis of the antibody titers was performed to minimize the inter-assay variation. Patients in group 2 were vaccinated with diphtheria, tetanus and Hib. Group 3 and controls were vaccinated also with measles. Post-vaccination antibody titers were also studied. All of the titer determinations were performed with the ELISA method. *Results.* Patients and controls had primary vaccination with diphtheria, tetanus and measles, but not with Hib. After chemotherapy median antibody levels against diphtheria, tetanus, measles and Hib were decreased but tetanus antibodies were still at the protective levels. Proportions of the patients with protective levels were 11.1%, 83.3%, 16.7% and 16.7% for diphtheria, tetanus, Hib and measles. Vaccination maintained protective antibody levels in 81%, 100%, 89.5% and 70% of the patients for diphtheria, tetanus, Hib and measles respectively. Vaccine responses during maintenance were also satisfying; 82.4%, 100% and 73.3% of the patients achieved protective antibodies for diphtheria, tetanus and Hib respectively (Table 1). *Summary and Conclusions.* Present study shows loss of immunity especially to diphtheria and measles after ALL chemotherapy. We recommend administration of Hib vaccine especially to children with no primary vaccination after the first three months of maintanence, followed by a second booster dose after cessation of the chemotherapy to increase immunity. Re-vaccination with tetanus, diphtheria and measles 3 months after cessation of chemotherapy seems more practical and economical instead of monitoring antibody levels.

Table 1. Proportions of patients and controls with complete protection.

groups	Diphtheria		Tetanus		Hib		Measles	
	Prevac	postvac	Prevac	postvac	Prevac	postvac	Prevac	postvac
At diagnosis n=20	80%	–	100%	–	35%	–	55%	–
During maintenance n=36	40%	82.4%	91.4%	100%	17.1%	73.3%	20%	–
After chemotherapy n=20	11.1%	85%	83.3%	100%	16.7%	89.5%	16.7%	70%
Controls n=13	46.2%	88.9%	100%	100%	15.4%	88.9%	38.5%	66.7%

0083

PROGNOSTIC SIGNIFICANCE OF ULTRASOUND-DETECTED BOWEL WALL THICKENING IN NEUTROPENIC ENTEROCOLITIS

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Background. Neutropenic enterocolitis is a life-threatening condition associated with severe neutropenia. It is most commonly seen in patients with acute leukemia but has also been described in patients with other malignancies. Ultrasonography and color Doppler of the bowel support the diagnosis by detecting the mural thickening and bowel perfusion. *Aim.* This study was designed to assess the prognostic value of bowel wall thickening detected by US and bowel wall perfusion in monitoring children with neutropenic enterocolitis with regard to the severity of the disease. *Results.* Our results revealed abnormal intestinal wall thickening of the ileoceacal region and ascending colon in 29 out of 42 patients treated from leukemia (69%), the mean wall thickness was (8.71±2.16; range 6-12.5 mm). Hypervascularity on color Doppler imaging was noted in the majority of patients whereas two out of eight deaths revealed absent flow within the bowel wall. Complete resolution of symptoms occurred in 21 (72.4%) of US-positive group with a mean duration of symptoms (9.83±2.78; range 7-15days) while it occurred to all patients without bowel wall thickening, mean duration of symptoms was (6.15±1.14; range 5-8 days). Serial sonographic studies documented a gradual decrease in bowel wall thickness in the surviving patients who eventually recovered (72.4% versus 100%, $p<0.05$). NE-related death was significantly higher in US-positive patients (8 vs. 0, $p=0.05$). Patients with bowel wall thickness >10 mm had significantly higher mortality rate than those with bowel wall thickness <10 mm (70% vs. 5.3%, $p<0.001$). *Conclusion.* In conclusion, neutropenic patients with sonographically detected bowel wall thickening have a poor prognosis. In addition, the degree of bowel wall thickening was significantly impact the outcome of those patients; so the US-detected mural thickness and bowel wall perfusion reflects the course of the disease and predictive for the clinical outcome.

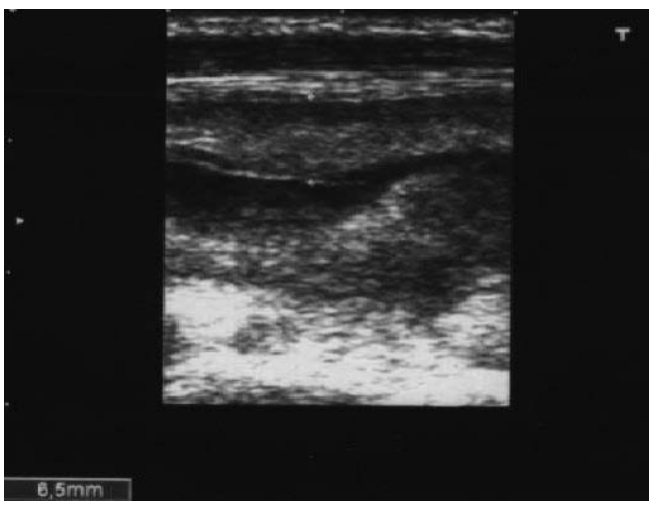

Figure 1. A case of NE in 10 yrs-old girl. (a&b) Ultrasound.

Hodgkin lymphoma

0084

EARLY AND LATE RESPONSE ASSESSMENT WITH FDG-PET AFTER BEACOPP-BASED CHEMOTHERAPY IN ADVANCED-STAGE HODGKIN LYMPHOMA PATIENTS HAS A HIGH NEGATIVE PREDICTIVE VALUE

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Background. Positron emission tomography (PET) is considered as a potential powerful prognostic factor in the treatment of Hodgkin lymphoma (HL) patients. *Aims.* To analyse the prognostic value of PET for early and late response to BEACOPP-based chemotherapy in patients with advanced-stage HL. *Methods.* Between January 2004 and April 2008, 69 patients with newly diagnosed HL in clinical stage IIB with large mediastinal mass or extranodal disease, in clinical stage III or IV with or without risk factors were treated according to the HD15 protocol of the German Hodgkin Study Group (GHSG). The treatment comprised of 6 - 8 cycles of BEACOPP-based chemotherapy and invoved-field radiotherapy in patients with PET positive rest-tumour after the end of chemotherapy. All patients received an early PET scan after 4 cycles of chemotherapy (early-PET) and a late PET scan after completion of chemotherapy (late-PET). For the calculation of the predictive value, death of any cause was counted as a treatment failure in addition to progression and relapse. *Results.* Of the overall group, 68 patients completed the scheduled chemotherapy and one patients died in the 8th cycle of chemotherapy due to bleomycin-induced pneumonitis. 18/69 patients had a positive early-PET (26%), while 51/69 had a negative early-PET (74%). The late-PET was positive in 9/68 patients (13%), and negative in 59/68 patients (87%). At a median observation time of 34 months, the negative predictive value for patients treated with BEACOPP only, was 96% for early-PET and 95% for late-PET. The positive predictive value for patients treated with BEACOPP with or without radiotherapy was 22% for both early- and late-PET. During the study-time 5 patients relapsed, 4 of them had a positive early-PET and 2 had a positive late-PET. Importantly, all patients who relapsed with negative late-PET had relapsed after 12 months follow-up. *Summary*. Advanced-stage HL patients with a negative PET in early and late response assessment have a very good prognosis, while PET positive patients have an increased risk for progression and relapse after BEACOPP-based chemotherapy. The sensitivity of a positive PET for treatment failure within the first year is very high, although later relapses can occur.

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0085

VEBEP REGIMEN AND HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) IN PATIENTS (PTS) WITH HD AND HIV INFECTION (HD-HIV): FINAL RESULTS OF THE ITALIAN COOPERATIVE GROUP ON AIDS AND TUMORS (GICAT) STUDY

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Background. The outcome of pts with HD-HIV is still poor, because the duration of complete remission (CR) is short. To improve the prognosis of HD-HIV, a feasibility study with the VEBEP regimen and HAART was started in previously untreated HD-HIV pts. *Methods.* CT included epirubicin 30 mg/m^2/day (days 1-3), cyclophosphamide 1000 mg/m^2 (day 1), vinorelbine 25 mg/m^2 (day 1), bleomycin 10 mg/m^2 (day 3) and prednisone 100 mg/m^2/day (days 1-3). *Results.* Since September 2001, 71 pts have been enrolled. The median age was 41 yrs. The median CD4$^+$ cell count was 248/mm^3 and 51% of pts had a detectable HIV viral load. Stage III-IV was present in 50/71 (70%) pts. Histologic subtypes were:

MC 70%, NS 20%, LD 4%, LP 2%, unknown 4%. Four toxic deaths were observed (septic shock, PCP, hepatic failure and pneumonia during neutropenia). An absolute neutrophil count <500 was noted in 60% of pts. Grade 3-4 anemia was observed in 38% of pts and severe thrombocytopenia in 22% of pts. Twenty-two per cent of pts had febrile neutropenia with 19 documented infections in 16 pts (4 varicella, 4 bacterial pneumonia, 3 bacterial sepsis, 2 PCP, 1 cerebral toxoplasmosis, 1 esophageal candidiasis, 1 HBV reactivation, 1 HCV reactivation, 1 prostatitis, 1 salmonellosis). CR was obtained in 47/71 pts (66%) and PR in 9/71 pts (13%). With a median follow up of 22 months, only 4 pts have relapsed. OS and TTF at 24 months are 69% and 59%, respectively. *Conclusions.* Our preliminary data demonstrate that VEBEP regimen in combination with HAART is feasible and active in pts with HD-HIV. This study was supported by ISS grants.

0086

18FDG-PET-NEGATIVE COMPLETE REMISSION PRIOR TO AUTOLOGOUS STEM CELL TRANSPLANTATION PREDICTS FOR SUPERIOR EVENT FREE SURVIVAL OF PATIENTS WITH RELAPSED OR REFRACTORY HODGKIN LYMPHOMA

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Background. In patients (pts) with Hodgkin lymphoma (HL) receiving first-line chemotherapy, interim restaging with 18F-FDG-PET scan (FDG-PET) after 1 or 2 cycles has been shown to predict event free survival (EFS) with high sensitivity and specificity overriding the clinical International Prognostic Score (IPS). The predictive value of FDG-PET in patients with relapsed or refractory HL undergoing high dose chemotherapy and autologous stem cell transplantation (ASCT) is less well established. *Aim.* We strived to determine the predictive value of FDG-PET in pts with HL planned to receive ASCT ± peri-transplant involved field radiotherapy (IFRT). *Methods*. A retrospective analysis was undertaken of 52 consecutive pts treated at three centres. Pts with primary refractory (n=25) or relapsed HL (n=27) underwent FDG-PET scanning after salvage chemotherapy and before ASCT. Remission status by FDG-PET post salvage, treatment details, including salvage type and peri-transplant IFRT, and clinical characteristics were recorded and EFS and overall survival (OS) post ASCT were evaluated. Survival analyses were performed using Kaplan-Meier estimates and cohorts were compared using the Log-rank (Mantel-Cox) and the Gehan-Breslow-Wilcoxon Test. The contingency of data between different groups was analysed using Fisher's exact test. *Results*. The median age of pts at the time of ASCT was 38 [18-61] years, 27/52 (52%) were male. The majority of pts received salvage chemotherapy with VIC (etoposide 100 mg/m^2 day (d)1-3, ifosfamide 5 g/m^2 d2, carboplatin AUC 5), n=24) or MADEC (methotrexate 400 mg/m^2 d1, cytosine arabinoside 75 mg/m^2 d1-5, dexamethasone 40 mg d1-4, etoposide 75 mg/m^2 d1-5, cyclophosphamide 750 mg/m^2 d2, n=13), other chemotherapy regimens used were BEACOPP, n=3, IGEV, n=3, FGIV, n=2, DHAC, n=2 or others, n=1 each. After salvage, 23/52 (44%) of pts were FDG-PET-negative and 29/52 (56%) were positive. With a median follow-up of 30 [4-115] months in surviving pts, the 6-year actuarial rates for EFS and OS for FDG-PET negative versus FDG-PET positive pts were 73% and 34% (p=0.03), and 95% and 64%, respectively (p=0.06). Overall, the addition of peri-transplant IFRT did not impact on EFS or OS. However, in 13 pts with post salvage FDG-PET-avid disease which was limited to an area entirely encompassed by IFRT, actuarial rates of 6-year EFS and OS were 51% and 66% which did not differ significantly from those obtained in FDG-PET-negative pts (p=0.47 and 0.39, respectively). Female gender was the only factor predictive for obtaining a complete FDG-PET-remission post salvage therapy (p=0.011). Female gender and duration of first remission of ≥12 months also independently predicted for superior EFS (p=0.017 and 0.039), respectively. Other characteristics including the presence of B-symptoms, extra-nodal disease prior to salvage, age ≥38 years, type of salvage or conditioning regimen used, or transplant centre did not influence EFS or OS. *Conclusions.* Our data show that FDG-PET-status after salvage chemotherapy for relapsed or refractory HL is a powerful predictor of EFS after ASCT demonstrating an excellent outcome for FDG-PET-negative pts. In pts with limited FDG-PET-avid disease following salvage chemotherapy, the addition of peri-transplant IFRT may reduce the poor prognostic impact of residual FDG-PET positivity in a subset of patients.

0087

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 one of the 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 become chemorefractory. 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 patients with localized Hodgkin's disease. *Patients*. From 2002, 163 new localized stage Hodgkin's lymphoma patients were consecutively admitted to nine Italian hematological centers. Patients with stage I-IIA according to Ann Arbor stage were considered for the study. FDG-PET was mandatory at baseline, after two cycles and at the end of therapy. We evaluated the progression free survival of patients starting from the time of diagnosis to relapse or progression of disease or last follow-up. Patients were candidate to receive 3 or 4 course of ABVD followed by involved field radiotherapy at 30 Gy, except in the cases in which physician decided to omit radiotherapy. No treatment variation based only on PET-2 results was allowed. *Results.* The median age was 33 years (16-75), 85 patients were female and 78 male, 15 patients presented stage I and 148 stage II, bulky was reported in 45 patients. One-hundred and fourty-eight patients were treated with combined modality (CT+RT) and 15 patients were treated with chemotherapy alone (all with 6 cycles). One hundred and fourty-seven patients attained CR while 16 were chemoresistent: 9 showed disease progression during CT and 7 showed an early relapse. The FDG-PET performed after two cycles (PET2) was positive in 23 patients (14%): 12 (52%) progressed or relapsed and 11 remained in CR. By contrast 130/140 (93%) patients with a negative PET2 remained in CR. Thus the positive predictive value of a PET2 was 52% and the negative predictive value was 93%. The sensitivity and specificity of PET2 were 55% and 92%, respectively. Seventeen patients showed disease progression during therapy or within 12 months after having reached CR, 11/17 (65%) were PET2 positive. The FDG-PET performed at the end of therapy was positive in 15 patients. Six patients died due to the disease, four were PET2 positive and two were PET2 negative. In univariate analysis negative FDG-PET performed after two cycles (p=0.0000), absence of bulky disease at diagnosis (0.004) were statistically correlated with a better progression free survival. In multivariate analysis only PET2 was independently predictive of relapse/progression probability (p=0.000). With a median follow-up of 31 months (range 6-87) 154 patients are alive and 141 (92%) are free from progression. The 2-yr FFS probability for PET2 negative and for PET2 positive patients were 94% and 58% respectively. *Conclusions.* This prospective and 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 evaluation methods in this subset of patients.

0088

PHASE II STUDY OF ORAL PANOBINOSTAT IN PATIENTS WITH RELAPSED/REFRACTORY HODGKIN LYMPHOMA AFTER HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANT

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Background. Panobinostat (LBH589) is a pan-deacetylase inhibitor

(pan-DACi) targeting epigenetic and non-epigenetic oncogenic pathways. *In vitro*, panobinostat decreases proliferation and induces apoptosis in HL cell lines at low nanomolar concentrations. Encouraging clinical activity, with a CT scan-based response rate of 42% in patients with HL, has been demonstrated in an ongoing Phase I study in patients with hematologic malignancies (Ottmann OG *et al.* ASH 2008 Abstract #958). *Aims.* The aim of this open-label Phase II study is to confirm the efficacy of panobinostat in HL. *Methods.* Utilizing a Simon two-stage design, patients with hodgkin lymphoma (HL), whose disease is refractory to, or has progressed after ASCT are being enrolled in the study. The primary objective of the study is to determine the overall response rate using the modified Cheson criteria. Secondary objectives include time to and duration of response, progression-free survival (PFS) and overall survival; clinical and laboratory safety data will be collected. To be included in the study, patients must have adequate organ function, no other significant medical conditions, and ECOG PS ≤2. Panobinostat is administered orally at a dose of 40 mg three-times weekly in a 21-day cycle. Treatment is continued until disease progression or intolerance. Dose delay and modification for toxicity is allowed. CT/MRI scans are conducted at the end of every two cycles. The *in vivo* effect of panobinostat on the expression of programmed cell death protein 1 (PD-1) on peripheral blood T-lymphocytes was evaluated by multicolor FACS analysis. *Results.* As of Feb 20, 2009, 30 patients have been enrolled, of whom 14 have completed at least 2 months of therapy and for whom preliminary data are available (median age 27.5 years [range 18-51]; six male, eight female; median number of prior regimens five [range one to six]). The more common AEs have been thrombocytopenia (n=6/14, all Grade 3 or 4), diarrhea (n=6/14), and nausea (n=8/14). Among the 295 ECGs analyzed, there have been no ≥Grade 2 QTc abnormalities. Efficacy data are available for six of the 14 patients who had tumor reductions ranging between 18% and 68%, including two partial responses. All these patients continue on study. Serial blood samples were obtained from four patients, and in all cases panobinostat significantly decreased the expression of PD-1 on $CD8^+$ T cells after 7 and 15 days of therapy. *Summary*. Panobinostat has encouraging clinical activity in heavily pretreated patients with relapsed classical HL. In addition to its direct antitumor effect, our data suggest that panobinostat may enhance the antitumor immune response by downregulating PD-1 on patients' T lymphocytes. Panobinostat is generally well tolerated, and thrombocytopenia is managed by dose delay or dose reduction. Enrollment into the study is ongoing; efficacy and safety data available at the time of the meeting will be presented.

0089

QUALITY OF LIFE AND FERTILITY IN HODGKIN DISEASE PATIENTS TREATED WITH ESCALATED BEACOPP

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Aims and Background. Quality of life and fertility was assessed in 173 patients with advanced hodgkin disease (HD) IIBX - IV CS, treated in a single institution with a medium follow-up of 5.5 years (2-12 years). After initial therapy with ABV, ABVD or escalated BEACOPP regimens (3 cycles) they were all assessed by CT or PET-CT. Good responders (defined as over 50% tumor mass reduction) were treated by ABV or ABVD (3 cycles), while other patients were subjected to escalated BEACOP. Involved field radiotherapy was considered in IIB patients, while second line regimens with ASCT in relapsing/ refractory cases. Average 5 year OS and EFS was significantly longer in patients treated with upfront escalated BEACOPP regimen: 94 and 89% (n=78) versus 90% and 74% respectively (n=95, $p<0.01$, D. Krochmalczyk, W Jurczak *et al.*, ASCO 2008). Intensive upfront therapy reduced the number of relapsing/ refractory cases (4 and 22 pts were subjected to ASCT respectively). *Methods and Results.* Quality of life (QoL) assessment addressing social, physical and emotional functioning performed 6-60 months after completing chemotherapy in 113 patients (177 completed questionnaires) revealed no important differences. Results were also similar in a direct comparison of ABV/ABVD and escalated BEACOPP regimen by 64 patients who were treated by both of them. However dacarbasine makes a difference: while 24 of 39 (61%) of patients preferred escalated BEACOPP to ABVD, only 8 of 15 (32%) preferred escalated BEACOPP to ABV. Hormonal and reproductive function of gonads was assessed in 106 patients 6-60 months after completing chemotherapy (women elder than 50 years were not included in analysis, those on hormonal replacement therapy discontinued the drugs, 2 weeks prior to analysis). Hypogonadysm was defined by low estradiol and testosterone levels (<30 pg/mL an 10 ng/dL respectively). Reproductive function of gonads was indirectly assessed by FSH levels (patients with FSH > 15 m IU/mL were regarded unfertile). As expected gonad function was not impaired in patients treated with ABV or ABVD regimens. The risk of infertility after escalated BEACOPP regimen was greater for males than females (over 70%, even if only 3 cycles were given). In females, it increased with the number of chemotherapy cycles, and strongly depended on dacarbasine: 21,4% in those with 3 x escalated BEACOPP/3 x ABV compared to 53% after 3 x escalated BEACOPP / 3 x ABVD (p=0.04, see Table for details). Hypogonadism, was on the contrary observed only in females. *Summary and Conclusions.* An upfront escalated BEACOPP regimen improves the outcome of HD patients, as proven elegantly by GHSG. It is efficient and well tolerated, so it should be offered as a standard I line regimen to all high risk HD patients. De-escalation of therapy in good responders to ABV/ABVD regimens may reduce it's side effects, however it doesn't solve the infertility problem. Every male should be therefore offered a possibility of cryopreservation, while eliminating dacarbasine may be considered in young females

Table.

		6 x ABV	6 x ABVD	3 x esc. BEACOPP + 3 x ABV	3 x esc. BEACOPP + 3 x ABVD	6 x esc. BEACOPP	2-nd line regimens (including ASCT)	Chi square Pearson test
Females N = 55	N	6	2	14	15	11	7	
	infertility	0%	0%	21,4%	53,3%	63,6%	42.86%	11,14 p=,04851
	hypogonadysm	0%	0%	21,4%	26,7%	54,5%	42,9%	14,03 p=,01542
Males N = 43	N	6	3	4	10	14	6	
	infertility	0%	0%	75,0%	70,0%	71,4%	50,0%	7,82 p=,16636
	infertility	0%		67,65%				13,08 p=,00030
	hypogonadysm	0%	0%	0%	0%	0%	0,0%	

0090

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 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.* Twentyseven HL patients (14M/13F) underwent RICT preceeded by autografting (ASCT). Median age at diagnosis was 27 (range 15-44), median n° of chemotherapy lines was 2.5 (range 2-4). All but one patient had disease at ASCT. Twelve patients were chemosensitive and 15 chemorefractory at ASCT and high-dose therapy consisted of Melphalan 200 mg/mq (n=7) and BEAM (n=20). The time interval between ASCT and RIC was 3 months (range 1.3-6.7). RIC consisted of fludarabine-cyclophosphamide (n=12) or fludarabine-melphalan (n=15). *Results.* The median time to neutrophils and platelets recovery was 10 days and 16 days, respectively. Chimerism studies indicated 100% donor-derived engraftment. Seven patients developed aGVHD (grade II-IV) and 9 cGVHD (2 limited and 7 extensive). At the last follow up 17 patients (63%) were alive, 12 (70.6%) in RC and 5 (29.6%) with disease. Ten patients expired (37%), 7 of disease progression, 1 of aGVHD, 1 of cGVHD and 1 of infection. With a median follow-up of 46 months (6-117 months), median OS was 47 months. *Conclusions.* These encouraging results suggest that GvLy may have a role on residual disease after ASCT. A prospective study based on genetic randomization (ASCT vs ASCT followed by RICT) would help to answer this important issue.

0091

QUALITY OF LIFE IN A 2-YEAR FOLLOW-UP: SIGNS OF PNP PREDICTING FATIGUE IN SURVIVORS TREATED IN THE HD10-12 TRIALS OF THE GERMAN HODGKIN STUDY GROUP

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Background. Hodgkin Lymphoma (HL) is one of the best curable cancers in adults. Thus, current research is focusing on the increasing proportion of HL survivors with respect to the long-term consequences of therapy. Especially health related quality of life (QoL) needs thorough investigation since its relevance for HL patients, complexity and limited knowledge up to now. QoL incorporates different aspects such as general QoL, fatigue, emotional, physical, role, social, sexual and cognitive functions. *Aims.* This analysis is focusing on QoL in the HD10-12 trials of the GHSG with special emphasis on fatigue and its predictors in a 2 year follow-up. *Methods.* Patients of the German hodgkin study group (GHSG) trials HD10-12 completed the QLQ-C30, the MFI20 and some additional items at the time of diagnosis, after chemotherapy, after radiotherapy and at follow-up examinations. We describe the courses of the QLQ-C30 scales with means and 95%-confidence intervals for each measurement point and in relation to the respective norm values. In multiple regressions we analyzed the role of the following predictors for fatigue 2 years after treatment: age, sex, disease stage, treatment modality, baseline score of fatigue and symptoms of peripheral neuropathy (PNP) before and after chemotherapy application. *Results.* In the sample of 3608 patients all scales showed abnormal values from the beginning, further deterioration to the end of chemotherapy and continuous recovery from the end of radiotherapy on. Most scales improved clearly beyond their pre-treatment values. Opposed to other scales, the scale for fatigue reached only a moderate improvement beyond the pre-treatment level. A first regression analysis showed a significant impact of sex, age, disease stage and symptoms of PNP before therapy on fatigue after 2 years. There was no effect of the treatment arms and the respective intensity of therapy within the 3 studies. When additionally fatigue baseline scores were included in the analysis, these baseline score were the best predictor of fatigue after 2 years and only PNP symptoms before chemotherapy (p<0.001) and age contributed additional significant information. *Conclusions.* In contrast to other QoL parameters, pathological values of fatigue occur frequently and show only a moderate improvement. The main finding from this large prospective study in a homogenous cohort of young cancer patients is that besides age and baseline values of fatigue, symptoms of PNP before therapy are specifically predictive of future fatigue complaints. Interestingly, we did neither find any significant effect of the type or intensity of therapy. With this knowledge, it is possible to define a patient cohort being at high risk for the development of chronic fatigue and to develop and test intervention strategies.

0092

EXPRESSION AND EPIGENETIC ANALYSIS OF KEY B-LINEAGE TRANSCRIPTIONAL PROGRAMMES IN HODGKIN / REED-STERNBERG CELLS

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Background. Classical Hodgkin Lymphoma (cHL) is characterised by the presence of Hodgkin / Reed-Sternberg (HRS) cells. Although derived from germinal centre B-cells, HRS cells have commonly down-regulated their B-cell phenotype due to mechanisms which are incompletely understood. *Aims.* 1. To determine the expression of the B-cell transcription factor Pax5 in cHL cell lines and primary tissue; 2. to investigate possible causes of Pax5 down-regulation: methylation of the Pax5 gene promoters and expression of proteins known to up-regulate Pax5 expression - EBF1, GABP and MTA1. *Methods.* Expression level of Pax5, EBF1, GABPα and MTA1 in cHL cell lines was determined using one or all of: - reverse transcriptase qPCR (Taqman primers and probes obtained from Applied Biosystems); - western Blotting of whole cell lysates; - immunocytochemistry of cytospin preparations. Expression of Pax5, EBF1 and GABPα in primary tissue from cHL cases was determined using immunohistochemistry on paraffin embedded samples subjected to antigen retrieval. Pax5 staining utilised the Envision kit (Dako) whereas EBF1 and GABPα staining was automated, using a Bond machine. Methylation analysis of the Pax5 α and β promoters was performed using bisulfite genome sequencing. DNA was bisulfite converted using the Epitect kit (Qiagen), amplified by PCR, cloned and sequenced. Demethylation was accomplished by culturing the cell lines in the presence of 5-aza-2-deoxycytidine +/- trichostatin A. *Results.* Pax5 protein expression was absent in L428 and KMH2 cell lines and weak in the L1236 cell line. 30 cHL cases were stained for Pax5 and staining of the HRS cells was compared with reactive B-cells: 3/30 cases were negative, 16/30 were weakly positive, 10/30 cases were moderately positive and only 1/30 of the cases was strongly positive. 10/21 Pax5α L428 cell line clones showed a high degree of CpG methylation. Only 4/21 L1236 α promoter clones showed a similar degree of methylation. The β promoters from both cell lines were largely unmethylated. Demethylation resulted in Pax5 mRNA upregulation in the L428 line. EBF1 protein in L1236 and KMH2 lines was not detected but it was strongly expressed in the L428 cell line. For primary tissue: 3/28 cases were negative for EBF1, 10/28 cases were weakly positive, 5/21 were moderately positive and 10/28 were strongly positive. GABPα was strongly expressed by L428, L1236 and KMH2 cell lines. 5/5 cases immunostained also showed strong HRS cell nuclear expression. MTA1 mRNA levels (relative to the control gene Abl1) in L428, L1236 and KMH2 were as high as those seen in B-cell non-Hodgkin Lymphoma lines. *Conclusions.* 1. Pax5 is down-regulated in cHL cell lines and HRS cells of primary tissue; 2. the Pax5α promoter is partly methylated in the L428 line and may be contributing to the down-regulation of Pax5 expression; 3. EBF1 is down-regulated in 2 of the cell lines tested and in the HRS cells of 13/28 cases tested suggesting this may underlie down-regulation of Pax5 expression in a proportion of cHL cases; 4. changes in GABP and MTA1 expression do not appear to be contributing to changes in Pax5 expression.

0093

PLASMA LEVELS OF CIRCULATING CELL-FREE DNA AND OF CYTOKINES AS POTENTIAL BIOMARKERS IN HODGKIN LYMPHOMA

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Background. Hodgkin lymphoma (HL) is characterized by an abundant microenvironment surrounding the malignant Hodgkin and Reed-Sternberg (HRS) cells. Production of cytokines and chemokines contribute to create the favourable conditions for the growth and survival of HRS cells. In particular, interleukin-10 (IL-10) and the chemokine CCL17/TARC appear to play an important role in the recruitment of tolerant T cells to the HL lesions. Proliferation of the malignant and the immune cells results in release of free DNA into the circulation. Cytokine levels have been reported as useful biomarkers in Hodgkin lymphoma (Casanovas, J Clin Oncol 2007; 25; 1732). *Aim.* To assess the relation between levels of free circulating DNA in the plasma to levels of other biomarkers as cytokine levels of IL-10, IL-6 and TARC and patient characteristics and their role for failure-free survival in Hodgkin lymphoma. *Methods.* We studied 58 patients with Hodgkin lymphoma (median age 34 years, range 13-74 years; 31 females and 27 males). Fifty-five patients were treated with standard chemotherapy regimens: 29 patients received ABVD, and 26 pts with advanced stage disease BEACOPP. DNA was extracted from plasma collected at diagnosis using the QIAamp DNA Blood MiniKit (Qiagen, UK) and DNA levels were determined using a quantitative PCR for the β-globin gene. Plasma levls of IL-10, IL-6 and CCL17/TARC were determined using ELISAs. Associations with patient characteristics and event-free survival (EFS) were analyzed using standard statistics (STATA 10). *Results.* Plasma DNA concentrations correlated to several patient characteristics as age >45 years, male sex, and the presence of B-symptoms. HL cases of the nodular sclerosis type were graded according to the BNLI classification, and patients with grade 2 disease had significant higher levels than patients with grade 1 disease (p=0.005). There was a weak, but significant correlation between plasma DNA levels and IL-6 levels (r=0.33, p=0.01), while IL-10 and TARC levels did not correlate to DNA plasma levels. The probability of failure-free survival at 4 years for patients with normal DNA levels was 92%, while it was 64% for patients with elevated DNA levels (95% C.I.,72-98, and 36-82, respectively; p=0.009). Elevated IL-10 and IL-6 levels were associated with failure-free survival as well (p=0.006 and p=0.04). Including the circulating DNA and cytokine levels into a multivariate analysis, circulating DNA and IL-10 proved to be independent predictors of survival (both p=0.03), while IL-6 and TARC were not of prognostic significance. *Conclusions.* We describe levels of circulating DNA in the plasma as a potential new biomarker in Hodgkin lymphoma, independent of other biomarkers as IL-10 and IL-6.

0094

THE INFLUENCE OF POLYMORPHISMS OF GLUTATHIONE S-TRANSFERASE MU 1 (GSTM1), THETA 1 (GSTT1) AND PI 1 (GSTP1) GENES IN THE OUTCOME OF HODGKIN'S LYMPHOMA

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Background. Glutathione S-transferases (GST) are involved in the conjugation of several anticancer drugs, including alkylating agents, anthracyclines and cyclophosphamide. The presence of at least one deletion in two of these enzymes, GSTT1 and/or GSTM1 is associated with an improved disease-free survival for patients with Hodgkin's Lymphoma (HL). In Brazil many patients present with advanced disease at diagnosis. *Aim.* To study the influence of the GST polymorphisms on the stage, response to chemotherapy and survival in HL. *Patients and Methods.* Our retrospective analysis included 110 patients, diagnosed between May 1993 and September 2007. Median age: 27 years (17-63); 58 males, 52 females. HL was diagnosed according to the WHO classification and staged by Ann Arbor criteria. All patients were treated with either ABVD or BEACOPP. In 55 patients, radiotherapy was included for consolidation. At diagnosis 67 patients had stages I / II and 42 had stages III / IV. Genomic DNA from peripheral blood of all individuals was analysed by the multiplex-PCR for identification of the GSTM1 and GSTT1 genotypes and PCR-RFLP for identification of genotypes of the GSTP1. *Results.* GSTP1 wild genotype and GSTM1 null type were higher in patients with stages III / IV (p=0.03 and p=0.05 respectively). The same was obtained for the International Prognostic Score. However, presence of bulky disease and bone marrow involvement was equally distributed in all genotypes. GSTT1 undeleted and GSTM1 null genotypes were associated with a lower percentage of complete remission (p=0.03), primary resistance and recurrence (p=0.08). After a median time of observation of 54 months (9-155) the overall survival in 5 years was 74%. Only GSTM1 null genotype was associated with a longer disease free survival (p=0.02). None of the examined polymorphisms was associated with the progression-free interval. A better overall survival was observed in patients with the GSTT1 null genotype (p=0.006). In the multivariate analysis for overall survival, comparing stage and International Prognostic Score with the polymorphisms studied, only failure to achieve a complete remission and GSTT1 undeleted genotype were independent risk factors. *Conclusions.* Detoxification enzyme polymorphisms may influence the outcome of HL by modifying the metabolism of cytostatic agents. Their study may help to identify patients that need intensification of chemotherapy.

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0095

ANTHRACYCLINE CARDIOTOXITY OCCURS EARLY DURING THERAPY IN CHILDREN WITH HODGKIN'S DISEASE

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The ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) regimen is the standard therapeutic regimen for treatment of Hodgkin's Disease (HD). However ,the therapeutic value of doxorubicin is limited by the cumulative dose-related cardiotoxicity. Echocardiography is used to serially assess left ventricular (LV) function in children, but the correlation between LV ejection fraction (EF) measured by echocardiography and cardiotoxicity assessed by endomyocardial biopsy grade is poor. *Objective*. We aimed to assess LV function in children with HD by radionuclide ventriculography (RVG) and compare it to simultaneous echocardiographic assessment of LV function. *Methods.* 39 children diagnosed with HD were included in the study, 10 patients early during the course of treatment (≤2 ABVD cycles) [Group I; mean age 8.9±3.4 years; 5 males, 5 females; doxorubicin cumulative dose: 75±27.3 mg/m^2; range: 50-100 mg/m^2] and 22 patients late during the course of treatment (≥6 ABVD cycles) [Group II; mean age 8.4±3.3 years; 17 males, 12 females; cumulative doxorubicin dose 328±64 mg/m^2; range: 210-485 mg/m^2].All patients were assessed prior to radiotherapy. Ten patients were stage II, 23 were stage III and 6 were stage IV HD. Following informed parental consent, all children underwent a full clinical assessment, 2D, M-mode and Dopper echocardiography and radionuclide ventriculography (RVG) for assessment of LV function. Seven of the children in Group I were reassessed after completion of 6 ABVD cycles and their data included in the analysis of group II (n=29).

Results. There was no significant difference between both groups as regards the stage of HD. The EF in Group I was 58.2±9.1% by echocardiography compared to 51.7±2.5% by RVG (p<0.05), and 7 patients had an EF<50% by RVG compared to only 1 patient by echocardiography. Group II had lower EF than group I by echocardiography (53±7.7%; p=0.09), but this was only significant when EF was assessed by RVG (44.2±4.5%; p=0.000). An EF<50% was present in 11 and 15 patients in group II by echocardiography and RVG, respectively. Paired analysis of the 7 children who were studied early and late during therapy showed a significant drop in EF by both echocardiography [58.7±7.3 vs. 52±52.44%; p=0.04] and RVG [51.4±2.6% vs. 47.2±3.1%; p=0.004]. There was a significant negative correlation between the cumulative dose of doxorubicin and EF measured by RVG (r=-0.531; p=0.001) but not with EF measured by echocardiography (r=-0.075; p=0.3). No correlation was found between EF measured by RVG and echocardiography EF(r=0.217; p=0.25). *Conclusions.* Doxorubicin cardiotoxicity occurs early and at relatively low cumulative doses in children with HD treated with the ABVD regimen. RVG is more sensitive than echocardiography in detecting early cardiotoxicity manifested as impaired LV EF in children receiving doxorubicin therapy. Baseline and serial assessment of LV function by RVG is recommended in children with HD receiving ABVD based protocols

0096

COMPLETE RESPONSE TO IGEV (IFOSFAMIDE, GEMCITABINE AND VINORELBINE) AND OUTCOME IN RELAPSED/REFRACTORY HODGKIN'S LYMPHOMA PATIENTS

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Background. High dose chemotherapy with autologous stem cells transplant (ASCT) is the gold standard in patients with relapsed/refractory hodgkin's lymphoma (HL). Response to induction chemotherapy (chemosensitive patients) plays a major role in prognosis, however the role of complete response (CR) status after induction therapy has not been established. *Aim.* Primary endpoint was to evaluate the role of CR versus no-CR to IGEV induction therapy on the outcome in terms of progression free survival (PSF) and overall survival (OS). *Methods.* One hundred twenty one patients with relapsed/refractory HL received 4 courses of IGEV followed by single (N=59) or tandem (N=19) ASCT (Santoro *et al.,* Haematologica 92, 2007). Response to IGEV was evaluated by Cheson criteria (1999). Statistical analysis was performed by using the Kaplan-Meier method and Cox proportional hazard model. *Results.* IGEV induced an overall response rate of 75% with 46% of CR. In the univariate analysis favourable factors for outcome were CR vs no-CR to IGEV (PFS: p<0.001, OS: p 0.002), A vs B symptoms (PFS: p 0.003; OS: p=0.05), limited vs advanced stage (PFS: p 0.03; OS: p 0.03), and 1 vs.≥2 previous chemotherapy lines (PFS: p 0.03, OS: p 0.02); response to last therapy (relapsed vs refractory) influenced PFS (p 0.03) but not OS (p 0.70). The multivariate analysis confirmed the favourable prognostic role of CR to IGEV (PFS HR: 2.5, CI 95%:1.3; 4.6 - OS HR 2.3, CI 95%:1.1;4.8) and of the number of previous chemotherapy lines (PFS HR:1.8, CI 95%:1.0;3.2 - OS HR 2.1, CI 95%:1.1;3.9). *Conclusions.* According to our data, we conclude that: 1. CR to IGEV is the strongest indicator of outcome in relapsed/refractory HL. 2. Achievement of CR to IGEV overcomes the role of initial disease status. 3. Efforts are warranted to increase the CR rate by induction therapy.

0097

THE PROGNOSTIC SIGNIFICANCE OF PET SCAN IN PATIENTS WITH RELAPSED/REFRACTORY HODGKIN LYMPHOMA TREATED WITH HIGH DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Approximately 50% of patients with relapsed/refractory hodgkin's lymphoma (HL) can be cured with high-dose therapy/autologous stem cell transplantation (HDT/ASCT). Response to salvage

chemotherapy, as assessed by clinical staging, prior to ASCT is one of the most powerful prognostic factors for outcome. On the other hand, the prognostic significance of interim PET scan in advanced HL patients treated with first-line ABVD chemotherapy is well established. However, its role in the ASCT setting for relapsed/refractory disease is not yet elucidated. *Aims.* To study the prognostic significance of PET scan before and after HDT/ASCT in patients with relapsed/refractory HL. *Methods.* Clinical staging with computed tomography and PET scan were performed after salvage chemotherapy just prior to ASCT and at 3 months post transplant and findings were correlated with failure free survival (FFS). PET scan was considered negative, when no uptake was present, positive, when any lesion was FDG avid with SUV equal to or greater than 4 and minimal residual uptake positive (MRUp), when any lesion showed abnormal uptake with SUV<4. *Results.* 33 patients were studied, 20 were males and 13 females, median age at ASCT was 26 years (18-53), 18 patients were treated for primary refractory disease, 12 at first relapse and 3 beyond first relapse. All patients received salvage chemotherapy and 22 had chemosensitive disease prior to ASCT. Among the 33 patients included in the study, 25 had a PET scan available pre ASCT, 29 post and 21 at both time points. Pre ASCT PET scan did not correlate with FFS. Five patients had a negative pre-ASCT scan and one of them relapsed, whereas 9 relapses were observed among 20 patients with a positive or MRUp pre-ASCT PET scan. One-year FFS was 100% and 48% respectively (p=non-significant). The figure of 48% remained unchanged for 3 years following ASCT. On the contrary, post-ASCT PET scan had a strong predictive value for outcome. Thus there were 2 relapses among 14 patients with a negative post-ASCT scan, vs 10 among 15 cases with a positive or MRUp post-ASCT PET scan. The corresponding 1-year FFS was 92% vs 34% (p=0.002). Pre-ASCT PET scan was positive or MRUp in 13/18 chemosensitive patients, vs 7/7 chemoresistant ones. Among the 21 patients who had a PET scan available at both time points, there were no relapses recorded for those who were pre-ASCT PET scan either positive or MRUp and became post-ASCT PETscan negative (0/10 patients), as shown in the Table. On the contrary, all patients with a pre-ASCT PET scan positive or MRUp, who remained or became post-ASCT PET scan positive, relapsed (7/7 patients), as shown in the Table. *Conclusions.* Pre-ASCT PET scan positivity does not preclude a positive outcome for patients with relapsed/refractory HL undergoing HDT/ASCT, since half of them remain relapse free for the three subsequent years. Patients who remain or become PET positive after ASCT have an extremely poor prognosis, whereas those who convert to negativity enjoy a favorable outcome.

Table. Relapses according to pre- and post-asct pet scan.

		PRE-ASCT PET SCAN		
		+	MRUp	-
POST-ASCT PET SCAN	+	5/5	2/2	-
	MRUp	0/2	-	0/1
	-	0/6	0/2	1/3

0098

LOW INCIDENCE OF PREMATURE OVARIAN FAILURE IN FEMALE PATIENTS TREATED FOR HODGKIN LYMPHOMA DURING PREPUBERTAL OR POSTPUBERTAL PHASE

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Background. Chemotherapy and radiotherapy may cause infertility in young women with Hodgkin Lymphoma (HL) because of the massive depletion of the ovarian follicle reserve resulting in premature ovarian failure (POF). Factors affecting the risk of POF include the age at the time of therapy, the types of drug used and the intensity of treatment. Without any ovarian protection, the expected rate of POF in postpubertal women is approximately 10-20%, 70% and 90-100% following the use of alkylating-free regimens (ABVD/ABVD-like), alkylating-containing regimens (MOPP/ABV, COPP/ABVD, BEACOPP) and ASCT, respectively. *Aims.* To evaluate the incidence of POF and infertility after chemoradiotherapy in prepubertal girls and in postpubertal women who received a Gonadotropin Releasing Hormone analogue (GnRHa) to prevent ovarian damage during treatment. *Methods.* From January 1991 to August 2008, 115 untreated female patients (pts) with HL aged 8-40 years (median 24) have been treated in our institution. Before HL treatment, 4 pts are prepubertal girls and 111 pts are postpubertal women. To protect ovarian function during chemo-radiotherapy, postpubertal pts received GnRHa monthly, while prepubertal girls not received ovarian protection. HL treatment included 4 to 6 courses of chemotherapy plus radiotherapy for CS I-IIA and 6 courses of chemotherapy plus radiotherapy to residual masses for CS IIB-IV. Overall, 90 pts received alkylating-free chemotherapy and 25 pts received alkylating-containing regimens (MOPPEBVCAD, COPPEBVCAD, COPP/ABV, BEACOPP) as first-line (20 pts) or salvage treatment (5 pts). Four of the 81 irradiated pts received subdiaphragmatic radiotherapy. Ten relapsed/refractory pts received salvage treatment including ASCT in 7 cases and allo-SCT in one case. *Results.* After a median follow-up of 144 months (range 5-204) 3 pts died of HL, 112 are alive and 103 are evaluable for treatment-related gonadotoxicity. All 3 evaluable pts treated during prepubertal phase have today normal menses. Only 7 pts (7%) developed POF, while 93 pts (93%) recovered a normal ovarian function in the group of postpubertal women treated with GnRHa. After treatment, 27 pts attempted pregnancy and conceived. Twenty-six healthy babies were delivered and 3 pregnancies are ongoing. We analyzed risk factors and found that the salvage treatment had a very significant impact on the incidence of POF (p<0.0001). The age (> 30 years) correlates with POF only in pts who received salvage treatment (p=0.05), while first-line treatment with alkylating drugs (p=0.2) and advanced stages of disease (p=0.18) were not significative. *Conclusions.* Our data confirm that prepubertal status may protect the ovaries from the toxicity of chemoradiotherapy. In agreement with this concept, we think that in postpubertal women who received GnRHa during HL treatment, the low incidence of POF following first-line therapy is due to the reversible induction of a prepubertal hormonal milieu during chemoradiotherapy. Unfortunately, in relapsed/refractory pts GnRHa does not seem to be very effective, and further experimental approaches are required for ovarian protection and fertility preservation. In this regard ovarian tissue cryopreservation represents a promising technology that may restore both complete ovarian function and fertility.

0099

COMPARATIVE STUDY OF TWO MOPP-DERIVED CHEMOTHERAPEUTIC PROTOCOLS FOR MANAGEMENT OF PEDIATRIC HODGKIN'S DISEASE: SINGLE CENTER 5-YEARS EXPERIENCE

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Background. In 1990s, countries with limited resources accounted for 86% of the world's children (<15 years), a figure projected to increase to more than 90% by 2030. These countries bear most of the global burden of childhood cancer (World Development Report, 1993). The inability to ensure most of the high-cost chemotherapeutic agents beside the world increment of drug cost with inability of local production of such high-technology medications obligate us among other underdeveloped countries to change the COPP-AV protocol to more available and less expensive COMP or OAP protocols. *Subjects and Methods.* A total of 119 newly diagnosed Hodgkin disease children were treated in the pediatric Hematology/Oncology Unit at Mansoura University Children's Hospital, in the period from January 2002 to December 2006. They were 74 males and 45 females with M/F ratio of 1.64:1 and median age of 8 years (range: 1-16 years). Median follow up was 587 days (19.5 months) with last follow up end at March 2008. Most of the patients were in stage I; 61 cases (51.3%), while stage II; 27 cases (22.7%), stage III; 24 cases (20.1%), and stage IV; 7 cases (5.9%). The histopathological type was mixed cellularity; in 67 cases (56.3%), Nodular sclerosis; in 26 cases (21.8%), Lymphocyte predominance; in 25 cases (21.0%), and lymphocyte depletion; in only 1 case (0.9%). The patients were randomized to receive either alkylating based COMP protocol (Cyclophosphamide 600 mg/m^2 IVI, Oncovin 1.4 mg/m^2 day 1,8 IV, methotrexate 40 mg/m^2 day 1,8 IVI and prednisone 40 mg/m^2 day days 1-14 PO for 8 cycles every 28 days) or anthracycline based OAP protocol (Oncovin 1.5 mg/m^2 day 1,8,15 IV, Adriamycin 60 mg/m^2 day 1,15 IVI and Prednisone 40 mg/m^2/day days 1-14 PO for 8 cycles every 28 days). Procarbazine was substituted in the 1st protocol and omitted in the 2nd one. Sixty patients received COMP protocol and 59 patients received OAP protocol. *Results.* Complete remission was achieved in a higher percentage of patients treated with COMP protocol 81.4% (48 cases), while only it was achieved in 50% of those received OAP treatment (32 cases) (p<0.001). Partial response was more

in those treated with OAP (23%) and less with COMP protocol treatment (5%). Relapse rate was almost equal in both treatment limbs but was earlier with OAP treated patients, as it occurred in 16 patients (27%) in COMP protocol at a duration of 106 days and in 18 patients (30%) in OAP protocol at a median duration of 60 days. The re-induction of remission after that relapse was successful in COMP limb in 7.5% vs. 3% in OAP limb. Acute drug toxicities were minor in both protocols of therapy. Chronic drug toxicities in the form of toxic hepatitis or liver cell failure were recorded in 3 cases only (5%) in COMP protocol patients. On the other hand, the complications were more prevalent in those treated with OAP protocol as 4 cases (6.8%) developed heart failure and 12 patients (20.3%) showed toxic hepatitis or liver cell failure. Five years survival was higher in those received COMP protocol (76.3%), versus 60% in those received OAP protocol ($p<0.01$). *Conclusions.* Patients treated with COMP protocol achieved better response, less relapse, higher survival and less toxicities when compared to those given OAP protocol .

0100

INTERLEUKIN-12 GERMLINE POLYMORPHISM AND OUTCOME OF PATIENTS WITH HODGKIN LYMPHOMA: A PROSPECTIVE STUDY OF THE GROUPE D'ETUDE DES LYMPHOMES DE L'ADULTE

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Background. Although Hodgkin lymphoma (HL) is a highly curable malignancy, about 15% and 30% of patients with respectively localized or disseminated disease do not respond or relapse after initial treatment. Several scoring systems using conventional biological and clinical parameters have been developed for limited and advanced stages in order to adapt the therapeutic strategy. However, the identification of patients with adverse outcome needs to be improved. Cytokines have an important role in the pathogenesis of HL. Among them, interleukin-12 had an important role in stimulating cytotoxic lymphocyte and natural killer cells. A recent study (Cozen *et al.* Blood 2008) showed that IL-12 level of HL patients and their twins was lower than matched controls and a IL-12 polymorphim (rs3212227) that modulate IL-12 expression was associated with an increase risk of HL. *Aims.* In this context, we evaluated the influence of this IL-12 germline polymorphism on response to treatment and outcome in patients with HL. *Methods.* Between 1998 and 2002, we prospectively investigated the prognostic role of plasma cytokines and soluble receptors in Hodgkin lymphoma patients (Casasnovas *et al.* JCO 2007). A sample was collected at diagnosis specifically to investigate single nucleotide polymorphisms (SNPs) in cytokine genes. DNA was extracted from venous blood samples. Genotyping experiments of IL12 rs3212227 (3'UTR +1188A>C) were performed in duplicate using Taqman technology (ABI Prism 7000, Applied Biosystems) in 259 patients. We estimated the prognostic value of this SNP for response to treatment, relapse and overall survival (OS). *Results.* Among the 259 studied patients, 25% were older than 45 years and 56% were male. At diagnosis, 187 patients (72%) were in Ann Arbor stage I-II and 116 (45%) presented with B symptoms. Histology was nodular sclerosis in 208 patients (80%). Treatment consisted of 4 to 6 courses of anthracycline-based chemotherapy (CT) followed by involved-field radiotherapy for stages I-II and 8 courses of anthracycline-based CT for stages III-IV. Complete response (CR) and uncertain CR (uCR) were observed in 228 patients (88%), partial response in 11 (4%) and stable and progressive disease in 20 (8%). Relapse occurred in 46 patients (18%) and 25 patients (10%) died, 18 of whom of HL. After a median follow-up of 4.2 years months, the 4-year progression free survival (PFS) and OS were 81.7% and 92.2%, respectively. Genotypes of IL12 +1188A>C were AA, AC and CC in 166 (64%), 73 (28%) and 20 (8%) patients, respectively. These distributions appear significantly different from what observed in the general population ($p<0.005$). No correlation was observed between initial characteristics of HL patients and IL-12 genotyping. Regarding treatment response, no specific IL-12 genotype was associated with a better CR or influenced OS and PFS. *Conclusions.* Whether HL patients presented a specific IL12 genotype distribution as suggested by our study and Cozen series need to be confirmed. However, we didn't observe in this large series of HL patients with a particularly favorable outcome that this SNP predict response to initial treatment or outcome of patients. The role of host immunogenetics as prognostic factors in HL deserves others studies.

Bleeding disorders (congenital and acquired)

0101

RECURRENT MUTATIONS IDENTIFIED ON LMAN1 AND COAGULATION FACTORS VII, X, XIII GENES

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Rare Bleeding Disorders (RBDs) as Factor (F)V, FV+FVIII, FVII, FX, FXI and FXIII deficiencies, afibrinogenemia and hypoprothrombinemia, are transmitted as autosomal recessive traits. RBDs are relatively rare in Europe (1:0.5-2 millions), but their frequency is increasing due to the high rate of immigration from the Middle East and North Africa where the incidence is significantly higher. In 2004 an International RBDs Database (RBDD: www.rbdd.org), structured to collect and to report phenotype, genotype, clinical and therapeutic information on each single disorder, was developed. This database contains data on 310 not related patients from all over of the world. Of them, 258 have been genetically characterized, with only 6% (16 patients) lacking a gene mutation in the coding region and 400bp of 5'-3'UTR. Out of 172 identified mutations, 52% (90) were missense, 12% (21) nonsense, 12% (21) splicing site, 21% (35) insertion/deletion and 3% (5) were located at the 5'UTR. A careful analysis of RBDD showed common mutations specific to some geographical areas: -p.Gln160Arg (originally reported as Gln100Arg) mutation on FVII gene was confirmed to be present only in Europeans, being found only in Italians (5 families out of 20, 25%) and in one Swedish although 62 families coming from different countries (Table 1) were characterized; -p.Arg40Thr (Arg-1Thr) mutation, on FX gene, was found only in Iranians (4 families out of 23, 17%), although 33 families coming from different countries (Table 1) were characterized; -p.Met1Thr mutation on LMAN1 gene was confirmed to be present only in Italians (4 families out of 8, 50%), although 26 families coming from different countries (Table 1) were characterized; p.Gly216Arg (Gly215Arg) and p.Arg78His (Arg77His) mutations on FXIII gene, were found respectively only in Serbians (100% of studied families) and in Iranians (6 families out of 20, 30%), although 30 families coming from different countries (Table 1) were characterized. The haplotype analysis in future will help to explain more on each genetic mutation distribution in different ethnic groups and eventual "founder" effect. Our results suggest the existence of recurrent mutations in specific geographic areas which could help for prevention of these disorders through prenatal diagnosis in families with already one severe affected child, particularly in those countries with low economic resources.

Table 1.

GENE	Recurrent mutations	N° characterized families	Countries	N° families in which the mutation was found and country of origin	% of families from the same geographical region with the same mutation
FVII	p.Gln160Arg (Gln100Arg)	20 25 4 1 1 3 3 1 1 1 1 1	Italy Iran Turkey Sweden Hong Kong India Serbia Greece Pakistan Romania Arabia USA	5 Italians 1 Swedish	25% -
FX	p.Arg40Thr (Arg-1Thr)	5 23 2 1 1 1	Italy Iran Turkey France India UK	4 Iranians	17%
LMAN1	p.Met1Thr	8 2 2 13 1	Italy Iran Turkey India Serbia	4 Italians	50%
FXIII	p.Gly216Arg (Gly215Arg)	3 20 2 1 3 1	Italy Iran Lebanon India Serbia Greece	3 Serbians	100%
	p.Arg78His (Arg77His)			6 Iranians	30%

0102

MANAGEMENT OF ACUTE HAEMARTHROSIS IN HAEMOPHILIA: A EUROPEAN SURVEY

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The treatment of haemarthrosis in a person with haemophilia may involve several different treatment modalities including factor replacement therapy, joint aspiration, ice, non-weight bearing interventions and/or immobilisation, analgesics including anti-inflammatory agents and rehabilitation. However, few data are available on the optimal management of acute haemarthrosis in practice. Such information may be critical in view of recent insights into the pathophysiology of haemophlilic arthropathy. Current management algorithms for acute haemarthrosis were surveyed among 23 haemophilia physicians representing 15 different European countries and responsible for the care of 3,633 patients. Three clinical scenarios of acute haemarthrosis in patients with haemophilia A were presented and management was recorded using 16 questions per scenario. For moderate haemarthrosis, a first dose of 30 U/kg FVIII was given by 75 % treaters once a day (88%) and repeated on day 2 (66%) and up to day 4 (22%). At presentation of a severe haemarthrosis, a first dose of 40-50 U/kg was given by 75 % treaters and repeated on day 1 (81 %). Replacement therapy was continued up to day 3 (77%) or 4 (54 %). Aspiration was considered by 19% of the physicians. Inhibitor screen was ordered by 27%, factor assays 70%, and imaging 57%. Active additional interventions in case of a severe bleed included immobilisation (splint or cast) (71%) and non-weight-bearing (85%). Analgesics were used in most cases, but steroids, NSAIDs and antifibrinolytic agents were used infrequently (<20%). In conclusion, this survey highlights potentially important variation in the management of acute haemarthrosis across the EU with respect to intensity and duration of replacement therapy as well as use of adjunctive therapies. Local practice and national guidelines may need to be revised in light of recent advances in the understanding of the pathogenesis of haemophilic arthropathy.

0103

CIRCULATING VERSUS PROGENITOR ENDOTHELIAL CELLS ARE ABNORMAL IN PATIENTS WITH DIFFERENT TYPES OF VON WILLEBRAND DISEASE AND CORRELATE WITH MARKERS OF ANGIOGENESIS: A COHORT STUDY OF 74 CASES

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Background. von Willebrand disease (VWD) is the most common inherited bleeding disorder and is caused by quantitative (VWD1 and VWD3) or qualitative (VWD2A, VWD2B, VWD2M, VWD2N) defects of von Willebrand factor (VWF). VWF, synthesized by endothelium and magakaryocytes, circulates in plasma and is present in sub-endothelium and platelets: therefore VWF is an ideal marker for endothelial formation/ damage and megakaryocytopoiesis. Circulating (CEC) and progenitor (CEP) endothelial cells have been also recently proposed as markers of peripheral and bone marrow-derived angiogenesis. *Aims of the study, Patients and Methods.* To evaluate the association of CEC/CEP with cellular and circulating VWF, we have measured the number of CEC/EPC together with VWF and cytokines involved in angiogenesis in a cohort of 74 patients with different VWD types. Seventy-four VWD patients were diagnosed according to the recommendations of the Scientific Standardization Committee on VWF of the ISTH. VWD cohort was composed by the following VWD types (number): VWD1 (22), VWD2A (9), VWD2B (19), VWD2M (17), VWD3 (7). Twenty healthy individuals were used as controls. CEC ($CD146^+$, $CD31^+$, $CD45^-$) and CEP ($CD34^+$, $CD133^+$, $CD45^-$) were evaluated by flow cytometry. Serum levels of VEGF, E-selectin, P-selectin, EPO and TPO were determined by ELISA. Both CEC/EPC and cytokines were analyzed by the same authors in blind, i.e. without knowing in advance VWD diagnosis. Continuous variables were expressed as median and range. CEC, EPC, VEGF, E-selectin, P-selectin, EPO, TPO were all left-skewed, so that a logarithmic transformation was performed before statistical analysis in order to approximate a normal distribution. Comparison between groups was made by Student's t-test or one-way ANOVA where needed. To evaluate the influence of VWF on CEC and EPC levels, a simple linear regression analysis was performed. Adjustment for age, sex and WBC levels was made by a multiple linear regression analysis. Correlation analysis was performed by using the Spearman's rank correlation test. $p<0.05$ was taken as a cut-off point for statistical significance. *Results.* CEC, E-selectin, VEGF and EPO tended to be higher in all VWD patients than in controls. TPO was particularly high in VWD3 patients but not in the other VWD types, while P-selectin serum levels were almost similar in VWD patients and in controls. Conversely, EPC were lower in all VWD patients than in controls. Considering VWD patients all together, there was a statistically significant difference between VWD patients and controls in mean levels of CEC, EPC, VEGF, E-Selectin and EPO ($p<0.01$ for all of them), while no statistically significant difference was found for P-Selectin and TPO. Dividing VWD patients into types (VWD1, VWD2A/2M, VWD2B, VWD3), a statistically significant difference was found for CEC (one-way ANOVA: $p=0.005$), EPC ($p=0.001$), E-Selectin ($p<0.0001$), EPO ($p=0.021$) and TPO ($p=0.004$). VEGF showed a trend towards significance ($p=0.061$), while for P-Selectin no significant difference was found ($p=0.952$). Considering only VWD1, we found a significant inverse relationship between CEC and VWF:Ag plasma levels ($p=0.048$; $R^2=0.19$). The relationship was still significant after adjustment for age, sex and WBC in a multiple linear regression analysis ($p=0.046$). *Discussion and Conclusions.* Based on these results, we can conclude that CEC are increased in VWD patients, especially in VWD2B and 3: high CEC are associated with increased levels of cytokines involved in angiogenesis (up-regulation). Conversely, CEP are always decreased in VWD patients, especially in VWD1 and VWD2A/2M, suggesting down-regulation of bone marrow-derived angiogenesis. This abnormal results on CEC/CEP in patients with inherited deficiencies of VWF might suggest a major role of VWF in peripheral and bone marrow-derived angiogenesis

0104

THE HEMOSTATIC POTENTIAL OF ACUTE PROMYELOCYTIC LEUKEMIA CELLS BY THE CALIBRATED AUTOMATED THROMBOGRAM ASSAY: MODULATION BY ARSENIC TRIOXIDE AND ALL-TRANS RETINOIC ACID

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Background. Acute promyelocytic leukemia (APL) cells highly express both procoagulant and anticoagulant activities. Overall these activities contribute to the coagulopathy typical of APL patients. Differentiation therapy with all-trans retinoic acid (ATRA) or arsenic trioxide (ATO) induces the APL molecular remission and affects the cellular hemostatic properties. *Aims.*We wanted to characterize the APL cell hemostatic potential by the calibrated automated thrombogram (CAT) standardized global assay, which reflects the net results of pro- and anti-coagulant forces. The endogenous thrombin potential (ETP), measured as the area under the thrombin generation (TG) curve, is a good indicator of overall plasma prothrombotic and hemorrhagic tendency. We evaluated 1) the sensitivity of NB4 cell TG potential to treatment with ATRA or ATO; 2) the correlation of CAT parameters to the levels of two known procoagulants, i.e. tissue factor (TF) and cancer procoagulant (CP), and 3) the association of global TG to cell differentiation, proliferation, and apoptosis/necrosis. *Methods.* NB4 cells TG was measured before and after 24h incubation with either 0.1 μM ATO, 1 μM ATRA, the combination 0.1 μM ATO/1 μM ATRA or the vehicle (control). TG potential of NB4 was evaluated in normal pool plasma (NPP) by CAT assay; TF by chromogenic, immunological, cytofluorimetric assays; TFmRNA by RT-PCR; CP activity by chromogenic assay; cell differentiation by cytofluorimetric analysis of surface CD11b expression; and cell apoptosis/necrosis by annexin V/propidium iodide staining. *Results.* Before any treatment the TG potential of NB4 cells (=1350±70 nM*min) was significantly increased compared to normal granulocytes ($p<0.05$). When the CAT was performed in the absence of factor VII (FVII), a significant decrease of ETP was observed, confirming a major role to global TG for TF, but also suggesting a role for other procoagulants (i.e. CP, phospholipids) to the remaining FVII-independent activity. Both ATRA

and ATRA/ATO significantly reduced the NB4 TG (980±100 nM*min, and 1090±90 nM*min, respectively) compared to untreated cells, whereas ATO alone had a minor effect (1200±120 nM*min). Results of specific assays to identify TF and CP confirmed a greater effect of ATRA and the combination ATO+ATRA in reducing both procoagulants compared to ATO alone. The observed modulation of TG potential by the two drugs paralleled the reduction of cell proliferation due to induction of apoptosis and cell differentiation (ATRA and ATO+ATRA) or to cell necrosis (ATO). *Summary and Conclusions.* The CAT assay appears to be a reliable and sensitive method for characterizing the TG potential of APL cells. It can be a valuable tool to characterize the overall hemostatic phenotype (both procoagulant and anticoagulant) of APL cells and ultimately to understand the role of different TG phenotypes in predicting the outcome of the APL coagulopathy.

0105

SINGLE CENTRE EXPERIENCE OF DESMOPRESSIN RESPONSE IN CHILDREN WITH MILD FVIII DEFICIENCY, VON WILLEBRANDS DISEASE AND PLATELET FUNCTION DEFECTS

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Background. Desmopressin(1-deamino-8-D-arginine-vasopressin) is a synthetic vasopressin analogue that increases factor VIII levels in mild Factor VIII deficiency, von Willebrand factor in von Willebrands disease(VWD), and improves bleeding time in some platelet function defects(PFD), thus avoiding the risk of acquiring blood-borne viral infections. Most clinical studies of Desmopressin (DDAVP) response have been in adults with only a few studies reviewing DDAVP efficacy in paediatric patients. It is suggested that response is lower and less predictable in children, particularly in younger children and those with lower baseline levels. *Aims.* We wished to evaluate the safety and efficacy of intravenous DDAVP in paediatric patients with mild factor VIII deficiency, VWD (type 1, type 2A and type 2M) and PFD, and determine if age and baseline values were predictors of response. *Methods.* A retrospective study was conducted of all children diagnosed with mild factor VIII deficiency (baseline FVIII:C <0.05iu/dL), VWD and PFD, who had a trial of treatment with intravenous DDAVP in an 11 year period between 1997-2008. Exclusion criteria included patients aged <2 years, history of heart disease or epilepsy, type 2B or type 3 VWD. Baseline diagnostic chromogenic factor VIII (FVIII:C) levels, von willebrand factor antigen(VWF:ag) and ristocetin co factor (VWF:RCo) and platelet function abnormalities were determined. All received 0.3 mcg/kg DDAVP over 30 minutes and further testing was performed on samples obtained prior to and one hour post DDAVP infusion. Response was defined as normalization of FVIII:C levels, VWF:ag, VWF:RCo levels or underlying platelet function defect. Adverse events associated with Desmopressin administration were recorded. *Results*. Two hundred and six children, aged 2-15 years (median 6.5yrs), were included. Fifty one (25%) had mild Factor VIII deficiency, 118 (57%) had VWD, {104 (88%) had type 1VWD, 5 (4%) had type 2A VWD and 9 (8%) had type 2M VWD}, and 37(18%) had a PFD. Thirty eight children (74%), aged 2-11 years (median 3.5yrs) with Factor VIII deficiency had a therapeutic response. Their baseline FVIII:C level was 0.18-0.47iu/dL (mean 0.33iu/dL). The age (2-11yrs, median 3yrs) of the non-responder group was similar but baseline levels were lower (mean 0.19iu/dL, range 0.09-0.47iu/dL). One hundred and one (85%) children, aged 2-15yrs median 5.5yrs, with VWD had a therapeutic response. Their mean baseline VW:ag level was 0.46iu/dL (range 0.18-0.46iu/dL) and VW:RCo level of 0.31iu/dL (range 0.09-0.47iu/dL). The non-responder group had a median age of 4.5 yrs (range 2-15 yrs), with mean baseline VW:ag levels of 0.28iu/dL (range 0.1-0.22iu/dL) and VW:RCo levels 0.22iu/dL (range 0.07-0.13iu/dL). VWD subtype did not influence response. Seventeen of thirty three (51%) children with a platelet function defect showed a therapeutic response. There was no age difference between the 2 groups. There were 6 adverse events, 4 children reported headaches and 2 developed hyponatraemia. *Conclusion.* Desmopressin is an effective and safe treatment modality for children with mild Factor VIII deficiency, VWD and PFDs but age, baseline FVIII:C, VW:ag or VW:RCo do not reliably predict response and a DDAVP trial should always be undertaken.

0106

GENZ-112638, AN INVESTIGATIONAL ORAL THERAPY FOR GAUCHER DISEASE TYPE 1: PHASE 2 CLINICAL TRIAL RESULTS AFTER ONE YEAR OF TREATMENT

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Background. Genz-112638 is a novel oral small molecule inhibitor of glucosylceramide synthase under development for the treatment of Gaucher disease type 1 (GD1). *Aim.* An open-label Phase 2 clinical study to evaluate efficacy, safety, and pharmacokinetics of Genz-112638 in patients with GD1 has recently completed 52 weeks of treatment. *Methods.* This clinical trial of Genz-112638, given 50 or 100 mg bid orally, treated 26 adults with GD1 (16F:10M; mean age 34 years, range 18-60; all Caucasian) at 7 sites in 5 countries. Eligible patients were required to have splenomegaly (volume≥10x normal) and either thrombocytopenia (platelets 45,000-100,000/mm^3) or anemia (hemoglobin 8-10 g/dL, female; 8-11 g/dL, male). None received enzyme replacement or substrate reduction therapy in the prior 12 months. The composite primary efficacy endpoint is based on improvements in ≥2 of the 3 main parameters: spleen volume (-15%), hemoglobin level (+0.5 g/dL) or platelet count (+15%) after 52 weeks of treatment. Liver volume, chitotriosidase, glucosylceramide are also assessed. Patients continue to be treated and monitored long-term. *Results.* Twenty-two patients completed the study to Week 52; 4 patients withdrew. The 52-week composite primary endpoint was met by 20/26 patients. Mean (±SD) changes from baseline to Week 52 were: hemoglobin +1.6 (±1.28) g/dL; platelet count +40.3% (±37.49%); spleen and liver volume (multiples of normal) 38.5% (±11.42%) and 17.0% (±10.48%), respectively; and chitotriosidase (median change) 51.3% (±17.16%). Plasma glucosylceramide levels normalized in all patients. Genz-112638 was well tolerated with an acceptable safety profile. In the first 52 weeks of treatment, the majority of AEs (91%) were reported as unrelated to study treatment. All of the AEs reported as treatment-related (7 AEs in 6 patients) were mild and transient. *Summary and Conclusions.* Results from this Phase 2 study indicate that Genz-112638 may be a safe, effective, and convenient oral therapy for patients with GD1. Clinical development of Genz-112638 is proceeding, and Phase 3 studies are planned.

0107

GAUCHER DISEASE DIAGNOSTIC AND DISEASE MANAGEMENT ALGORITHMS: A GUIDE FOR HEMATOLOGISTS

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Background. Gaucher disease (GD) is an inherited deficiency of acid β-glucocerebrosidase leading to abnormal glycolipid accumulation in macrophage lysosomes and consequent hematologic, visceral, and skeletal abnormalities. GD is rare (estimated prevalence 1:60,000, though more common in individuals of Ashkenazi Jewish descent) and clinically heterogeneous, and therefore can present a diagnostic challenge. *Aims.*GD patients often present to haematologists when unexplained haematologic abnormalities (thromobocytopenia, anemia) are identified, often in conjunction with enlarged spleen. Recent studies have found that GD does not have a high index of suspicion (Mistry *et al.* 2007) and is often only considered when other possibilities excluded;

hematologic malignancy is more often suspected. Prompt diagnosis is critical as diagnostic delay increases the risk of disease progression, inappropriate intervention (such as splenectomy) and sub-optimal treatment. *Methods.* In order to raise awareness about GD and facilitate diagnosis, an international group of GD experts was convened to review GD management guidelines and to determine under which circumstances GD should be given greater priority in differential diagnosis. *Results.* Algorithms for diagnosis specifically geared toward haematologists were created. Separate algorithms were developed for individuals of both Ashkenazi Jewish and non-Ashkenazi Jewish background. The algorithms include a review of the differential diagnosis and guidance on diagnostic procedure when GD is suspected. GD can be confirmed or eliminated through a simple enzyme activity assay performed on peripheral blood leucocytes. DNA analysis and bone marrow biopsy can provide additional information about disease burden but should not be considered diagnostic. Diagnostic algorithms are accompanied by disease management algorithms focusing on assessment and monitoring, which reflect published therapeutic goals and monitoring guidelines, to simplify decision making in the evaluation, treatment and ongoing monitoring of GD. *Summary and Conclusions.* The identification of symptoms highly suggestive of Gaucher disease and creation of diagnostic algorithms geared specifically toward haematologists may help decrease the time to diagnosis for patients affected by this rare, heterogeneous but treatable disorder. Associated guidelines for disease assessment and monitoring may simplify the development of patient-specific management plans.

0108

THE SEQUENTIAL COMBINED BYPASSING THERAPY IS EFFECTIVE AND SAFE IN THE TREATMENT OF UNRESPONSIVE BLEEDS IN HAEMOPHILIA PATIENTS WITH INHIBITORS

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Background. Development of inhibitors is today the most severe and challenging event in the treatment of patients with haemophilia. Replacement therapy with the missing coagulation factor is ineffective in high-responders and bypassing agents are required. Unfortunately, whatever by-passing agent is initially used, some bleeds (10-20%) cannot be controlled. When all treatments have failed the sequential administration of 2 bypassing agents (SCBT) has been recently reported to have a positive synergistic effect. *Aims.*To provide a deeper insight on efficacy and safety of SCBT. *Methods.* A survey on each course of SCBT given was conducted by the European Haemophilia Treatment Standardization Board by means of a web-based database. SCBT was defined as the administration of recombinant factor VIIa (rFVIIa) and activated prothrombin complex concentrate (APCC) within 12 hours from each other. *Results.* SCBT was used in 2 children (aged 8 and 14 year-old) and 4 adults (mean age: 34, range: 24-45 years) with haemophilia A (6 patients) and B (1 patient), all patients with high-responding inhibitors and unresponsive to APCC and rFVIIa. The children were suffering from joint bleeds refractory to high doses of rFVIIa (up to 270 µg/Kg/2h) and to high doses of APCC (up to 80 U/Kg/8h). Three adults had undergone major surgery (removal of knee prosthesis, knee arthrodesis, laparotomy for kidney rupture), initially treated with rFVIIa from 120 up to 270 µg/Kg/2h, followed by significant bleed. One of these patients was switched to APCC 80 U/Kg/8h without success. The fourth adult was suffering from lower limb compartmental syndrome and had no response after 5 administration of 180 µg/Kg every 3h and two infusions of FEIBA 75 U/Kg. SCBT was administered in children and adults alternating one APCC dose (range 50-80 U/Kg) to one or two rFVIIa doses (range 90-270 µg/Kg) every 4-12 hours. Complete bleeding control was achieved in 12-24 h in all patients. SCT was discontinued after 2-15 days. All patients underwent prophylaxis with FEIBA or rFVIIa thereafter. No clinical adverse event was observed, but a rise of D-dimer levels occurred in 3 of 6 patients, without a consumption of platelet and/or fibrinogen. *Conclusions.* SCBT might represent a valid salvage treatment of unresponsive bleeds. A much larger and prospective clinical trial is needed to confirm these findings.

0109

PREVALENCE OF VWD IN IRAN

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Background. vWD described by Dr Erik von willebrand in 1962. It is the most frequent congenital bleeding disorder, Caused by the quantitative deficiency (type I&III) or dysfunction (type II) of vWF. The disease inherited in an autosomal dominant/ recessive pattern. The principle clinical symptoms of vWD are prolonging bleeding after surgeries and mucosal bleeding. Excessive bleeding at the time of menstruation and during childbirth is a frequent problem in women of childbearing age. vWD is classified into 3 different types; Type I, the most frequent type of vWD, (~ 80-85%) inherited in an autosomal dominant pattern. Type II vWD (Subtype: 2A, 2B, 2M, 2N) also transmitted in an autosomal dominant trait (~15%). Type 3 is rare (o.5-5%), but the most severe form of vWD, inherited in an autosomal recessive pattern. Clinically significant vWD is estimated 50-100 per million populations. *Aims.* To evaluate prevalence of vWD and distribution of different types, pattern of bleeding and type of complications associated with pregnancy and childbirth. *Methods.* Data collected from Tehran University, Imam Khomeini Hospital, Hemophilia Center retrospectively. *Results.* 900 patients with vWD are registered in our National Registry includes 426 females & 474 Males. Distribution of types of vWD in Iran: Type I: 118 Pts (13%), type II: 142 Pts (16%), type III: 460 Pts (71% of all cases). *Conclusions.* Menorrhagia is one of the most frequent complications in women with vWD and has a negative effect on their quality of life. Management of menorrhagia in women with vWD should be provided by a multidisciplinary team including a Haematologist and Gynacologist. There was no evidence of reduced fertility in married women. *Discussion.*Epistaxis and menorrhagia are the most frequent bleeding symptoms especially in typeIII vWD. Frequency of type 3 vWD and other bleeding disorders which inherited in an aoutosomal recessive trait increased by 5-10 times where consanguineous marriage is high, such as Iran and Soutern India. In Iran, prevalence of type III is 9 per million populations (Iran has 70 million population). The high prevalence of Type III and a low prevalence of type1 vWD in developing countries, suggests the low awareness of disease as also under-diagnosis of the mild cases.

Table 1. Types of (%) bleeding symptoms in 900 Iranian patients with vWD.

No	Bleeding symptoms	Type III (640 Pts)	Type II (142)	Type I
1	Epistaxis	77	63	61
2	Menorrhagia	69	36	32
3	Post dental extraction bleeding	70	39	31
4	Hematomas	35	14	13
5	Bleeding from wound	52	40	36
6	Gum bleeding	55	35	31
7	Post –surgical bleeding	41	23	20
8	Post partum bleeding	16	18	17
9	Gastrointestinal bleeding	20	8	5
1	Joint bleeding	37	4	3
1	Haematuria	1	5	2
1	Cerebral bleeding	5	2	0

0110

COMPARATIVE STUDY OF CLINICAL, X-RAY AND MRI SCORES IN THE FOLLOW-UP OF HAEMOPHILIC ARTHROPATHY

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Arthropathy is the most frequent cause of morbidity in patients with severe haemophilia. Once established, arthropathy is considered as an irreversible or progressive complication, even in children on prophylaxis. To estimate the role of clinical, x-ray and MRI scores in the evaluation of progression of haemophilic arthropathy, 84 joints of 24 boys with severe (n=18) and moderate (n=6) haemophilia (A: 22, B: 2) were investigated with clinical examination, x-rays and MRI at two time periods (Time 0 and 1). Patients' age at Time 0 was 10.5±3.6 years and time elapsed to time 1 was 3.8±1.4 years. At Time 0: All investigated joints had a history of more than three bleeds. 16 boys were on secondary prophylaxis for 5.4±2.8 years. Clinical score: 2.0±3.6, Pettersson score: 2.1±2.8, Denver score: 4.5±3.8. After the first evaluation prophylaxis was intensified in 11 children and initiated in 4. At Time 1: Clinical score: 1.5±3.1, Pettersson score: 1.7±2.7, Denver score: 5.1±4.1. The analysis of results provided evidence that the information carried by the three scores could be divided into two parts: a) overlapping information given by one score that was explained by the information given by the other, b) information given by one score that was not explained by the other. At both examinations, the x-ray (Pettersson) score was acting as a mediator, correlating well both with the clinical and the MRI (Denver) score while the last two were more alienated from each other. The correlation of x-ray with the Denver score was significantly reduced at the second examination, while it was slightly improved with the clinical score. Comparing the findings, deterioration was found in 15.3%, 15.3% and 34.1% and improvement in 25.9%, 40% and 16.5% of the joints with clinical, x-ray and MRI scores, respectively. Besides, in 58.8%, 44.7% and 49.4% of the joints no progress of arthropathy was found with the three scores, respectively. Moreover, the number of haemarthroses per joint per year was reduced (0.7±1.76 vs. 2.0±1.8) (p<0.01). Initiation or intensification of prophylaxis resulted in three fold reduction of haemarthroses (p<0.01) and significant improvement of clinical and Pettersson scores (p<0.01). On the contrary, MRI findings were mostly progressive with the exception of 16.5% of joints with reversible elements (mild or moderate synovitis). *Conclusions.* Apart from the information shared by the clinical, x-ray and MRI scores, each of them provide different and additional information which should be estimated separately by the physician in the follow-up of evolution of haemophilic arthropathy.

0111

MUTATION SPECTRUM OF SEVERE VON WILLEBRAND DISEASE TYPE 3 IN INDIA

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Background. von Willebrand disease (VWD) is the most common autosomal inherited bleeding disorder caused by defects of von Willebrand factor (VWF). Severe type 3 VWD is characterized by complete absence or presence of only trace amounts of non-functional VWF. Severe VWD is caused by small insertions and deletions, splice site, missense and nonsense mutations of the VWF gene respectively but large deletions are a rare cause of type 3 VWD. *Aims.* To evaluate the VWF gene mutation spectrum in severe type 3 VWD patients from India. *Methods.* This study includes 20 unrelated Indian patients previously diagnosed with severe VWD type 3 by conventional tests and multimer analysis. Mutation screening was done by PCR and direct sequencing of the coding region (exon 2-52) of VWF including intronic flanking regions. Characterization of breakpoints of two novel large deletions was done by using walking primer pairs. *Results.* Twenty-five different mutations were identified including 17 novel mutations. Ten patients were homozygous, six were compound heterozygous, one patient had a gene conversion with the VWF pseudogene on chromosome 22 comprising the mutations V1279I, Q1311X, A1317, I1343V, V1360A and F1369I. In one patient no candidate mutations were identified. In a second patient only one mutation was found. A third patient was homozygous for a silent mutation in exon 26 (N1138N) that might possibly affect splicing. Six different nonsense mutations were detected. Five patients had homozygous nonsense mutations, four patients were compound heterozygous including two patients with an additional third mutation. Six patients carried deletions including four small novel deletions (1bp or 2bp) while two carried large novel deletions of ≈81 kb and ≈2.3 kb, respectively. *Conclusions.* Our results reveal that VWD type 3 is caused by a broad spectrum of mutation located in the whole VWF gene. Large homozygous deletions were found in two unrelated patients. Nonsense mutations and deletions were found to be a common cause of severe type 3 VWD in India.

0112

INCIDENCE OF LUPUS ANTICOAGULANT IN HEMOPHILIA PATIENTS

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Background. Two types of pathological inhibitors of coagulation have been developed in hemophilia patients: inhibitor against factors VIII(IX) and lupus anticoagulant. *Aims.* To investigate the incidence of lupus anticoagulant in hemophilia patients depending on severity of the disease, titers of inhibitors against factors VIII (IX) and type of responsiveness of patients' immune system (low-responder or high-responder). *Patients and methods.* 82 hemophilia patients were investigated (69 -hemophilia A, 11- hemophilia B, 1 - hemophilia C and 1 - hemophilia (A+B)). Their age ranged from 10-77 years. There were 46 hemophilia patients with inhibitors and 36 persons with suspicion of LA presence or clinical signs of resistance to substitution therapy. All patients underwent general clinical and laboratory tests, coagulological investigations necessary for diagnostics, as well as determination of coagulation factors and activity of inhibitor of the deficient procoagulant. Investigation of LA activity was performed using three-step complex of tests according to the international criteria. *Results.* In the investigated group of patients LA activity was detected in 24 (29.3%) patients with hemophilia, 20 of them (24.4%) had hemophilia A and other 4 (4.9%) - hemophilia B. Particularly, laboratory signs of anticoagulant were found in 39,1% of patients with specific inhibitors and in 16,7% of patients without inhibitors of coagulation factor VIII (IX). The proportion of LA-positive persons in the group of patients with immune inhibitors of coagulation factors was significantly larger than in patients without inhibitors (χ^2=3.897, p<0.05). There was no LA activity detected for patients with hemophilia C and hemophilia (A+B). No influence of age on the incidence of LA in hemophilia patients was found, as well as connection between the anticoagulant and the disease type and severity. The main clinical manifestations of LA presence were relapsing hemorrhagic complications and resistance to the substitution therapy with plasma products. The signs of thrombosis were detected in 1 patient during the substitution infusion therapy. *Conclusion.* Effects of the lupus anticoagulant were revealed in 29.3% of hemophilia patients, particularly - 24.4% with hemophilia A and in 4.9% with hemophilia B. In patients with inhibitor form of hemophilia the antiphospholipid activity was diagnosed significantly more frequently than in patients without specific inhibitors. Age of patients, disease type and severity had no influence on the incidence of lupus anticoagulant in patients with hemophilia. Clinical consequences of the occurrence of lupus anticoagulant activity in hemophilia patients may be represented by hemorrhage relapse as well as thrombotic complication.

0113

INDIVIDUALISED ORAL VITAMIN K FOR REVERSAL OF WARFARIN EXCESSIVE ANTICOAGULATION

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Background. Warfarin over-anticoagulated patients present a wide range of international normalized ratio (INR) values and may respond differently to fixed doses of vitamin K. Consequently a high proportion of patients still remain outside their target INR 24 hours after vitamin K treatment, being prone to either hemorrhage (if the patient is still over-anticoagulated) or thromboembolism (if the INR is over-corrected). *Aims.* 1. To assess the effectiveness of a *tailored* vitamin K dosing regimen

based on individual patient INR, in the reversal of warfarin over-anticoagulation. 2. To determine the impact of patient age, gender, weight, height, body surface area, warfarin daily dose, target INR and CYP2C9 and VKORC1 polymorphism on the rate of INR reversal. *Methods.* Patients on warfarin, either asymptomatic or with minor hemorrhagic complications, and with a venous INR >6.0 were recruited into the study. Written informed consent was obtained. Oral vitamin K was administered to the patient and unless already taken the day's dose of warfarin was omitted. Vitamin K dose was calculated using a regression equation produced from a previous study and it is based on initial INR and target INR. Venous INR was determined again 24 hours after vitamin K administration. Regression analysis was used to evaluate the impact of patient characteristics and genetic polymorphisms on INR reversal. *Results.* Eighty seven events (38M/49F) were included in the study. Total number of patients was 69, with 14 of them having more than one episode requiring vitamin K (one event in 55 patients, two events in 11, three events in 2 and four events in 1). Initial INR ranged from 6 to 16 (median 7.4), median vitamin K administered dose was 1.7mg (range 1.1-3.4) and median INR after 24 hours was 2,4 (range 1.3-6.4). Out of the 87 analysed events, INR after 24 hours was in range in 33 (37.93%), above range in 17 (19.54%) and below range in 37 (42.53%). Change in INR (initial INR - INR after 24 hours) showed a statistically significant correlation with dose of vitamin K, body surface area, not omitting warfarin on day zero and target INR. Genetic variants for CYP2C9 and VKORC1, tested in a subgroup of 66 events, did not show any impact on INR reversal although, as previously described, they correlated with warfarin daily dose. *Conclusions.* The individualized vitamin K dose did not result in a significant improvement on the percentage of patients reaching their target INR after 24 hours compared to previously published results. Factors that influence INR reversal are vitamin K dose, body surface area, target INR and whether the patient takes warfarin on the same day that vitamin K is administered. Those factors should be used in subsequent studies for accurate vitamin K dose calculation. Genetic variants of CYP2C9 & VKORC1 do not seem to affect response to vitamin K, although due to the low number of patients it needs confirmation in larger studies.

0114

NOVEL ASSOCIATION BETWEEN ACQUIRED TYPE 2 VON WILLEBRAND DISEASE AND GLYCOGEN STORAGE DISEASE IA: A REPORT OF 3 AFFECTED SIBLINGS

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Background. Glycogen storage disease type Ia (GSD Ia) is an autosomal recessively inherited disorder of carbohydrate metabolism caused by defects of the glucose-6-phosphatase complex which results in inadequate glucose production. An associated bleeding diathesis has been recognized since the 1970s and was initially attributed to a platelet function defect but defects in von Willebrand function (VWF) have since been described. Defects have been found in 60% of screened patients with GSD Ia but published cases are almost exclusively mild type 1 von Willebrand disease (VWD). Correction of the glucose with continuous gastric feeding or intravenous glucose infusions for 24-48 hours has been shown to correct the coagulopathy and is often recommended prior to elective surgery. We describe three female siblings affected by GSD 1a who developed acquired type 2 VWD. VWF parameters were not corrected by glucose infusion. *Patients and Methods.* A 13 year old girl with GSD Ia presented with severe periorbital bruising following mild blunt trauma. She had previously undergone portacath insertion without significant bleeding or bruising. Investigations revealed a prolonged APTT 40 sec (26.7-37.6), FVIII:C 0.43U/mL (0.50-1.50), VWF:Ag 0.15U/mL (0.46-1.46), VWF:RCo 0.05U/mL (0.50-1.72), compatible with type 2 VWD. The mulitimer pattern was normal. A 48 hour trial of 10% glucose infusion resulted in an improvement in FVIIIC and APTT but there was no effect on VWF:Ag or VWF:RCo. Desmopressin (DDAVP) resulted in FVIII:C 1.18U/mL,VWF:RCo 0.6U/mL at 1 hour post infusion and has been used for minor bleeding. Intermediate purity factor concentrate (Haemate P) has provided adequate haemostasis for minor surgery. An 8 yr old sister, also with GSD Ia, was found to have similar results to her sister: APTT 40.7 sec VWF:Ag 0.18U/mL, VWF:RCo <0.05U/mL, FVIII:C 0.43U/mL with normal VWF multimers. She bruised easily but had previously undergone tonsillectomy and portacath insertion without complication. A 20 yr old sister, also affected with GSD Ia, was found on testing to have results consistent with type 2 VWD but with a less marked defect of VWF than her two sisters: FVIII:C 0.63U/mL, VWF:RCo 0.23U/mL. She had undergone dental extractions, a liver biopsy and multiple central venous catheterisations without abnormal bleeding. Both parents had normal coagulation profiles, FVIII:C, VWF:Ag, and VWF:RCo. We conclude that all three siblings have acquired type 2 (probably 2M) VWD in association with GSD Ia. We discuss the possible mechanism of this association. *Conclusions.* Although acquired type 1 VWD in association with GSD Ia is reported, this may not be widely recognised even amongst paediatric haematologists. This report is the first, to our knowledge, of affected siblings with acquired type 2 VWD associated with GSD Ia. Previous surgery without bleeding complications does not exclude a severe coagulopathy and we recommend that all patients with GSD should be investigated for VWD prior to surgical procedures. Acquired VWD in association with GSD Ia may require DDAVP or intermediate purity factor concentrate to manage haemostatic challenges since the haemostatic defect may not be corrected by glucose infusion alone.

0115

BONE STATUS OF CHILDREN WITH HEMOPHILIA A ASSESSED WITH DUAL ENERGY X-RAY ABSORPTIOMETRY AND QUANTITATIVE ULTRASONOGRAPHY: COMPARISONS AND CORRELATIONS

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Background. Adult patients with hemophilia A are known to be at risk for developing osteopenia or osteoporosis due to multiple factors, the main cause being recurrent hemarthrosis and subsequent reduced physical activity. Recent reports have demonstrated decreased bone mineral density (BMD) values even to younger hemophilic patients. *Aims.* To evaluate bone status of hemophilic children using two different *Methods.* Dual Energy X-ray Absorptiometry (DXA) and Quantitative Ultra-Sonography (QUS) and to determine the degree of correlation between these two techniques. *Methods.* Seventeen patients with hemophilia A, aged 11.87±4.91 years (range: 4.94-17.62 years) participated in the study. The majority of patients had a severe bleeding phenotype and were receiving either primary or secondary prophylaxis. Patients were HCV and HIV negative. With regards to study methods, weight and height were measured using standard techniques. Pubertal status was determined according to Tanner staging. Body Mass Index (BMI) was calculated as the ratio weight/height2 (kg/m^2). For every auxological parameter, Standard Deviation Scores (SDS's) were calculated according to sex- and age-matched normal greek population. BMD at lumbar spine (L2-L4 vertebrae) was determined by DXA technique using Cronos' bone densitometer (DMS, France). Results were expressed as grams per centimetre squared (g/cm^2), whereas Z-scores were calculated based on BMD measurements of normal sex- and age-matched Caucasian population, provided by the DXA device's manufacturer. QUS measurements (Speed Of Sound, SOS) were performed using Omnisense 7000 P (Sunlight Medical Ltd, Israel) at two peripheral sites: distal third of the radius (SOSR) and midshaft tibia (SOST), both at the patient's non-dominant and dominant side. Z-scores were calculated according to normative data derived from sex- and age-matched Greek population. Levels of intact parathormone (iPTH), fT4, fT3, TSH, calcium (Ca), phosphate (P) and alkaline phosphatase (ALP) were evaluated using commercial assays. Finally, joint evaluation was performed using the Hemophilia Joint Health Score (HJHS), a validated 11-item scoring tool scale assessing six index joints (elbows, knees and ankles).

Table 1.

	Weight SDS	BMI SDS	HJHS	BMD_L Z-score	QUS_R Z-score	QUS_T Z-score
Age	r=-0.264 p=0.153	r=0.165 p=0.264	r=0.402 p=0.077	r=0.408 p=0.052	r=-0.127 p=0.314	r=-0.304 p=0.118
Weight SDS		**r=0.832 p<0.001**	r=-0.053 p=0.428	r=0.349 p=0.085	r=0.188 p=0.235	r=0.287 p=0.132
BMI SDS			r=0.019 p=0.475	**r=0.448 p=0.036**	r=0.069 p=0.396	r=0.291 p=0.129
HJHS				**r=0.473 p=0.044**	r=0.048 p=0.435	**r=-0.541 p=0.023**
BMD_L Z-score					r=0.150 p=0.283	r=-0.197 p=0.224
QUS_R Z-score						r=-0.216 p=0.202

Results. All patients had normal pubertal development for age whereas biochemical profile, intact PTH concentrations and thyroid function tests were normal. Mean BMD Z-score was -0.12±1.08 g/cm^2, whereas 3 and 2 patients were classified as having osteopenia and osteoporosis respectively. Mean SOSR Z-score and mean SOST Z-score were -0.08±0.83 m/sec and -0.10±1.6 m/sec, respectively. No correlation was observed between DXA values and QUS-derived measurements. No agreement was recorded between the two methods in identifying hemophilic patients at risk for osteoporosis (kappa value = -0.25, p=0.17). SOS values at the dominant side were significantly correlated to SOS values at the non-dominant side both at radius (r=-0.541, p=0.01) and at tibia (r=0.45, p=0.04). Finally, the HJHS was negatively correlated with the SOST Z-scores (r=-0.541, p=0.023), whereas it was, surprisingly, positively correlated to BMD Z-scores (r=0.473, p=0.044). Correlations between studied parameters are demonstrated in Table 1. *Conclusions.* DXA detected a significant number of hemophilic children with impaired bone status; however, these findings were not confirmed by QUS measurements.

0116

FUNCTIONAL CHARACTERIZATION OF FIBRINOGEN POZNAN: A PREMATURE TRUNCATING MUTATION IN FGG ASSOCIATED WITH HYPOFIBRINOGENEMIA AND MILD BLEEDING TENDENCY

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We report here a novel nonsense mutation in FGG exon 4 identified in heterozygosity in three members of a Polish family causing hypofibrinogenemia and mild bleeding tendency in two out of three individuals. A 25-year-old male was referred due to lifelong history of mild bleeding symptoms i.e. bleeding from minor skin wounds, epistaxis, easy bruising, and bleeding after tooth extraction. Fibrinogen antigen level (immunonephelometry) was 1.12 g/L and functional fibrinogen (von Clauss method) was 1.0 g/L. Thrombin time (TT) was prolonged (21.1 s [N10-17.5 s]). Liver function tests showed normal results. The patient's 18-year-old brother reported prolonged bleeding after tooth extraction. Fibrinogen level was reduced to 1.37 g/L, with functional fibrinogen 1.0 g/L and TT 23.6 s. Their parents and sister as well as her 2 children had no bleeding history and normal fibrinogen levels. All exons and intron-exon junctions of the FGA, FGB and FGG genes were analyzed by PCR amplification followed by sequencing. We identified a novel nonsense mutation in FGG exon 4 c.331A>T (AAG>TAG) p.Lys111X (Lys85X in the mature chain lacking the signal peptide) in heterozygosity in the two brothers with low fibrinogen levels (Fibrinogen Poznan). Interestingly, despite normal fibrinogen levels, the father was also heterozygous for the Lys111X (Lys85X) mutation, which might be due to differential allelic expression, a widespread phenomenon affecting the expression of around 20% of human genes. The Fibrinogen Poznan mutation is predicted to encode a severely truncated fibrinogen gamma chain, lacking part of the coiled coil domain necessary for fibrinogen intermediate formation and hexamer assembly and the conserved globular C-terminal domain, also important for fibrinogen assembly. Co-transfection of the mutant FGG cDNA with the wild-type FGB and FGA cDNAs in COS cells followed by Western blot analysis demonstrated that the mutant is incapable of assembling a functional hexamer, since no hexamer was detected in the cells nor in the media.

0117

DIFFERENT REGIMENS OF PROPHYLAXIS TREATMENT IN YOUNG SEVERE HEMOPHILIA A PATIENTS: COMPARISONS ON EFFICACY, FVIII CONSUMPTION AND THERAPY COMPLIANCE

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Background. Primary and secondary prophylaxis is the emerging standard treatment for severe hemophilia A patients. The routine administration of FVIII is effective in children as prophylaxis against hemophilic arthropathy. Nevertheless, the costs and the patient's compliance represent barriers to prophylaxis treatment and to date the most efficacious, cost-effective regimen has not yet been determined. *Aims.* We retrospectively evaluated our severe hemophilia A patients, aged ″18 years, treated with different prophylaxis regimens to compare efficacy, FVIII consumption and patient/family's compliance. *Methods.* Nineteen patients (median age 13 years, range 3.6-17.8) with severe hemophilia A) on prophylaxis treatment were evaluated. Three regimens of therapy were implemented: administration of FVIII once a week in 1 patient, twice a week in 12, three times a week in 6. Prophylaxis was initiated because of increasing hemorrhages and presence of target joint. Results are shown in the Table. Before prophylaxis, 4 patients presented a low titer inhibitor that disappeared during treatment. All patients are HCV, HIV, HBsAg seronegative and are treated with rFVIII. In patients on the "twice a week regimen" treated with rFVIII at a dosage of 50 IU/kg, the level of FVIII before the administration was always between 0.5-1.5%. *Conclusions.* When the two most used prophylaxis regimens (twice and three times a week) were evaluated in young patients with severe hemophilia A, an important reduction of hemorrhagic episodes on prophylaxis versus on demand treatment was observed; however, no significant differences were recorded between the two prophylaxis regimens. There is no difference in the total amount of concentrates administered between the two prophylaxis regimens, but there is a very important increase in the consumption of FVIII concentrate during prophylaxis. There is no difference in the orthopedic score before (median 0; 0-2) and during prophylaxis (median 0.5; 0-2), probably due to the young age of patients. The twice a week prophylaxis regimen should be a good alternative treatment to the classic one, and preferable especially for young children because of the reduction in the number of venipunctures. The consequences are a better compliance and a greater adherence to treatment by patients and their families.

Table.

	Once a week	Twice a week	Thrice a week
Duration of prophylaxis (months)	9.6	Median 60 (18-111)	Median 113.5 (35.7-141.6)
rFVIII Dosage (IU/kg)	50	Median 42.5 (35-50)	Median 29 (25-30)
Number of hemorrhages in the year before prophylaxis	4	Median 9 (4-30)	Median 4 (0-65)
Number of hemorrhages/year during prophylaxis	0	Median 0 (0-1)	Median 1 (0-2)
Number of hemarthroses in the year before prophylaxis	1	Median 7 (1-18)	Median 5.5 (2-26)
Number of hemarthroses/year during prophylaxis	2	Median 0 (0-2)	Median 0.25 (0-2)
rFVIII units/kg/year before prophylaxis	300	Median 1200 (200-5760)	Median 850 (200-900)
rFVIII units/kg/year during prophylaxis	1800	Median 4320 (2900-5200)	Median 4320 (4032-4800)

0118

PHYSICAL ACTIVITY AS PREVENTION OF OSTEOPOROSIS IN PATIENTS WITH SEVERE HAEMOPHILIA ON LONG TERM PROPHYLAXIS

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Background. Physical activity is considered as an important factor for bone density and as prevention of osteoporosis. Physical activity confers the greatest benefit when initiated in prebubertal, promotes greater maintenance of bone density in adult. However, normal bone density during adulthood is an important protective factor against osteoporosis and related fractures in later life. *Aims.* The purpose of this study was to examine the relation between different aspects of physical activity and bone mineral density (BMD) in patients with severe haemophilia on long-term prophylaxis. *Methods.* The study group consisted of 39 patients with severe haemophilia (mean age 30.2 years). All patients with severe haemophilia receive prophylaxis to prevent bleeding (≥2 times per week). The median age at start of prophylaxis was 2 years. The bone density (BMD g/cm^2) of the total body, lumbar spine (vertebrae L1-L4), total hip, femoral neck and trochanter was measured by dual energy x-ray absorptiometry (DXA). Physical activity was assessed using the self-report Modifiable Activity Questionnaire (MAQ), an instrument which collects information about leisure and occupational activities for the prior 12 months. Physical activity was scored as duration in hours/week (h/wk) and as metabolic physical activity score by weighting the intensity (MET. h/wk). *Results.* There was only significant correlation between duration and intensity of vigorous physical activity (i.e.: activity with high intensity >6 MET) and bone density at lumber spine L1-L4; for

duration (R=0.429 and p=0.020) and for intensity (R=0.430 and p=0.019); whereas no significant correlation between vigorous physical activity and bone density at total hip, femoral neck, trochanter and total body. However, there was no significant correlation between other aspects of physical activity (i.e.: weight bearing activity, non weight bearing activity, walking activity, total leisure activity + walking, occupational activity and total activity + walking) and bone density at all measured sites. Moreover, there was no correlation between all aspects of physical activity and bone mineral content (BMC) at all measured sites. *Conclusions.* we could not find a significant correlation between all aspects of physical activity and BMD at different measured sites, except for vigorous activity and bone density at lumber spine. These result may support that the responsiveness to either an increase or a decrease in mechanical strain is probably greater in growing bones than in those of adults and also supports the importance of starting prophylaxis early in life so that the children can play and lead active life and normal bone density when they grow up.

0119

AN INVESTIGATION INTO THE EFFECT OF STORAGE TIME ON THE STABILITY OF SAMPLES FOR INR DETERMINATION AND THE RELIABILITY OF RESULTS THROUGH DELAYED INR DETERMINATION

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Background. The distinct increase in samples for International Normalised Ratio (INR) determination being received from sites distant to the laboratory and the related delay in INR determination for up to 48 hours has raised uncertainty over the stability of these samples on which the quality of anticoagulant care rests. *Objectives*. To determine the effect of delayed INR determination on the stability of samples through repeated testing at timed intervals over a 48-hour period. In addition these results will be further substantiated by the monitoring Factor VII (FVII) activity. *Patients and Methods.* A total of 214 subjects were included in the study, 197 had a baseline INR result <4.0 and 17 had a baseline INR of ≥4.0. Samples were then analysed at 4, 12, 24, and 48-hours. FVII activity (n=20) was measured at baseline and at 24 hours. Statistical analysis included One-way repeated measures ANOVA, linear regression analysis. *Results.* The mean overall difference and % change after 48 hours for samples within the therapeutic range was -0.04 INR and -1.4% respectively. For over anticoagulated samples the mean difference and % change was -0.2 and -4.1% respectively. Any deviations outside the 10% limit set were not deemed clinically important in either group. There was a significant mean increase in levels of FVII activity over the 24-hour period, though this was not considered of any clinical significance to results obtained. *Conclusions.* Samples undergoing delayed INR determination show no clinically significant changes over 48 hours and produce accurate and dependable results.

0120

PREOPERATIVE EVALUATION OF PLATELET FUNCTION WITH THE PFA-100 IN ORTHOPEDIC PATIENTS RECEIVING ASPIRIN AND/OR CLOPIDOGREL

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Background. Aspirin and clopidogrel are antiplatelet compounds that are widely used in patients suffering from coronary artery disease, stroke or peripheral artery disease and are likely to result in a higher risk of increased perioperative bleeding. Although these agents are often stopped 7 days before elective orthopedic surgery, little is known regarding management of patients undergoing emergency surgery. In such patients, the risk of bleeding has to be balanced against the risk of delaying emergency surgery. PFA-100 (platelet function analyzer) is a relatively simple tool for the investigation of primary hemostasis and its results could be useful in this setting. *Aims.*The objective of this study was to assess platelet function (using the PFA-100) in patients who were treated with aspirin and/or clopidogrel in whom the antiplatelet agent was discontinued in order to undergo major orthopedic surgical procedure. *Methods.* The study included 380 consecutive patients (146 men and 234 women; age range: 45-95 years, mean: 76 years) who were admitted in the orthopedic departments of our hospital from June 2007 to January 2009 on an emergency basis (mostly due to fractures). Among these patients, 261 were under treatment with aspirin, 99 were under clopidogrel and 20 patients were receiving both aspirin and clopidogrel. In all of them, platelet function was evaluated preoperatively using the PFA-100. *Results.* C/EPI and C/ADP closure times (CT) in patients under aspirin or clopidogrel are shown on Tables 1 and 2. The sample of patients receiving both aspirin and clopidogrel was too small (n=20) to draw any conclusions. Though, it must be mentioned that no patient had normal C/EPI-CT and C/ADP-CT 0-1 day after discontinuation of the drugs and only one patient had normal C/EPI-CT and C/ADP-CT 2-4 days after discontinuation. On the other hand, in patients receiving either aspirin or clopidogrel the following were noticed: a) only 2 patients in the aspirin group had normal C/EPI-CT and 1 patient in the clopidogrel group had normal C/EPI-CT and C/ADP-CT 0-1 day after the drug was stopped b) a significant proportion of patients under aspirin or clopidogrel (74% and 68.5% respectively) displayed normal closure times 2-4 days after cessation of antiplatelet treatment c) there were still 17 patients in the aspirin group and 7 patients in the clopidogrel group who had prolonged closure times more than 7 days after the treatment was stopped. *Conclusions.* Assessment of platelet function is very important in patients under antiplatelet treatment who need major orthopedic surgery on an emergency basis. According to the results of this study, there are a significant number of patients with normal PFA-100 values 2-4 days after discontinuation of aspirin or clopidogrel. Nevertheless, all PFA-100 results should be interpreted with caution, given the fact that this method certainly has its limitations when it comes to monitoring antiplatelet therapy. These results should probably be co evaluated with other parameters (e.g. severity of trauma), when a decision is made about the timing of surgery.

Table 1. Patients under treatment with apirin. *normal; **prolonged.

Day after cessation	0-1	2-4	5-7	>7
EPI-CT:(n*)	2 (10%)	82 (74%)	52 (73%)	42 (71%)
EPI-CT:(p**)/ADP-CT:(n)	13 (65%)	21 (19%)	10 (14%)	6 (10%)
EPI-CT:(p)/ADP-CT:(p)	5 (25%)	8 (7%)	9 (13%)	11 (19%)
Total number of patients	20	111	71	59

Table 2. Patients under treatment with clopidogrel.

EPI-CT:(n)/ADP-CT:(n)	1 (50%)	24 (68.5%)	26 (67%)	16 (69.5%)
EPI-CT:(n)/ADP-CT:(p)	0	5 (14%)	1 (2.5%)	2 (9%)
EPI-CT:(p)/ADP-CT:(n)	0	2 (6%)	3 (7.5%)	1 (4%)
EPI-CT:(p)/ADP-CT:(p)	1 (50%)	4 (11.5%)	9 (23%)	4 (17.5%)
Total number of patients	2	35	39	23

0121

ACQUIRED HAEMOPHILIA; A COHORT OF 26 PATIENTS DIAGNOSED AND TREATED IN THE SOUTH EAST OF ENGLAND BETWEEN JAN 1996-2009

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Background. Acquired haemophilia is a rare disorder caused by autoimmune destruction or inhibition of a coagulation factor, usually FVIII. It is seen most commonly in the elderly and is associated with other autoimmune disorders, pregnancy, and malignancy. Clinically life threatening bleeding into muscle, soft tissue, gastrointestinal & urological tracts is seen with mortality between 8-42% within weeks of presentation. Treatment is directed at eradicating the inhibitor using immunosuppressive agents and treating the bleeding with haemostatic agents. There is a lack of randomised studies; most reports in the literature are from findings of single centre cohorts. *Aims.*To review the treatments and outcome of all patients diagnosed with acquired haemophilia between Jan 1996-2009. *Methods.* All patients who presented with recent bleeding with a low FVIII caused by an inhibitor from South London and the South East were identified by retrospective analysis of medical notes, blood results and product usage between this period. Response to treatment was defined as FVIII level >50 iu/dL and undetectable inhibitor. Relapse was defined as re-emergence of inhibitor and / or a FVIII level <30 iu/dL. *Results.* 26 patients were identified of which 64% were female. At presentation the median age was 72yr, with FVIII level ranging from <1-14 iu/dl. The quantified inhibitor levels from presentation to peak ranged from 2 - 3800

Bu/mL. Two patients had a peak inhibitor above 400 Bu/mL. The majority of patients presented with subcutaneous bleeds, 3 had retroperitoneal bleeds. The 2 patients who presented with forearm bleeds developed compartment syndrome which required surgical decompression. One patient presented with a post partum bleed. Most patients (89%) received steroids alone, 68% had steroids + cyclophosphamide. One received Cyclosporine and another Azathioprine. 2 patients who failed to respond to steroids alone received Rituximab. 52% of patients also received intravenous immunoglobulin. The majority (52%) of patients received FEIBA as a bypassing agent, only 26% received rFVIIa, the rest (21%) did not need any treatment. One patient was switched from rFVIIa to FEIBA due to lack of response. The shortest duration of bypassing product usage was 1 day, the longest was 30 dys. Half of the patients needed >2 units of blood. Relapse was seen in 26%, 3/5 of these received Rituximab. In 3 patients the inhibitor was never eradicated. 4 patients had positive auto antibodies, 5 had raised malignancy markers, 2 had a paraprotein identified, and 2 were diagnosed with Pemphigus. 1 patient died from a sudden massive GI bleed soon after control of initial presenting bleed. *Summary and Conclusions.* Findings from this cohort are very similar to those previously reported. There is no improvement in outcome in patients treated with steroids in combination with Cyclophosphamide and ivIG. FEIBA was given preferentially for convenience of use. The majority of patients needed prolonged use of a low dose of steroids to sustain remission. Patients on combined immunosuppressive therapy did not show an increased rate of infections.

0122

COAGULATION SCREENING IN AN IRISH TEACHING HOSPITAL

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Background. Coagulation screening using prothrombin time (PT) and activated partial thromboplastin time (APTT) is widely used. However these tests have significant limitations. They have not been shown to predict bleeding during surgery or invasive procedures. Furthermore mild prolongation of PT or APTT has not been shown to predict increased bleeding risk. They may miss some clinically significant bleeding disorders and they may be abnormal in conditions not associated with bleeding. *Aims.* To audit requests for coagulation screening in an Irish teaching hospital. *Methods.* We analysed prolonged PT and/or APTT during normal working hours during a one-week period in our hospital. Abnormal results due to anticoagulants were excluded from further study. In samples with PT longer than 15.5 seconds and/or APTT longer than 42 seconds we proceeded to 1:1 mixing studies if the PT was prolonged and 1:1 mixing studies, Factor XII assay and Lupus screen if the APTT was prolonged. We also obtained referral source for all samples and clinical details for abnormal samples. *Results.* 671 coagulation requests were received during the study period. 318/671 (47.4%) coagulation requests were for monitoring of anticoagulation. 353/671 (52.6%) requests were for coagulation screening rather than anticoagulant monitoring. 24/353 (6.8%) coagulation screens were abnormal. Of the coagulation screening requests 21 were from ICU/HDU and 8/21 (38.1%) were abnormal. 332 requests were received from outside ICU/HDU and 16/332 (4.8%) were abnormal. In the coagulation screens received PT was prolonged in 19/353 (5.4%), median 17secs, range 16-48 seconds. PT was longer than 20 seconds in 4/353 cases (1.1%). PT corrected using 1:1 mixing studies in 18/19 (94.7%) cases of prolonged PT. Referral sources for prolonged PT were: ICU/HDU-8; Medical-8; Surgical-3. APTT was prolonged in 19/353 (5.4%), median 46 seconds, range 43-59. APTT was longer than 50 seconds in 4/353 (1.1%). APTT corrected using 1:1 mixing studies in 16/19 (84.2%) cases of prolonged APTT. 3/19 (15.8%) cases of prolonged APTT had Factor XII levels less than 50%. 1/19 (5.3%) cases of prolonged APTT had lupus anticoagulant. Referral sources for prolonged APTT were: Medical-9; ICU/HDU-8; Surgical-2. The 24 abnormal coagulation screens were from 21 different patients. 5/21 (23.8%) patients had prolonged PT and normal APTT. 5/21 (23.8%) patients had prolonged APTT and normal PT. 11/21 (52.4%) patients had prolonged PT and APTT. *Conclusions.* Unregulated coagulation screening has a low yield of abnormal results and the majority of these abnormal results are mild prolongation of PT or APTT of uncertain clinical significance. Requests for coagulation screening outside the ICU setting have an even lower yield of abnormal results. We believe many of the coagulation requests received are not indicated. We plan to introduce evidence-based guidelines on indications for coagulation screening in our hospital. We will repeat this audit following the introduction of these guidelines to see if selected testing based on clinical criteria can reduce unnecessary coagulation screening.

Quality of life, palliative care and ethics

0123

VALIDATION OF THE BRAZILIAN PORTUGUESE VERSION OF THE MEDICAL OUTCOMES STUDY - SOCIAL SUPPORT SURVEY IN HODGKIN LYMPHOMA SURVIVORS

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Background. Studies in the last three decades have documented the effects of social support on physical and psychological well-being, mainly in chronically ill patients or on the onset of a stressful event. There are few studies about social support in cancer survivors. *Aims.* To assess the psychometric properties of the "Medical Outcomes Study - Social Support Survey (MOS-SSS)" in Hodgkin Lymphoma (HL) survivors. *Methods.* The Brazilian Portuguese version of the MOS-SSS, already validated in a healthy population, was applied to a sample of 122 HL survivors treated in five institutions in Rio de Janeiro, Brazil. All the participants were contacted by telephone. The questionnaire was self-administered at the treatment center or at home, and sent by traditional or electronic mail, according to individual choice. An informed consent was obtained from each participant. *Results.* The median age of the patients at diagnosis was 32.5 years (16-77) and the median follow-up was 6.8 years (3.5-11.7) since diagnosis. Among the 122 individuals, 51% were female, 71% had a good International Prognostic Score (less than two factors), 61% had advanced HL, 35% had bulky disease, and 92% were treated with ABVD chemotherapy. Responses to the 19 social support items were skewed toward positive evaluations (item means of 3.27 to 3.8, in a 0-4 possible range of responses). Pearson correlation coefficients between items varied from 0.11-0.71, with most values higher than 0.3. Correlation coefficients between items and their dimensions varied from 0.44-0.71. The internal consistency was evaluated with Cronbach's alpha, and was 0.93 for the overall scale, ranging from 0.73 to 0.86 for the five subscales. The factor analysis yielded a 4 factor solution that explained 68% of the variance. These 4 functional support subscales measured affection, material aspects, emotional/informational aspects and positive social interaction. *Summary and Conclusions.* The psychometric properties of the Brazilian version were similar to those obtained with the original English version of the MOS-SSS. The Brazilian Portuguese version will now be used to evaluate social support and its association with structural support, long-term disease outcomes, socioeconomic status and the quality of life of Hodgkin lymphoma survivors.

0123a

QUALITY OF LIFE IN PATIENTS WITH VWD

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Background. Only few studies compared to haemophilia are published related to health-related quality of life (HRQoL) in patients with von Willebrand (VWD). In none of those studies a disease-specific HRQoL questionnaire was applied. The recently in Italy developed VWD-specific questionnaire (VWD-QoL) was administered to VWD patients during their annual patient meeting. *Methods*. During the annual meeting of the German VWD patients' association patients and parents of VWD children filled in the preliminary version of the German VWD-QoL questionnaire and were asked to evaluate the questionnaire concerning relevance and comprehensibility of items. *Results*. 22 adult patients (A) with VWD and 7 parents of children (C) with VWD filled in the questionnaire with a mean age of adult patients (M=55.86+13.4 years). All types of VWD patients were represented; children suffered mainly from Type 3 and were severely affected (71.4%), while the major part of adults had Type 1 (35%). Most patients were treated with factor concentrate (A: 59.1% vs. C: 85.7%). 66.7% reported often/always unpleasant sensations or strange sensation after injection (50%) due to treatment. 63.6% adults had bleeds in the last 4 weeks (C: 57.1%), mainly epistaxis (31.8% vs. 42.9%), followed by teeth bleeds (27.3% vs. 42.9%). Patients reported days lost at work/school in the past 12 months (A: 13.6%, Median No of days 52; C: 71.4%, Median No of days 27). Mean age of diagnosis in adults was 32.6+17.3 years (range 1-62), in children 2.4+3.41 years (range 0-10). When receiving the diagnosis 38.1% were frustrated and worried about their future while 28.6% felt calmer and more relaxed. 50% of adult patients worried about VWD and were afraid to hurt themselves (16.7% of children). More children reported about bruises (66.7% vs.

45.5%), while more adults stated that they could not walk as far as they wanted (50% vs. 16.7%). *Conclusions.* Time of diagnosis is quite different between children and adults which has an impact on their quality of life. Adult patients are more impaired in different aspects of their quality of life then children. Quality of life assessment is a new area in VWD which should be implemented in the routine care of those patients.

0124

PAIN RATE AND SOCIAL CIRCUMSTANCES RATHER THAN CUMULATIVE ORGAN DAMAGE DETERMINE THE QUALITY OF LIFE IN ADULTS WITH SICKLE CELL DISEASE

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Background. Sickle cell disease (SCD) is characterized by chronic haemolytic anemia and recurrent microvascular vaso-occlusion resulting in progressive organ damage and reduced life expectancy. Previous analyses have demonstrated a strongly reduced quality of life (QoL) in sickle cell patients which however could not be explained by the severity of pain only. Therefore, other factors like chronic organ damage and socioeconomic circumstances may play a role. Recently, we systematically screened consecutive patients with SCD for chronic organ damage and determined their social-economic circumstances. *Aim of the study.* In the present study, we analysed the specific contribution of chronic organ damage and social circumstances to the QoL in patients with SCD. *Methods.* Consecutive adult sickle cell patients in a teaching hospital were included. Genotype, pain rate, rate of acute complications (acute chest syndrome, stroke, priapism) in the last five years and cumulative organ damage (pulmonary hypertension, microalbuminuria, retinopathy, renal failure and osteonecrosis) were assessed during routine visits. Occupation and level of education were determined by interview. QoL was assessed with SF-36 forms and represented in a physical (PCS) and mental component (MCS) which was compared with the normative score of the general Dutch population and related to pain rate, organ damage, occupation and education level. Differences in continuous data were tested with the Mann-Whitney test and in categorical data with the Chi-square test. *Results.* QoL was analysed in 94 adult patients with SCD and was significantly reduced as compared to the Dutch population. Linear regression showed that pain rate was associated with MCS (p=0.021) demonstrating the lowest scores in patients most frequently admitted. The PCS of the QoL was not related to pain rate. With respect to social circumstances, 35% of the patients were unemployed as compared to 6% of the general population and 16% of immigrants without SCD. In contrast to pain rate, both occupation and the level of education were significantly related to PCS while no relation with MCS was found. Genotype and presence of chronic organ damage did not affect QoL. *Conclusion.* Patients with SCD have significantly reduced QoL-scores which are determined by the pain rate, occupation and education level. Frequent painful crises appear to have a major impact on the mental component of the QoL in patients with SCD while the physical component was related to occupation and the level of education. Chronic organ damage, although responsible for limited life expectancy, was not related to QoL. With respect to QoL, comprehensive care for patients with SCD should not only focus on reducing pain rate and organ damage but also on factors determining social wellbeing.

0125

HEALTH-RELATED QUALITY OF LIFE AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION: A MULTICENTRE PROSPECTIVE STUDY IN THE CZECH REPUBLIC (FINAL RESULTS)

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Quality of life is an important parameter evaluated not only as part of clinical studies but also as a routine indicator in the case of expensive and/or sophisticated therapeutic approaches. Since 2004, a prospective health-related quality of life (HR-QOL) assessment in patients with autologous stem cell transplantation (ASCT) has been in progress. Since July 2005, most Czech transplant centres (Olomouc, Pilsen, Prague, Brno and Hradec Kralove) have participated in the study. Inclusion criteria: A signed informed consent form, age 18 years and over, compliance, haematological malignancies, planned ASCT. *Methods.* Two questionnaires for assessing quality of life are used - the EORTC QL Q-C30 v. 3.0 (European Organisation for Research and Treatment of Cancer) and EQ-5D (EuroQol Group) - as well as other parameters (Hb, Plt, Leu, BMI, sex, age, duration of hospitalization etc.). As QOL assessment may change over time, the patients are followed at T0 (admission to the transplant unit), T1 (10 days after ASCT), T2 (100 days after ASCT) and T3 (1 year after ASCT). Group characteristics: A total of 148 patients (84 males, 64 females) who underwent ASCT. The patients were diagnosed with multiple myeloma, diffuse large B-cell lymphoma, follicular lymphoma, Hodgkin's disease and other non-Hodgkin's lymphomas. The median age was 54 years in both males and females (average ages 51.2 and 51.3 years, respectively). *Results.* It is obvious that at time T0, i.e. prior to transplantation, physical functioning, role functioning and social functioning of oncology patients is found to be impaired in comparison to the control group. Of the symptoms listed, the degree of financial independence is much lower than in the control group. The other studied symptoms and domains show no significant differences when compared with the healthy population. The most severe impact on quality of life (QOL) is observed at T1. In nearly all studied parameters (with the exception of dyspnoea and constipation), patients perceive their quality of life as decreased. At T2, however, the vast majority of the patients' parameters are comparable with those in the reference population (similar to T0). In physical functioning, role functioning and social functioning, the outcomes are significantly worse than in the reference population. The T3 assessment results show that in fact all studied QOL parameters remain at a level comparable with the reference population. Financial difficulties are constant throughout the studied period, confirming the fact that in formerly active population, oncological disease results in decreased financial independence. The overall QOL (1y after ASCT) is better than prior to transplantation (p=0.02). Other parameters are statistically significantly better as well. None of the quality-of-life parameters were found to be worse at one year after autologous transplantation than before it. According to the EQ-5D questionnaire, three-quarters of patients perceive their health status at 1 year post-transplant as better than a year earlier. Better overall global quality of life correlates with higher Karnofsky scores. *Conclusions.* ASCT and especially the preparatory regimen and previous intensive chemotherapy remain demanding and not always successful therapeutic regimens. The study results suggest that transplantations used to treat blood diseases do not imply prolonged decrease of health-related QOL. According to the patients, the most burdensome symptom is fatigue. Surprisingly, more than half of oncology patients are overweight or obese (BMI over 25 in nearly 60% of the studied patients). It seems crucial to prolong the time of monitoring the QOL parameters after transplantation (optimum time of 3-5 years) and to include the patients' occupational status.

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0126

INFLUENCE OF ANEMIA ON QOL, CLINICAL SYMPTOMS AND COGNITIVE FUNCTIONS IN NEWLY DIAGNOSED (NOT TREATED) HEMATOLOGIC PATIENTS

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Introduction. Anemia, bad Qol and cognitive disfunctions are commonly present in hematologic patients but often underreported by patients and underdiagnosed by health care professionals. Previous studies mainly evaluated these problems during chemotherapy or under influence of erythropoetic agents (Epo) which has been known to have various effects on QoL and cognition besides their influence on anemia. *Aim.* We evaluated influence of anaemia per se on QoL, clinical simptoms and especially on cognitive functions in hematologic patients before starting any chemotherapy and measured changes in short time period to see if correction of anemia by standard therapy could improve QoL and cognition. Pts on Epo has been excluded from this study. *Patients and Methods.* In Clinical Hospital Center of Rijeka, Croatia 137 pts in very early phase of diagnostic procedure which finally resulted in diagnosis of hematologic malignancies were evaluated by FactAn QoL Questionary and five point scale for subjective clinical symptoms. Cognitive functions have been measured by Complex Reactiometer Drenovac (CRD), a PC based psyhodiagnostic laboratory based on chronometric aproach which examine: perceptive abilities (detection, identification, visual orientation, spatial visualisation), memory (short term memory, maze learning, actualisation of memorized contents),thinking (operative thinking, problem solution, convergent thinking), psychomotor reactions (simple and complex), dynamic features of CNS function

(excitability, agility, stability, balance, endurance, reliability), attention (attention span, concentration, vigilance) and functional disturbances (rigidity, agitation, perseverance, regression). All parameters have been measured twice: T0 -basal measurement ,T1 -after one month (± 7 days). Among T0 and T1 patients received therapy for their anaemia according to anemia type and Hb level but no chemo, radio or immunotherapy for their disease or Epo agents. *Results.* Hemoglobin level significantly influence QoL and cognitive functions in all pts (so called anemic and non anemic).When statistically partialized , the effects of gender, age, education and Hb level showed the Hb level as the most effective variable on cognition analysed by β weights (anaemia has a 10,5% influence on IQ). Correction of anemia significantly improved QoL and cognitive functions but not in all categories (visual orientation and memory). Subjective feeling of cognitive disturbancies are not in correlation with real cognitive achivement measured by CRD. *Discussion.* Impairment of brain function caused by anaemia profoundly affects cognition, psychological well-being, the ability to perform the usual activities of daily living and in general affects their QoL esspecially in pts with malignant diseases. We concluded that changes in hemoglobin level during phase of diagnostic procedure before any treatment (immuno, chemo, irradiation therapy) in hematology patients greatly positively influence their QoL and esspecially their cognitive functions.

0127

HOME CARE FOR UNFIT ELDERLY PATIENTS WITH MYELODISPLASTIC SYNDROMES: AN ITALIAN SINGLE-CENTER EXPERIENCE

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Background. Myelodisplastic syndromes (MDS) are an heterogeneous group of bone marrow disorders, with a higher incidence in old age. In the elderly setting main clinical needs are transfusion support and regular follow-up appointments, commonly provided on outpatient basis, often resulting in discomfort arising from driving distances and long waiting times. Hospital admissions, usually due to worsening of comorbities and infectious events, are also frequent. *Aims.* In our department a home care (HC) service sustained by the fundraising organization A.I.L. (Italian Association against Leukemia-Lymphoma-Myeloma) is active in order to manage fragile hematology patients outside the standard in-hospital assistance, to improve their quality of life and optimize healthcare resources. Here we describe our experience in 40 consecutive elderly patients with MDS referred to a domiciliary service of supportive care in the period 2000-2008. *Methods.* MDS diagnosis was formulated according to WHO classifications. The Severity of Illness Index was calculated to evaluate the impact of comorbidities. All 40 patients fulfilled inclusion criteria for HC (poor performance status, appropriate home logistics, caregiver availability, distance from hospital ″30 km). Home transfusions were requested on the basis of hemoglobin levels (Hb ″ 8 g/dL) and platelet count (PLT ″10.000/mcl) and/or in presence of symptoms related to anaemia and thrombocytopenia. HC duration, generally corresponding to the remaining lifetime, was compared among different patients' groups divided by age, MDS subtype and comorbidity burden. *Results.* Median age was 83 years (range 64-97), with 70% of patients aged ≥80 years. Gender was equally distributed (19 male, 21 female). 22 patients presented refractory anaemia (RA), 2 patients had refractory anaemia with ring sideroblasts (RARS); refractory anaemia with excess of blasts type 1 (RAEB-1) and type 2 (RAEB-2) were respectively diagnosed in 4 and 7 patients. 5 patients with chronic myelomonocytic leukemia (CMML) were also included in this report. 15 out of 40 patients (37.5%) developed leukemic transformation. The median number of severe non-hematologic comorbidities was 1.57, with prevalence of cardiac and pulmonary diseases. Median duration of HC was 309 days, with significant differences only according to the number of severe comorbidities (417 days if ″1, 146 days if ≥2). Home transfusion support was provided, without relevant adverse events, in 26 patients (65%), for a total of 385 units of packed red blood cells and 92 of platelet concentrates. 27 patients (67.5%) were admitted as inpatients for a total of 44 hospital admissions, mainly caused by cardiac failure, severe infections, caregivers' burn-out and end-of-life care. *Summary*. In our operating model HC for unfit elderly patients affected by myelodisplastic syndromes is feasible, sustainable and safe. Severe comorbidities are correlated with poorer survival, irrespective of MDS subtype and age distribution. HC represents a valid resource to implement the management of patients with blood malignancies, especially if there is a consultant hematologist fully dedicated to domiciliary patients, allowing an efficient integration among hospital division, general practitioners and community health services. Cost-effectiveness analysis and economic recognition in public health systems are some of the open issues to be shortly explored.

0128

PAIN MANAGEMENT OF BORTEZOMIB-INDUCED NEUROPATHY WITH ORAL CONTROLLED-RELEASE OXYCODONE

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Background. In nearly one third of patients treated with the proteasome-inhibitor bortezomib it has been reported the occurrence of an axonal sensitive neuropathy, which is responsible of severe neuropathic pain to the lower extremities, a very distressing symptom for patients. Strong opioids have been already employed for the treatment of neuropathic pain provoked by other causes. *Aims.* To evaluate in a group of patients with bortezomib-induced painful neuropathy: i) the effectiveness of controlled-release (CR) oral oxycodone to reduce the pain intensity and its interference with some daily activity functions; ii) the tolerability and the safety of this strong opioid. *Patients and methods.* Twenty-three patients with a Karnofsky performance status > 50 (median age 62 yrs.) affected by multiple myeloma (22), and non Hodgkin's lymphoma (1) with moderate-to-severe pain unresponsive to previous analgesic treatment for the bortezomib-related painful neuropathy were enrolled in the study. The continuous and breakthrough pain intensity was evaluated at baseline, on day 3, 7 and 14 with the Short-Form Brief Pain Inventory (SF-BPI), scored on an 11 point numerical rating scale (NRS), where 0 = *no pain* and 10 = *pain as bad as you can imagine*, along with the evaluation of pain interference with some functions such as quality of sleeping, appetite, walking ability, self care, daily activity, mood and concentration, the global patient evaluation of efficacy (GPE). Good pain relief was defined as >30% reduction of the previous NRS score. Side effects such as nausea and vomiting, constipation , drowsiness, confusion, and dry mouth were rated using a scale from 0 to 3 (not at all, slight, mild, severe). Patients received oral CR-oxycodone at the starting dose of 10 mg bid. During the study period, 14 days, no dose reduction of bortezomib has been carried out. *Results.* The daily average dose of CR-oxycodone administered was 28,46 mg (range 20-80 mg).CR-oxycodone reduced dramatically the pain intensity, from a mean NRS value of 7.6 at baseline, to 3.9 on day 3, 1.7 on day 7, and 1.3 on day 14. Similar trend to decreasing values was observed for all the daily life functions. Breakthrough pain events, observed at baseline in the 61% of patients, reduced their frequency and intensity: on day 14 the NRS value corresponded to 3,1 and these events occurred in the 47,% of patients. 11 patients used rescue drugs for breakthrough pain. Side effects occurred in 12 patients (51%), all of them of slight or mild intensity. *Summary*. CR-oxycodone was effective for pain relief caused by the bortezomib-related neuropathic pain and it was well tolerated. The pain control improved also the quality of the daily life functions, such as the walking ability, usually compromised in this clinical condition. A good pain control allowed the continuation of the cancer treatment.

0129

HOW HAEMOPHILIA IMPACTS THE QUALITY OF LIFE OF ELDERLY PATIENTS

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Rationale. Due to improvements of haemophilia care over the last 3 decades the life expectancy of people with haemophilia (PWH) has increased enormously resulting in more PWHs reaching an old age. However, only few information are available on health status and health-related quality of life (HR-QoL) in elderly PWHs. Therefore a retrospective case-control study was carried out in Italy. *Methods.* Patients ≥65 years with severe haemophilia were enrolled matched to male controls without bleeding disorders for age, geography and social status. Emotional well-being was assessed with the Geriatric Depression Scale and various HR-QoL instruments, namely the generic EQ-5D, WHOQOL-BREF, WHOQOL-Old and

a disease-specific questionnaire (Haem-A-QoLElderly). *Results.* 84.6% of the in Italy registered elderly PWHs were enrolled (n=39) with a median age of 68 years (65-78). PWHs suffered significantly more from viral infections (chronic hepatitis C: 87% vs. 5%; HIV: 13% vs. 0%), hypertension, arthropathy (OJS: M=18.1 vs. M=1.19) and had more impairments in physical functioning and showed a trend to mild depression (M=9.9 vs. M=5.3). By contrast more controls suffered from hypercholesterolemia and cardiovascular diseases, but had similar cognitive functioning. PWHs showed significantly higher impairments in their generic HR-QoL compared to matched controls (WHOQOL-BREF: $p<0.0001$; WHOQOL-OLD:$p<0.041$; EQ-5D:$p<0.0001$). In regression models including infections, no of total bleeds, invalidity, OJS, marital status and depression the latter could explain up to 67% of the variance in HR-QoL. In turn depression correlated significantly with orthopaedic status, physical and mental functioning. *Conclusions.* Elderly PWHs show not only impairments in their health status and daily activities, but reveal to have as well significantly more emotional problems compared to controls. Since impairments in physical and mental functioning are correlated to depression and HR-QoL special care should be provided to this patient population.

0130

SINGLE DOSE PALONOSETRON IN PREVENTING CHEMOTHERAPY-INDUCED NAUSEA AND VOMITING IN PATIENTS WITH AGGRESSIVE NON HODGKIN'S LYMPHOMAS WHO UNDERWENT MODERATELY EMETOGENIC CHEMOTHERAPY

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Background. The standard approach in the antiemetic therapy in non hodgkin's lymphomas (NHL) is represented nowadays by antiserotoninergic drugs. Palonosetron is a selective and potent serotonin antagonist with a distinct pharmacological profile, different structure and enhanced binding affinity. Clinical studies in solid tumours have defined the improved efficacy of palonosetron compared to older 5-HT3 receptor antagonists. *Aims.* The objective of the study was to evaluate the efficacy and safety of a single dose of Palonosetron in preventing chemotherapy-induced nausea and vomiting (CINV) induced by moderately emetogenic chemotherapy (MEC) agents used for the treatment of aggressive NHL. *Methods.* This is an open-label, multicenter phase II study assessing the efficacy of a single i.v. dose of palonosetron (0.25 mg) prior to the administration of chemotherapy in the first day of treatment. The primary end point is the overall rate of patients achieving a complete response (CR-defined as no emetic episode and no use of rescue medication) during the whole study period (0-120 h). Drug activity was evaluated based upon a one-stage Fleming study design for determination of response rates based on a single treatment group. Relevant secondary endpoints included: CR in the acute (0-24h) and delayed (24-120) phases, no emesis and no nausea rates. Adverse events were also recorded. *Results.* In ten Italian centers eighty-six patients, affected by aggressive NHL, mostly undergoing CHOP±R (74.4%), were evaluated for the study. The primary endpoint was achieved with a CR rate of 86%. The CR in the acute and delayed phases was 90.7% and 88.4% respectively. No emesis rates were 91.9% (0-24h), 89.5% (24-120h) and 88.4% (0-120h). Similarly no nausea rates were 84.9%, 75.6% and 74.4% in the acute, delayed and overall period respectively. No serious drug related adverse events were reported. *Conclusions.* Palonosetron alone given as antiemetic treatment is effective and safe in preventing acute and delayed CINV in patients with aggressive NHL treated with moderately emetogenic chemotherapy.

0131

PHYSICIANS AND PATIENTS PERCEPTION OF A PROBLEM AS ETHICALLY RELEVANT IN ONCOHEMATOLOGY: ONLY A QUESTION OF PERSPECTIVE?

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Background. Ethical problems are connatural to the medical activity. Ethical problems regarding some medical specialities as obstetrics or pre or perinatal medicine are well known. Nevertheless few is known about ethical problems perceived as remarkable from physicians and patients in oncohematology. *Aims.* Aim of this study is to recognize, in oncohematology, what are problems perceived as ethically relevant not only from physicians, but also from patients. It might consent to ameliorate medical practice and the quality of care perceived from patient. *Methods.* Ethical problems regarding oncohematologic patients were recognized consulting clinical diary of 200 patients treated in our institution. Patients have casually been selected in the temporal period from january 2004 to june of 2008. Ethical problems were listed in two groups: those perceived as remarkable for the physicians and those remarkable for the patients. This study is a retrospective monocentric descriptive study. *Results.* Median age was 58.5 years (R 33-86). M/F was 126/74. 123 patients were affected by chronic or indolent hemopaties. 143 patients received Ist line therapy, instead 67 IInd line or superior. 150 patients had licence of primary school, 40 of secondary, 10 degree. 150 patients were assisted from a relative. 60 patients perceived a problem as ethically relevant (9 compliance to treatments, 11 refusal of treatment, 15 intolerance to hospitalization, 17 scarce trust in caregivers, 8 difficulty to understand therapeutic plain). In 82 cases physicians perceived a problem as ethically relevant (19 over treatment, 14 type of treatment to adopt, 18 correct timing of therapy, 24 correct use of resources, 8 adequacy of treatment). Physicians perceived these problems mainly about patients requiring IInd line treatment or further (56 cases on 67). In 52 cases only patients perceived a problem, in 51 cases only physicians perceived a problem, while only in 39 cases a problem was perceived as ethically relevant from physicians and patiens simultaneously. *Summary andconclusion.* In our study, in oncohematology the main problem perceived from patients as ethically relevant is the scarce trust in caregivers, while for physicians is the correct use of resources. Physicians perceive a bioethic problem mainly considering patient candidate to IInd line treatment or further. In 25% of cases a problem perceived as ethically relevant from patients is underestimated from physicians. In this contest the possibility of bioethical counseling might create a convergence between physician and patient perspective.

0132

COST-EFFECTIVENESS OF RIVAROXABAN VERSUS ENOXAPARIN FOR PREVENTION OF VENOUS THROMBOEMBOLISM AFTER TOTAL HIP AND KNEE REPLACEMENT IN EUROPE

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Background. Rivaroxaban, an oral direct Factor Xa inhibitor, was compared with enoxaparin after total hip replacement (THR) and total knee replacement (TKR) in four large randomized controlled trials. Two of these trials compared equal treatment durations of rivaroxaban (10 mg once daily [od]) and the most commonly used European regimen of enoxaparin (40 mg od). In RECORD1, patients undergoing THR received 35 days' prophylaxis with rivaroxaban or enoxaparin. Rivaroxaban reduced total venous thromboembolism (VTE; composite of any deep vein thrombosis, nonfatal pulmonary embolism and all-cause mortality) by 70% versus enoxaparin after 35 days' prophylaxis. In RECORD3, in patients undergoing TKR, 12 days' rivaroxaban reduced total VTE by 49% and symptomatic VTE by 66% versus 12 days' enoxaparin. Major bleeding was similar between rivaroxaban and enoxaparin regimens in both studies. *Aims.* To assess the cost-effectiveness of oral rivaroxaban versus subcutaneous enoxaparin for the prevention of VTE after THR and TKR in key European countries. *Methods.* An economic model was developed to assess the cost-effectiveness of rivaroxaban versus enoxaparin over equal durations of prophylaxis in several key European countries. RECORD1 and RECORD3 data were used to populate the model for THR and TKR, respectively. Risks of VTE and post-thrombotic syndrome (PTS) beyond the post-surgery trial period were estimated from published data and extrapolated out to 5 years. Costs to the healthcare sector were derived from published sources in Spain, Italy, Sweden and the UK. Weights of quality of life were also derived from published literature. The model estimated the cost-effectiveness of rivaroxaban versus enoxaparin over the 5-year time frame, which is sufficient for the long-term burden of venous thromboembolic events, such as PTS and recurrent VTE, to be incorporated. In the UK and Sweden, 8% and 10% of enoxaparin patients, respectively, require daily nurse visits to administer enoxaparin after hospital discharge; these costs were included in the analysis. Outcomes were expressed in quality-adjusted life years (QALYs). *Results.* Thirty-five days'

rivaroxaban was less expensive and improved health outcomes versus 35 days' enoxaparin after THR. Rivaroxaban was associated with a gain in QALYs (up to 0.004 QALYs) and per-patient cost savings over 5 years ranging from €4 to €46 in the UK. For TKR, 12 days' rivaroxaban is consistently less expensive than 12 days' enoxaparin and consistently improves health outcomes. Improved health outcomes were similar between countries (up to 0.0215 QALYs), whereas per-patient cost savings over the 5-year period ranged from €82 in Sweden to €145 in Spain. Extensive sensitivity analyses showed these findings to be robust. *Summary and Conclusions.* This analysis suggests that after both THR and TKR, rivaroxaban thromboprophylaxis consistently improves health outcomes and produces overall cost savings across a range of European settings. With more than 400,000 procedures being performed in Europe annually, rivaroxaban has the potential to produce substantial savings for European healthcare systems.

0133

ANALYSIS OF COSTS ASSOCIATED WITH REMOBILIZATION OF HEMATOPOIETIC STEM CELLS (HSC) USING CHEMOTHERAPY, IN PATIENTS WITH MULTIPLE MYELOMA FAILING ADEQUATE HSC COLLECTION FOR AUTOLOGOUS STEM CELL TRANSPLANTATION WITH G-CSF ALONE

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Background. Peripheral blood (PB) hematopoietic stem cells (HSC) can be mobilized with cytokines alone or chemotherapy plus cytokines. The HSC yield is higher and the failure rate is lower with chemo mobilization. Although a cell dose of 2 million CD34$^+$ cells/kg is considered adequate, some authors have shown that patients who receive <5 million CD34$^+$ cells /kg have higher resource utilization after transplant. *Aim.* the purpose of this study was to analyze the costs associated with remobilization of HSCs using chemotherapy in patients with multiple myeloma (MM) who collected <2 million CD34$^+$ cells/kg in 4 leukapheresis procedures in a prior mobilization attempt with G-SCF alone. *Methods.* This is an IRB approved retrospective chart review of 302 patients with MM who underwent HSC mobilization at our institution between 1/05 and 10/07. The estimated costs were divided into mobilization, pre apheresis, apheresis, and post apheresis costs. (Source: Cleverly and Associates; www.drugstore.com; Redbook'; Thompson PDR)

Table.

Procedure	National Average Hospital and Clinic Charges ($)	N, Median (Range)	Average Cost Per Patient ($)
Chemotherapy mobilization*			
Daily room charge	863.00	2.5 (0-36)	2158
Cyclophosphamide (350 mg/m^2 every 12 hours x 8 doses)	54.62/g	4.928 g	269
Mesna (400 mg/m^2 CIV over 24 hours daily x 4 days)	223.1/0.4 g	2.82 g	1570
Vincristine (0.4 mg/m^2 CIV over 24 hours daily x 4 days)	10/1 mg	2.81 mg	28
Doxorubicin (10 mg/m^2 CIV over 24 hours daily x 4 days)	44.4/10 mg	70.4 mg	313
Dexamethasone (40 mg IV daily x 4 days)	11.45/40 mg	160	45.8
Daily CBC, diff	93	2.5 (0-36)	233
Blood transfusions (n = 21)	494	1 (0-4)	494
Platelet transfusions (n = 12)	494	0 (0-3)	0
G-CSF	528	18.5 (11-32)	9768
Prophylactic oral antibiotics (valacyclovir/ fluconazole/ levofloxacin)		12.5 (5-29)	144/124/158
Readmission			
Daily room charge	863.00	6.5 (5-15)	5610
Daily CBC, diff	93	6.5 (5-15)	605
Apheresis			
Pre-apheresis			1800
Apheresis Procedure	1,917.73	3 (0-6)	5753
PB CD34 analysis	135	2 (estimated)	270
Post-Apheresis (Cryopreservation)	1246.42	3 (0-6)	3739
Average Total Cost of Remobilization/ Patient			**33,081**

**Chemotherapy dose based on median body surface area of 1.76 (range, 1.47-2.44); n=16*

Results. Twenty-six patients (9%) failed the first mobilization attempt. Of these, 21 patients were remobilized with chemotherapy. The most commonly-used chemotherapy regimen was modified hyper-CVAD (fractionated cyclophosphamide, mesna, vincristine, doxorubicin, and dexamethasone) in 16 of 21 patients. Two patients failed the second mobilization attempt also, and did not get an autologous transplant. Both received further salvage therapy for their MM. In patients who were successful remobilizers, the average number of apheresis procedures required to reach a target HSC dose of 6 million CD34$^+$ cells/kg was 3 (range, 0-6). The average in patient stay for chemotherapy administration during remobilization was 2.5 days (range, 0-36). The number of days on G-CSF was 18.5 days (range, 11-32). All patients received IV hydration while in the hospital and were discharged on prophylactic oral antibiotics (antibacterial, antiviral, and antifungal) to be continued till ANC > 500/mm^3. The median time on oral antibiotics was 12.5 days (range, 5-29). Platelet and RBC transfusions were administered in 7/16 and 10/16 patients, respectively. Six patients required readmission (for fever and/or infections) ranging from 5-15 days. The average charges per patient for chemotherapy administration, transfusions, prophylactic antibiotics and G-CSF were $15,304.Average pre-apheresis charge per patient was $1800 (clinic visit, laboratory evaluation, insertion of central venous catheter, and chest x-ray to check placement). The average cost of apheresis per patient was $6,023 (includes cost of apheresis and analysis of PB CD34$^+$ cells). This is an underestimation as it does not include the costs associated with supportive care, intravenous fluids, professional fees etc. The average post-apheresis charges were $3,739. The average cost of readmission per patient was $6,215. *Conclusions.* Although only a minority of patients with MM fail to collect adequate HSC with cytokines alone, for patients who do fail the costs of remobilization with chemotherapy are substantial. Accordingly, the financial implication for transplant centers where reimbursement charges are DRG (disease related group) or case rate based is also significant. Interventions that may reduce the failure rate or reduce the number of apheresis procedures required to reach the target cell dose without an increase in toxicity may ultimately result in cost savings.

0134

DEVOLOPMENT OF A TIME-DRIVEN ACTIVITY-BASED COSTING MODEL FOR ASSESSING THE SOCIETAL ACQUISITION COST OF ERYTHROCYTE CONCENTRATES IN EUROPE

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Background. The cost of blood products is regularly underestimated by public health decision makers, hospital administrators and clinicians since the acquisition cost does not represent the total cost for obtaining and producing blood components. Historical accounting attempts to assess the acquisition cost of erythrocyte concentrate have varied in scope, perspective and methodology, leading to incomparable and criticised conclusions. *Aims.* To assess the true acquisition cost of erythrocyte concentrate in different European health care systems. *Method.* We derived a time-driven activity-based costing model taking into consideration blood centre costs and donor costs. Input from 3 (Swedish) blood centres was consolidated to develop a country specific model. Together with transfusion specialists, the transfusion chain is described in main processes chronologically and further broken down into activities. A division is made between direct *production*, quality assurance and supporting processes. Hereafter, all necessary resources and outputs of the activities are identified and quantified. All cost information is collected from the blood centre's general ledger. A capacity cost rate, representing a monetary value for each minute of available practical capacity, is calculated for the resources actually performing the work. The consumption of each resource for all identified activities is quantified based on time estimates from transfusion professionals. To quantify donor costs, we obtained figures on transport method, time and moment of donation by questionnaires which were administered to donors. The national value of productivity is used to quantify donor time in monetary terms. All transport costs are obtained from national databases. *Results*. The main activity components and their relations are illustrated in Figure 1. Within each main process, between 5 and 24 activities are described. Outputs are represented by statistics on donors, blood collections and erythrocyte concentrate units. Practical capacity includes all direct labour functions and equipment performing automated work in the direct "producing" processes. Costs include expenditures for direct and indirect resources necessary to perform the activities. We were able to determine a simplified cost formula: Societal cost of erythrocyte concentrate = $\sum$(direct labour time x labour capacity cost rate) + $\sum$(direct equipment time x equipment capacity cost rate) + $\sum$donor time x national value of production + $\sum$cost donor transport. Dividing the obtained cost by the number of units erythrocyte concentrate obtained in the same period of time, shows the total societal cost of 1 unit erythrocyte concentrate. The model presents the respective costs, driven by time consumption, for each of the identified processes where costs are occurred. *Summary and Conclusions.* The detailed process description and analysis reveal many activities and a significant amount of resources that have been excluded in previous accounting attempts to assess the acquisition cost of erythro-

cyte concentrate. The described model provides a complete and documented scope with a straightforward and transparent cost allocation methodology, respecting the societal perspective. Reporting a detailed cost structure allows insights in how the total cost is occurred and comparisons of results from different health care systems. Assessing the total cost of erythrocyte concentrate is important for cost-effectiveness analyses. It will support public health decision makers in evaluating the effectiveness of alternative therapies. Furthermore, this true cost calculation should be beneficial for institutions seeking adequate financing of erythrocyte concentrate.

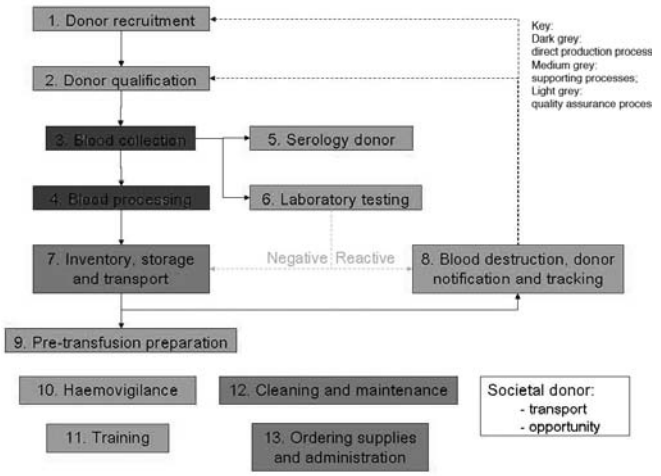

Figure 1. Main processes.

0135

HEALTH ECONOMIC IMPACT OF INTRAVENOUS IRON SUPPLEMENTATION IN ANEMIA TREATMENT WITH ERYTHROPOIESIS-STIMULATING AGENTS

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Background. Currently, erythropoiesis stimulating agents (ESA) are widely used in the treatment of anemia, e.g due to chronic kidney disease (CKD) or due to chemotherapy induced anemia (CIA). Not only due to recent safety concerns but also due to their high acquisition price, the usage of ESAs has come under severe pressure from health care payers. By optimising iron stores and iron bioavailability, the initiation of ESA therapy can be delayed and their efficiency be maximized. The combination of ESA therapy with a systematic iv iron supplementation may hence lead to significant cost savings which are likely to be markedly higher than the expenditures for IV iron. *Aims.* This study aimed at establishing the economic benefit of using IV iron in anemia treatment. A cost neutral price for IV iron was determined by calculating cost savings due to increased ESA efficacy. In addition we tested whether simplified handling of intravenous administration of iron might yield additional cost-savings. *Methods.* We determined the cost neutral price of IV iron as the price of IV iron at which the intervention is cost neutral to the Swiss healthcare system. This price equals the costs of spared ESA per responding patient. Given this definition, any IV iron priced below cost neutrality would be cost saving to the system. Using a third party perspective, we analysed the economic benefits in the following areas: (1) hemodialysis-chronic kidney disease (HD-CKD), (2) non-dialysis-chronic kidney disease (ND-CKD) and (3) chemotherapy-induced anemia (CIA). *Results.* The cost-neutral price of IV iron per 1000 mg in different therapeutic areas was: €500 in HD-CKD, €420 in ND-CKD and €2090 in CIA. The total costs of administering 1000mg iron, including IV iron plus the ancillary costs of administration were €270 for ferric carboxymaltose, €370 for iron sucrose, €374 for iron dextran and €389 for iron gluconate. The corresponding costs of the iron preparations per 1000mg are €250, €137, €124, and €69 respectively. *Summary and Conclusions.* Even though ferric carboxymaltose is the most expensive IV iron product, the associated savings are that significant, to become the most economically viable option in three different treatment settings.

Stem cell transplantation – GvHD, graft rejection, infection

0136

PREVALENCE OF HUMAN HERPES VIRUS 6 CHROMOSOMAL INTEGRATION (CIHHV-6) IN ITALIAN ALLOGENEIC STEM CELL AND SOLID ORGAN TRANSPLANTATION PATIENTS

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Background. Human herpes virus (HHV) 6, an ubiquitous β-herpesvirus, may reactivate and cause diseases upon immunosuppressive state. Half of stem cell (SC) and/or solid or-gan transplant (SOT) recipients experience HHV-6 viremia. Bone marrow (BM) suppression, encephalitis, pneumonitis and hepatitis are being increasingly associated clinical manifestations. A lesser recognized form of HHV-6 latency is the integration of the viral genome in a host chromosome (CIHHV-6). This phenomenon, characterized by a high viral copy number in blood or sera, may confound laboratory diagnosis of HHV-6 active infection, but apparently neither affects host health status nor requires clinical intervention. *Aims.* We have studied either SCT or SOT patients to identify cases of HHV-6 related-diseases and the prevalence of CIHHV-6. *Methods.* 78 allogeneic BMT and 345 SOT were evaluated. HHV-6 loads have been quantified by a commercially available quantitative real time polymerase chain reaction diagno-stic kit (Nanogen Advanced Diagnostics, Turin, Italy). *Results.* 70 out of 78 (90%) SCT and 135 out of 343 (39%) SOT patients were screened for HHV-6 either on body fluids or on tissue specimens. 16 out of 70 (23%) SCT patients and 52 out of 135 (39%) SOT patients presented at least one positive sample for HHV-6. 3 (18%) out of 16 SCT and 7 (13.4%) out of 52 SOT patients presented signs and symptoms consistent with HHV-6 related-diseases, namely four neutropenia and thrombocytopenia, two thrombocytopenia, two fever, one fever and lymphadenitis and one syncytial giant cell hepatitis. 2 (0,9%) out of 205 patients, one SCT and SOT respectively, demonstrated high value of HHV-6 DNAemia. The SOT patient presented a mean HHV-6 viral load >3.5 log10 copies/mL in plasma, and >6 log10/mL in blood during the entire clinical course. The alloSCT patient, presented high BM HHV-6 DNAemia (>5 log10 copies/mL) soon after transplantation, reducing to <4 log10 copies/mL with increasing chimerism, while presented fluctuating HHV-6 viremia, from 2 to >4 log10 copies/mL in plasma, unparalleled to white blood cell en-graftment and associated with graft versus host disease episodes. HHV-6 load in hair follicles in both patients revealed ≥1 HHV-6 copy/cell, indicating that the virus had been inherited in the germ line and is found in all cell of the body. Both patients had been heavily treated with antivirals without changes in HHV-6 loads. *Conclusions.* In Italian transplant patients, CIHHV-6 may occur at a frequency of 0.9%, while the frequency of HHV-6 related-diseases is approximately 5%. The results of molecular studies for HHV-6 should be critically evaluated and the distinction between HHV-6 active infection and CIHHV-6 should be pursued by the comparison of viral loads on different biological fluids. The search for HHV-6 on hair follicles, nails or other tissue specimens may confirm the occurrence of CIHH-6. The pretransplantation screening of donors and recipients could prevent misinterpretation of CIHHV-6 phenomenon in the recipients after transplantation, and preclude the administration of unnecessary and potentially toxic antiviral treatments.

0137

GENETIC VARIATIONS IN HEPARANASE GENE ARE ASSOCIATED WITH INCREASED RISK OF GRAFT VS. HOST DISEASE FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Complications of allogeneic hematopoietic stem cell transplantation (HSCT) halt its wider application for a variety of diseases. Graft-versus-host disease (GVHD) is the most common cause of overall mortality and

morbidity 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. Therefore we investigated the possible association of HPSE gene SNPs with the risk of post HSCT GVHD and transplantation outcome. Assessment of heparanase gene SNPs among healthy individuals demonstrated a significant correlation between HPSE gene SNPs and the expression level of heparanase, SNP rs4693608 being the most prominent (p=0.00043). This approach allowed distribution of all possible HPSE genotype combinations into three groups (LR, MR and HR) correlating with low, intermediate and high heparanase mRNA and protein expression levels. In the group of HSCT patients we found a highly significant correlation between HPSE gene SNPs rs4693608 and rs4364254, their combinations and risk of acute GVHD. The cumulative incidence of acute GVHD, grade II-IV, was 54.5% (95% CI 43.2-68.6) in the recipient group HR, while in recipient groups MR and LR, the cumulative incidences were 34.7% (95% CI 26.1-46.2) and 20.5% (95%CI 12.4-34.0), respectively (p=0.0001). Moreover, mismatching between recipient and donor in these SNPs combinations significantly increased the risk of acute GVHD. This association was statistically significant when recipients possessed genotype combinations HR and MR correlating with high heparanase mRNA levels, while their donors possessed the MR or LR genotype combinations, respectively (mismatched I group: HR-MR, MR-LR, and HR-LR pairs) (p=0.0000). The cumulative incidence of acute GVHD was 72.9% (95% CI 59.3-89.5) in the group of mismatched I, while in groups of matched and mismatched II (LR-MR, LR-HR, and MR-HR pairs) the cumulative incidences were 33.7% (95% CI 24.6-46.1) and 29.6% (95% CI 17.1-51.2), respectively (Figure 1). In addition, HPSE gene SNPs correlated with incidence of extensive chronic GVHD, transplantation-related death, and overall survival. The study demonstrated significant correlation between HPSE gene SNPs and heparanase expression levels, resulting in increased risk of acute and extensive chronic GVHD. Our findings may imply the involvement of heparanase in the pathogenesis of GVHD. Differences in heparanase expression between recipient and donor may lead to aggressive phenotype of donor T lymphocytes followed by infiltration and destruction of patient tissues.

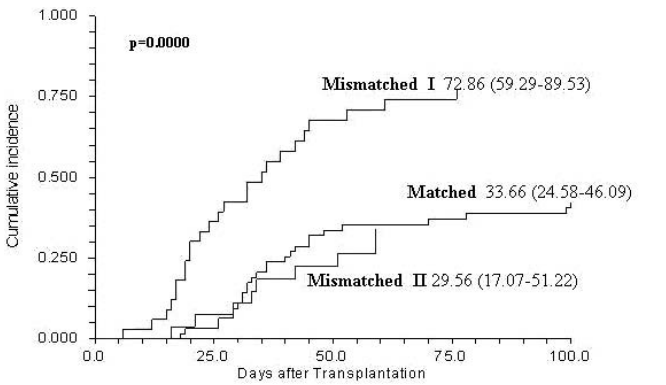

Figure 1. The cumulative incidence of acute GVHD, grade II-IV, following hematopoietic stemm cell transplantation according to matching or mismatching between HPSE gene SNPs combinations of recipient and donor.

0138

IMMUNE RECONSTITUTION AND RISK OF CYTOMEGALOVIRUS INFECTION IN PATIENTS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: IMPLICATIONS FOR PREEMPTIVE AND PROPHYLACTIC STRATEGIES

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Background. Infection or disease by cytomegalovirus (CMV) is still a major cause of morbidity and mortality after allogeneic stem cell transplantation (allo-SCT). *Aims.*We prospectively analyzed immune reconstitution kinetics and risk factors for CMV infection in 89 patients after allo-SCT. *Methods.* The serum IgG concentration and counts of CD4$^+$ T cells, CD8+ T cells, CMV-specific (pp65) CD8$^+$ cytotoxic T cells (CTLs) and NK cells were monitored monthly, starting from day +30 and continued up to the maximum of one year after allo-SCT. CMV was monitored by the pp65 antigen assay or an real-time PCR. *Results.* The recipient/donor CMV serostatus and the type of *in vivo* T cell depletion (TCD) had a significant influence on the cumulative CMV infection incidence in univariate and multivariate analysis. The CMV infection incidence was 83% after *in vivo* TCD with alemtuzumab, but 43% and 36% in patients who received antithymocyte globulin (ATG) or no *in vivo* TCD (p=0.011 and 0.002). The conditioning type (non-myeloablative with 2 Gy total-body irradiation (TBI), fludarabine-based reduced intensity (RIC) or conventional) had no significant impact on the estimated cumulative CMV infection incidence (60%, 45% and 35%). Day +30 counts of CD4$^+$ T cells, CD8$^+$ T cells, CMV-specific CTLs, NK cells and the serum IgG concentration did not significantly influence the occurrence of CMV infection. However, the day +60 NK cell count was identified as the most important risk factor for CMV infections beside the recipient's CMV seropositivity, whereas patients who showed any CMV infection or disease prior to day +60 were excluded from this analysis (CMV infection incidence 6%, 17% and 38% for patients with a day +60 NK cell count of >200/μL, 100-200/μL and <100/μL). The mean percentage of CMV-specific CTLs was low at days +30 and +60 (0.09% and 0.92%), reached the maximum with 2.24% (range 0-7.1%) at day +90 and decreased again thereafter. At day +30, CMV-specific CTLs were detected in 4 of 7 patients (57%) allografted from a CMV seropositive donor, but in none of 10 patients who had a CMV seronegative donor (p=0.01). Alemtuzumab strongly depressed reconstitution of CD4$^+$ and CD8$^+$ T cells, whereas ATG only delayed CD4$^+$ T cell reconstitution. CD4$^+$ and CD8$^+$ T cell reconstitution was improved after non-myeloablative conditioning in comparison to fludarabine-based RIC and conventional conditioning. Neither the type of *in vivo* TCD nor the conditioning type had any significant influence on immune reconstitution profiles of NK cells. *Summary and Conclusions.* The type of *in vivo* TCD (alemtuzumab or ATG) influences differentially both the CMV infection risk and CD4$^+$ and CD8$^+$ T cell reconstitution kinetics in patients after allo-SCT. Therefore, preemptive strategies could be adequate for patients after *in vivo* TCD with ATG, but patients after *in vivo* TCD with alemtuzumab might require antiviral prophylaxis. Our data further suggest that NK cells play an important role in preventing CMV reactivation beyond day +60 in patients who underwent allo-SCT. This observation might be the basis for clinical trials evaluating the efficacy of adoptive NK cell transfer.

0139

EXPANSION AND SUPPRESSIVE FUNCTION OF UMBILICAL CORD BLOOD REGULATORY T CELLS

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Background. Cord blood (CB) stem cells are now broadly used in the unrelated stem cell transplant setting and comparative studies with different stem cell sources have shown that CB transplant is characterized by a lower risk of graft-versus-host disease (GVHD). The immaturity of CB T cells is considered the major contributing factor accounting for this phenomenon; the possible role played by CB regulatory T cells (Tregs) for the suppression of the allogeneic T-cell response is now under investigation, but very scarce data are so far available. *Aims.*Aim of this study was to analyze and compare the expansion and suppressive functions of Tregs obtained from CB units with those obtained from the peripheral blood (PB) of adult normal donors. *Methods.* Tregs were purified from mononuclear cells obtained from CB units or from the PB of normal donors using the CD4$^+$CD25$^+$ regulatory T-cell isolation kit (Miltenyi Biotec) and expanded for 6 days in 96-well U-Bottom plates coated with anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) MoAbs in the presence of IL-2 (100 U/mL). To assess their suppressive functions, expanded Tregs were seeded with autologous effector T cells stimulated with allogeneic dendritic cells (DC) pulsed with apoptotic leukemic blasts, then incubated with [3H]-thymidine and counted in a β-counter. Suppressor activity was measured as [3H]-thymidine incorporation in the presence or absence of Tregs. The IL-10 production capacity was also tested using an ELISA assay. *Results.* Tregs expanded from CB units (n=15) and from the PB of normal donors (n=8) presented similar immunophenotypic features in terms of expression of surface CD4, CD25, CD62L, CCR5 and CD45RO, and cytoplasmic CTLA-4 and Foxp3; also, they both were negative for the CD45RA antigen. On the contrary, Tregs obtained from CB units presented a more than double expansion capacity: [mean fold increase (range): CB 10.089 (1.76-24), normal donors 4.59 (1.5-10), p 0.05], and exerted a potent suppressive function on the proliferative reac-

tion of T cells stimulated by allogeneic DC (n=6) [mean fold reduction (range), CB units 7.8 (2.5-15.1)]. Tregs expanded from CB units also presented a high *in vitro* IL-10 production capacity. *Summary and Conclusions.* These results indicate that Tregs contained in CB units loose their naïve phenotype following expansion and may exert a potent suppressive function, possibly accounting for the reduced incidence of GVHD that characterizes CB transplants. Our data offer further insights into the understanding of the biology of CB transplant and suggest possible therapeutic options for the prevention and cure of GVHD based on the infusion of expanded CB Tregs.

0140

THE MODULATORY PROPERTIES OF REGULATORY T CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WHO HAVE UNDERGONE AN ALLOGENEIC STEM CELL TRANSPLANT CORRELATE WITH THE PRESENCE OF GRAFT-VERSUS-HOST DISEASE

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Background. Graft-versus-host disease (GVHD) represents the major cause of morbidity and mortality after an allogeneic stem cell transplant (SCT). Studies in mouse models of SCT have shown that the infusion of culture-expanded regulatory T cells (Tregs) can be effective in preventing and suppressing GVHD, while apparently retaining the graft-versus-leukemia effect. *Aims.* Aim of this study was to verify whether the modulatory properties of Tregs expanded from the peripheral blood (PB) of patients who have undergone an allogeneic SCT correlate with the presence of acute and/or chronic GVHD. *Methods.* Tregs were purified from mononuclear cells obtained from PB using the $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec) and expanded for 6 days in 96-well U-Bottom plates coated with anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) MoAbs in the presence of IL-2 (100 U/mL). To assess their suppressive functions, expanded Tregs were seeded with autologous effector T cells stimulated with allogeneic dendritic cells (DC) pulsed with apoptotic leukemic blasts, then incubated with [3H]-thymidine and counted in a β-counter. Suppressor activity was measured as [3H]-thymidine incorporation in the presence or absence of Tregs. IL-10 production was also measured using an ELISA assay. *Results.* Tregs purified from the PB of patients with (n=7) or without (n=9) acute and/or chronic GVHD showed an equivalent expansion capacity and no immunophenotypic differences, in terms of expression of surface CD4, CD25, CD62L, cytoplasmic CTLA-4 and Foxp3. However, Tregs expanded from the PB of patients without signs of GVHD exerted a higher suppressive function on the proliferative reaction of T cells stimulated by allogeneic DCs compared to Tregs expanded from the PB of patients with GVHD: mean fold reduction (range), PB patients without GVHD 9.8 (5.28-21.82); PB patients with GVHD 3.9 (1.49-6.34), p 0.05. Moreover, Tregs expanded from patients without GVHD showed a significantly higher *in vitro* IL-10 production capacity: mean pg/mL (range), patients without GVHD 626 (461-731), patients with GVHD 245 (125-355), p 0.02. *Summary and Conclusions.* These observations indicate that the functional activity of Tregs in the PB of patients who have undergone an allogeneic SCT may protect from GVHD and support the design of therapeutic and/or preventive GMP protocols based on the *in vivo* infusion of ex vivo expanded and activated Tregs, aiming at modulating the activity of the immune system after an allogeneic SCT.

0141

DIVERSITY AND DIFFERENTIAL INVOLVEMENT OF HLA CLASS I AND II RESTRICTED MINOR HISTOCOMPATIBILITY ANTIGENS IN GRAFT-VERSUS-LEUKEMIA REACTIVITY

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Background. Donor lymphocyte infusion (DLI) can be an effective cellular immunotherapy for patients with hematological malignancies after HLA-matched allogeneic stem cell transplantation (alloSCT). The effect of DLI is mediated by donor derived T-cells recognizing minor histocompatibility antigens (mHags) on malignant cells of the recipient. These T-cells may also induce Graft-versus-Host Disease (GvHD) when directed against mHags with broad expression on non-malignant tissues. *Aims.* The aim of the study was to investigate T-cell diversity and specificity in Graft-versus-Leukemia (GvL) reactivity. *Methods.* $CD8^+$ and $CD4^+$ T-cell clones were isolated from a patient successfully treated with DLI for relapsed chronic myeloid leukemia (CML) more than one year after HLA-matched alloSCT. GvL reactivity in this patient was accompanied with mild GvHD of the skin. Reactivity of the T-cell clones was analyzed in detail and HLA-class I and II restricted mHags were characterized by screening plasmid and bacteria cDNA libraries, respectively. *Results.* The isolated T-cell clones were shown to be directed against 13 different mHags. HLA-class I mHags included HA-1 and HA-2 in HLA-A2 and 5 unknown mHags in HLA-B8 and -B60. HLA-class II mHags were presented by HLA-DQ and -DR. By screening plasmid and bacteria cDNA libraries, we identified an HLA-B60 mHag encoded by the TRIP10 gene, an HLA-DQ mHag encoded by the PI4K2B gene and four HLA-DR mHags encoded by the PTK2B, MR1, LY75 and MTHFD1 genes. Analysis of public microarray databases revealed ubiquitous expression of TRIP10 and PI4K2B, whereas the other mHags were selectively (HA-1, HA-2, LY-75) or predominantly (PTK2B, MR1, MTHFD1) expressed in hematopoietic cells. The T-cell clones were analyzed in detail for reactivity against hematopoietic cells, including non-malignant peripheral blood cells (monocytes, B- and T-cells), malignant $CD34^+$ CML progenitor cells, professional antigen presenting cells (APC) and acute myeloid and lymphoblastic leukemias (AML and ALL). All $CD8^+$ and $CD4^+$ T-cell clones recognized (subsets of) peripheral blood cells, except for the T-cell clone for TRIP10. Moreover, all $CD8^+$ T-cell clones, except for the T-cell clone for TRIP10, recognized $CD34^+$ CML cells. Differential recognition of $CD34^+$ CML cells was observed for $CD4^+$ T-cell clones. The T-cell clone for MTHFD1 recognized $CD34^+$ CML cells, whereas no reactivity was observed for the T-cell clones recognizing LY-75, PTK2B and MR1. All T-cell clones strongly recognized professional APC, including monocyte-derived dendritic cells and *in vitro* differentiated $CD34^+$ CML cells with APC phenotype. Finally, all T-cell clones were capable of recognizing primary AML and ALL, except for the T-cell clone for TRIP10, which showed restricted recognition of AML-M4 and -M5 of monocytic origin. *Conclusions*. Our data show a detailed analysis and characterization of mHags recognized by T-cells induced in a patient successfully treated with DLI for relapsed CML and provide evidence for differential involvement of mHags in the onset and execution of GvL reactivity. All identified mHags were shown to be expressed on (subtypes of) primary leukemic cells, and may therefore be appropriate targets for T-cell based immunotherapy.

0142

CYCLOSPORINE, METHOTREXATE AND METHYLPREDNISOLONE FOR GRAFT-VERSUS-HOST DISEASE PROPHYLAXIS IN CHILDREN WITH HEMOGLOBINOPATHIES: A PROSPECTIVE STUDY

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Background. Although children are at less risk for GVHD than adults its incidence is still significant despite drug prophylaxis. Unlike hematological malignancies where the high rate of GVHD may be offset by lower relapse rates there is no benefit of GVHD for non malignant diseases. In a retrospective study we have previously demonstrated a decrease in the incidence of acute GVHD in class 3 patients who received cyclosporine (CSA), a modified short course of methotrexate (MTX) [Cyclophosphamide 7.5 mg/kg on day +1] and methylprednisolone (MP) [CSA/CY/MTX/MP)] as compared to class 1 and 2 patients treated with a CSA/MP combination. Therefore in an attempt to reduce the incidence of GVHD in this patient population we used CSA/MTX/MP prophylaxis in all patients apart from risk classes. This is the first study prospectively evaluating a three drug regimen in children with hemoglobinopathies to date. *Aims.* to evaluate the efficacy and safety of CSA/MTX/MP regimen for GVHD prophylaxis in children with hemoglobinopathies. *Methods.* 101 children with median age of 8 years (range, 1.6-17) affected by thalassemia (n=95) or sickle cell anemia (n=6) were given a bone marrow graft from HLA matched family donors between 2004 and 2008. The conditioning regimen for class 1 and class 2 patients (n=52) consisted of BUCY200 ± thiotepa, class 3 patients (n=43) of BU/CY160 ± thiotepa (preceded by hydroxyurea, azathioprine and fludarabine) and for SCA patients of BUCY200 and thymoglobulin. Forty six patients received CSA, a short course of MTX (10 mg/m^2 on days +1, +3, +6) and low-dose MP (0,5 mg/kg/d) [CSA/MTX/MP], while 55 patients were given CSA/CY/MTX/MP as GVHD prophylaxis. The median NC, CD34 and CD3 cell dose was 4.4×10^8/kg (range 1.3-10.8), 7.1×10^6/kg (range 0.78-34.7) and 54×10^6/kg (range 3.8-208) respectively.

Thirty male patients received stem cells from female donors. Thirty seven patients were transplanted with marrow from ABO major or minor mismatched donors. *Results.* Overall 33 out of 96 evaluable patients have developed grade II-IV acute GVHD with the probability of 33% (95% CI: 24-42). The probability of grade III-IV GVHD was low 4% (95% CI 1-9). The incidence of aGVHD was similar in patients who received CSA/MTX/MP or CSA/CY/MTX/MP (p=0.8). In logistic regression analysis female donor for male patients (OR 2.8; p=0.005), the number of $CD3^+$ cells >54×10^6/kg (OR 2.2; p=0.025) and ABO mismatch (OR 2.3; p=0.021) predicted grade II-IV aGVHD. The probability of chronic moderate or severe GVHD was 9% (95% CI 4-16) and 3% (95% CI 1-8) respectively. Previous acute GVHD (OR 2.4; p=0.002) and patient age >8.8 years (OR 6.9; p=0.005) predicted chronic GVHD. Eight patients have died 3 of them from GVHD o related complications. *Conclusion.* This study showed that the incidence of grade II-IV acute GVHD following CSA/MTX/MP prophylaxis is similar to those observed after CSA/MP prophylaxis (our previous study). These data suggest that the addition of MTX to CSA/MP was not associated with a low incidence of aGVHD in this patient population. The incidence of extensive chronic GVHD in patients with hemoglobinopathies is low.

0143

ALLOANTIGEN-SPECIFIC *DE NOVO* INDUCED FOXP3⁺ REGULATORY T CELLS FAIL TO PREVENT GVHD DUE TO *IN VIVO* INSTABILITY

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Background. Thymus-derived, natural Tregs (nTregs) have been shown to protect mice from lethal GvHD. Induced antigen-specific FoxP3+ T cells (iTregs) are discussed as a promising alternative to polyclonal natural FoxP3+ T cells for cell-based therapies, particularly to achieve more specific transplantation tolerance. *Aims.* To establish a reliable protocol to induce *de novo* alloantigen-specific iTregs *in vitro* and to test their ability to prevent GvHD *in vivo*. *Methods.* Using FoxP3-reporter mice (C56BL/6), we established an efficient protocol to induce and expand alloantigen-specific iTregs from $FoxP3^-CD4^+$ T cells with allogeneic cluster-disrupted dendritic cells (BALB/c). Interestingly, syngeneic or LPS-matured dendritic cells fail to induce FoxP3 in allogeneic $CD4^+$ T cells. Functionality of iTregs was tested *in vitro* and in a C57BL/6 into BALB/c mouse model of acute GvHD. *Results.* After *in vitro* induction $FoxP3^+$ induced Tregs were mainly $CD62L^+$ and $CD25^+$ and their immunosuppressive capacity *in vitro* was similar to those of natural Tregs. Induced and natural Tregs were retrieved from recipient mice after transplantation at similar levels, but, in contrast to nTregs, iTregs failed to prevent lethal GvHD. Within irradiated recipients, the majority of adoptively transferred $FoxP3^+$ iTregs but not $FoxP3^+$ nTregs had quickly reverted to $FoxP3^-CD4^+$ T cells (70% after four days and 80% after 8 days). *Conclusions.* Our data suggest that therapeutic approaches to treat GvHD should rely on nTregs whereas the use of *de novo* induced alloantigen-specific iTregs must be handled with caution since the stability of the regulatory phenotype of the iTreg appears to be of major concern. Thus, when exposed to a lymphopenic, recently conditioned environment after irradiation or chemotherapy, the use of iTregs may turn out to set a fox to keep the geese.

0144

RECOVERY OF SPECIFIC CMV CD8+ T LYMPHOCYTES AND T REG AFTER ALLOGENEIC PERIPHERAL STEM CELL TRANSPLANTATION: A SINGLE CENTRE EXPERIENCE

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Background. Recovery of cytomegalovirus (CMV)-specific T-cells after allogeneic stem cell transplantation (alloSCT) is critical for protection against CMV disease; in humans the CMV-specific $CD8^+$ must be regenerated after SCT in order to obtain long-term protection against CMV infection and disease. Moreover the recovery $CD4^+CD25^+Foxp3^+$ regulatory T cells (T reg) are a major regulator of adaptive immunity. The aim of our study was to evaluate the recovery of CMV-specific $CD8^+$ and T reg in the peripheral blood after alloSCT. AIMS 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) in 60 patients after alloSCT. *Patients and Methods.* Patients were transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=53) or an HLA identical unrelated donor (n=7). Median age was 36 years (range 18-61); diagnoses were acute myeloid leukaemia (n=48), acute lymphoblastic leukaemia (n=8), chronic myeloid leukaemia (n=3), myelofibrosis (n=1). *Results.* Median CMV-specific CD8+ T lymphocytes were significantly higher in patients without than with CMV infection/disease at 1 (2 cells/mcL vs 0 mcL, p=0.05), 2 (5 cells/mcL vs. 1 mcL, p=0.05), 3 (12 cells/mcL vs 2, p=0.03) and 6 months (22 cells/mcL vs 3 , p=0.03), respectively. Tetramer analysis showed that 31/60 (51 %) patients reconstituted CMV-specific $CD8^+$ at 2 months after transplantation; CMV infection/disease was observed in 0/12 (0%) patients with CMV-specific $CD8^+$ recovery and without GvHD, in 3/28 (10%) patients with recovery of CMV-specific $CD8^+$ T-cells with GvHD and in 19/20 (95 %) patients without recovery of CMV-specific $CD8^+$ T-cells. In our experience no CMV infection/disease was observed in cases with recovery of CMV-specific $CD8^+$ T-recovery cells > 5 mcL. Moreover, we observed a good correlation between the recovery of CMV-specific $CD8^+$ lymphocytes and of $CD4^+/CD25^{high}$ regulatory T cells at 2 (p=0.05, r=0.8) and 3 (p=0.06, r=0.7) months after alloSCT. However, median T reg values were significantly higher in patients without than with CMV infection/disease at 2 (15 mcL vs 3mcL, p<0.03) and 3 months (22 mcL vs 6mcL, p=0.05). Acute GvHD grade II-IV was observed in 24/60 patients (40 %); at univariate analysis, T reg was significantly higher in patients without than with aGvHD (17 mcL vs 4 mcL, p=0.05). *Summary*. In conclusion we suggest a good correlation between the recovery of CMV-specific $CD8^+$ T-cells and the recovery of T regs; T regs mediate the protective effects toward the aGvHD and the maintenance of an optimal microenviroment for the reconstitution of functional immunity. This supports further consideration of T regs immunotherapy in clinical alloSCT.

0145

PROTEOMIC PATTERNS AND GRAFT VERSUS HOST DISEASE AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The application of allogeneic hematopoietic stem cell transplantation (alloSCT) is limited due to severe life threatening complications such as severe acute GVHD (Graft Versus Host Disease). To date, diagnosis of GVHD is mainly based on clinical features. Recently the application of proteomic tools, allowing screening for differentially expressed or excreted proteins in body fluids, could allow detecting specific biomarkers, and, in this respect, whole saliva is highly attractive for the non-invasive specimen collection. Aims:in this aim, the application of proteomic analysis to salivary specimens of 14 patients (pts) submitted to alloHSCT in our Institution 2008 is here described. Pts characteristics' were: 7M/7F, median age 35 years (range 14-66). Underlying diseases were: 8 AML, 3 ALL, 2 MM, 1 HD. Nine pts were submitted to standard dose conditioning regimen while 5 to reduced intensity regimens. *Methods.* Samples of whole saliva were collected between 10.00 and 12.00 am by using a soft plastic aspirator. All specimens were mixed immediately in a 1:1 (v/v) ratio with aqueous 0.2% trifluoroacetic acid solution and centrifuged at 8,000 g at 4°C for 5 min. The obtained solutions were either immediately analyzed by High-Performance Liquid Chromatography (HPLC) coupled to ElectroSpray-Ionization Mass Spectrometry (ESI-MS) or stored at -80°C before analysis. *Results.* Seven out of 14 pts (50%) developed aGVHD involving oral mucosa. The chromatograms of the acidic soluble fraction of salivary proteins of pts with aGVHD were compared to the asymptomatic ones. Different expressions of calgranulin A, part of an etero-dimeric leukocyte-derived protein called calprotectin, over-expressed in oral mucosal inflammation were observed, as a preliminary result. Six pts out of 7 with GVHD showed in the chromatograms the presence of calgranulin A (M average 10834.6; elution time 38.5 min), while the protein was not detectable in the chromatograms of pts without GVHD (Fisher's exact test p=0.0047). *Conclusions.* Despite the small number of pts until now examined, highly significant statistic between the presence of calgranulin A and the development of aGVHD involving sequelae of oral mucosa was found. Further studies should clarify if this protein could be considered either a marker of GVHD or an index of mucosal inflammation. However, this protein is not detected in early phase after transplantation, when oral mucositis is present.

0146

CD8 DEPLETED DLI RECONSTITUTE FULL DONOR T-CELL CHIMERISM AND REPLENISH THE CD52-POSITIVE CD4 T CELL POOL AFTER ALEMTUZUMAB-BASED T-CELL DEPLETED ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Donor lymphocyte infusions (DLI) are frequently used preemptively for mixed hematopoietic chimerism after allogeneic hematopoietic stem cell transplantation (HSCT) although they carry a substantial risk of inducing graft-versus-host disease (GVHD). Removal of CD8 T cells from DLI has been shown to reduce this risk while preserving beneficial DLI effects. We investigated on the application of prophylactic DLI heavily depleted of CD8 T cells early after anti-CD52 (alemtuzumab) mediated T-cell depleted allo-HSCT with regard to T-cell chimerism (TCC) and immune-reconstitution. Peripheral blood mononuclear cells of patients following conditioning with fludarabine, melphalan and high-dose alemtuzumab (100mg) were monitored for TCC and phenotype in the course after HSCT. Monitoring TCC, we identified 20 individuals with mixed chimerism beyond day +60; 13 of them received DLI while 7 did not. The 13 DLI patients showed an increase of donor TCC, 12 converted to complete T-cell chimeras. In contrast, only 1 patient of the non-DLI group spontaneously converted to full donor TCC. Interestingly, we observed successful conversion by DLI even in cases with donor TCC less than 20%. When we analyzed the phenotype of reconstituting T cells, a substantial proportion of CD4 T cells did not express CD52, the target-antigen of the T-cell depleting antibody alemtuzumab. We followed the CD52-status on T cells of patients with and without CD8-depleted DLI and found significantly higher percentages of CD52+ CD4 T cells after DLI. After 1 year, CD4 T cells in DLI patients were mainly CD52 positive while without DLI, significant proportions of CD4 T cells remained negative for CD52. The CD52-expression of monocytes, B cells, and NK cells remained unaltered after transplantation and was not influenced by the application of CD8-depleted DLI. When we treated purified human CD4 T cells with Alemtuzumab *in vitro*, we observed down-regulation of CD52. But this loss of CD52-expression was transient with a minimum after 3-7 days of culture. In contrast, the CD52-expression remained negative over more than 6 weeks when we separated $CD52^-$ from $CD52^+$ CD4 T cell populations from the peripheral blood of our patients and expanded them *in vitro*. We did not find any evidence for a transcriptional regulation of CD52 by RT-PCR. Since CD52 is a glycosylphosphatidylinositol (GPI)-linked surface protein, we stained for other GPI-anchored proteins and found that $CD52^-$ CD4 T cells also had lost expression of CD55 and CD59 *in vivo* as well as in our *ex vivo* culture. In summary, we demonstrate that CD8-depleted DLI can be used to reliably convert mixed to full donor TCC after T-cell depleted allo-HSCT. Their impact on immune reconstitution is also verified by replenishment of the $CD52^+$ CD4 T-cell compartment. The *in vivo* CD52-downregulation after alemtuzumab-mediated T-cell depletion remains stable even if cells are transferred to cell culture and is associated with the loss of further GPI-anchored surface molecules.

0147

CYCLOSPORINE DOSE INTENSITY IS A CRITICAL DETERMINANT OF OUTCOME AFTER A REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANT

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Background. The ability of allogeneic transplants performed using a reduced intensity conditioning (RIC) regimen to deliver long term disease free survival is dependent on the genesis of an immunologically mediated graft-versus tumor (GVT) effect. Post-transplant immunosuppression plays an important role in limiting graft-versus-host disease (GVHD) but also modulates a GVT effect. Cyclosporine A (CsA) is the most commonly utilised form of GVHD prophylaxis after a RIC allograft but its impact on transplant outcome has not been studied. *Aim.* We wished to examine the impact of CsA dose intensity on disease relapse and overall survival (OS) in patients transplanted using an alemtuzumab based RIC regimen. *Methods.* CsA exposure in the first 21 days post-transplant was measured in 132 patients and correlated with overall survival, and relapse risk. 68 patients were transplanted for a myeloid malignancy (acute myeloid leukemia (n=41), myelodysplasia (n=17)) and 74 for a lymphoid malignancy (Non-Hodgkin's lymphoma (n=51) or Hodgkin's disease (n=23)). All patients with a myeloid malignancy were transplanted using a regimen consisting of fludarabine, melphalan and alemtuzumab (FMA). Patients with an underlying lymphoid disease were transplanted using FMA (n=31) or a regimen consisting of BCNU, etoposide, cytosine arabinoside, melphalan and alemtuzumab (BEAMA) (n=43). 39 patients had chemoresistant disease at the time of transplant. All patients received intravenous CsA at a loading dose of 5 mg/kg on day -1 followed by 2.5 mg/kg b.i.d. Patients were switched to oral CsA prior to discharge. Trough CsA levels were measured thrice weekly for the first three weeks after stem cell infusion and the dose of CsA adjusted to achieve levels in the region of 200-300 μg/L during this period. Trough levels obtained during the first 21 days post-transplant were used to calculate the CsA area under the curve (AUC) for each patient. *Results.* 71 patients were transplanted from HLA identical siblings and 61 from volunteer unrelated donors. The median age of the whole group was 48 years (range 17-68). The incidence of acute GVHD (Grades 2-4) was 34%. The median CsA AUC in the first 21 days post-transplant was 3682 microg.hr/l (range 2162-8084). In univariate analysis the presence of chemoresistant disease at the time of transplant and a high CsA AUC were both associated with a decreased OS. In multivariate analyses chemoresistant disease (HR=2.60, 95% CI 1.44-4.65, p=0.002) and linearly increasing CsA AUC were associated with an increased hazard of death (HR=1.1, 95%CI 1.02-1.24, p=0.02). The two year OS for patients with a CsA AUC less than 3682 microg.hr/l was 77% compared to 30% for patients with CsA greater than 3682 microg.hr/l (p<0.0001). Increased CsA AUC and the presence of chemorefractory disease at the time of transplant were associated with an increased risk of relapse in univariate analysis. In multivariate analyses only increased CsA AUC was significantly associated with a higher risk of relapse (OR=4.1, 95% CI 2.50-6.81, p<0.0001). Decreased CsA AUC and the use of an unrelated donor were associated with an increased risk of acute GVHD in univariate analysis. Multivariate analyses demonstrated that the use of an unrelated donor (HR=2.9, 9%% CI 1.36-6.36, p=0.006) and linearly decreasing CsA AUC were significantly associated with an increased risk of acute GVHD (HR=1.2, 95% CI 1.05-1.43, p=0.01). *Conclusions.* These data identify CsA exposure as a critical and manipulable determinant of outcome after a T depleted RIC allograft. They support a randomised trial aimed at identifying the optimal CsA dose schedule in this population of patients. It will be important to determine whether post-transplant CsA exposure plays a similar role in determining outcome after a T replete RIC allograft.

0148

IMPACT OF MINOR HISTOCOMPATIBILITY ANTIGEN MISMATCHES ON GRAFT-VERSUS-HOST DISEASE IN NON-MYELOABLATIVE OR REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. In a fully molecular HLA-matched setting, mismatches in minor histocompatibility antigens (mHAs) between donor and recipient might impact the incidence of graft-versus-host disease (GVHD) and transplant survival after allogeneic stem cell transplantation (alloSCT). *Aims.* The aim of this study was to assess whether mHAs mismatches could affect GVHD incidence (acute and chronic, aGVHD and cGVHD), overall survival (OS) and progression free survival (PFS) in patients undergoing alloSCT for lymphoid malignancies. *Methods.* Fifty-two consecutive patients who underwent alloSCT were studied. Nine patients had chronic lymphocytic leukemia (17%), 13 had Hodgkin's lymphoma (25%) and 30 had multiple myeloma (58%). All the patients received peripheral blood stem-cell allograft after non-myeloablative (11 patients, 21%) or reduced intensity (RIC, 41 patients, 79%) conditioning. GVHD prophylaxis included cyclosporine plus methotrexate or micomofetil fenolate. Median age was 51 years (range 17-65). Twenty-nine patients were male (56%) and the median number of previous chemotherapies was 3 (0-7). Nineteen patients were in complete remission (CR, 37%), 27 in partial remission (PR, 52%) and 6 had progressive disease (PD, 11%). Karnofsky performance status (PS) at transplant was >80% in 41 patients (79%). Forty patients were allografted from HLA-matched siblings (77%), 12 from matched unrelated donors (23%): all were matched at allelic level for HLA-A, -B, -Cw, -DRB1 and -DQB1 loci. Allelic mHAs typing was performed by PCR with sequence-specific primers for 14 autosomic mHAs and H-Y. OS and PFS were analyzed with Kaplan-Meier method and log-rank test. aGVHD and cGVHD were analyzed with a multivariate logistic regression model including as covariates age,

sex, disease diagnosis, PS and mHAs mismatches. Grade >=2 aGVHD and extensive cGVHD were considered events. *Results.* Median follow-up was 42 months (range 2-83). One-year OS was 83%, 2- and 3-years OS were 79% and 74%. One-year PFS was 58%, 2- and 3-years PFS were 47% and 43%. OS and PFS were significantly affected by disease status (CR vs PR vs PD, $p<0.001$ and $p=0.003$) and PS (≤80 vs. >80, $p<0.001$ and $p=0.001$). Donor-vs-recipient (DR) mHAs mismatches did not have a significant impact on OS and PFS. The presence of at least one DR mHAs mismatch and the number of DR mHAs mismatches correlated with aGVHD ($p=0.02$ for both). Broad DR and H-Y mHAs mismatches did not influence aGVHD ($p=0.49$ and $p=0.27$, respectively). A correlation of DR hematopoietic-restricted mHAs mismatches with aGVHD was found ($p=0.01$). DR LB-ADIR mHAs mismatches had a significant influence on aGVHD ($p=0.04$) whereas DR HA-1, HA-2 and HA-8 mHAs mismatches did not ($p=0.42$, $p=0.31$ and $p=0.33$, respectively). Recipient-vs-donor (RD) mHAs mismatches, as well as the other factors considered in multivariate model did not significantly influence aGVHD. DR mHAs mismatches had no impact on cGVHD incidence whereas the presence of at least one RD mHAs mismatch correlated with cGVHD ($p=0.02$). *Conclusions.* This study showed that in non-myeloablative and RIC setting mHAs mismatches may have a significant role in determining aGVHD. Assessing mHAs mismatches may be a useful tool to choose patient-specific GVHD prophylaxis in conditioning regimens and in post transplant follow-up.

0149

CHARACTERIZATION OF T CELL RECEPTOR BETA REPERTOIRE IN T CELL POPULATION ASSOCIATED WITH GRAFT-VERSUS-HOST AND GRAFT-VERSUS-LEUKEMIA EFFECT

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Background. Graft-verus-tumor (GVT) effect of the donor T cells in hematopoietic stem cell transplantation (HSCT) is usually complicated by the ineligible alloreactivity of these T cells which leads to acute graft-versus-host (GVH) disease. GVT and GVH reactions are proved to be mediated by different T cell clones and can be distinguished by virtue of their T cell receptore β sequence. *Aims.* The objective of this study was to identify and characterize T cells clones with specific antitumor activity without mediating GVHD in T cell populations activated by leukemia and myeloma antigens. *Methods*. We have performed primary mixed leukocyte reaction (MLR) *in vitro* using patient non-leukemic irradiated peripherial blood mononuclear cells (PBMC) as stimulators and donor PBMC as responders. To prepare GVT specific T cells, activated alloreactive T cells were first selectively depleted with an anti-CD25 immunotoxin. Allodepleted T cells were then stimulated in secondary MLR using irradiated leukemia or myeloma cells from the same patient. Activated tumor-reactive cells were purified by immunomagnetic selection or by FACS based on INF-γ or CD25 expression, respectively. Clonotypic assay was used for identification of individual tumor-specific T cell clones. This highly sensitive assay is based on detailed analysis of T cell receptor β VDJ unique sequence (TCRB-VDJ). mRNA was extracted from sortred activated cells and cDNA synthetized by anchored reverse transcription. Target TCRB-VDJ gene sequence was amplified by anchor PCR and used to transform bacteria. Bacterial colonies were picked for plasmid isolation and subsequent direct automated sequencing of the TCRB-VDJ sequences. We assume that the frequency of particular TCRB-VDJ sequences among bacterial clones after transformation is proportional to the frequency of those sequences in the original population of T cells activated by GVH or GVT reaction. *Results.* We investigated the presence of individual antileukemic T cell clones in T cell populations derived from MLR using tumor cells from patients with acute myeloid leukemia (AML), chronic lymphatic leukemia (CLL) a and multiple myeloma, and defined them by the TCRB VDJ unique sequence. The sequences that occured in more than 10% bacterial colonies are likely to represent the most immunodominant clones. The immune responses against tumor cells were oligoclonal, i.e. we observed limited number of individual immunodominat clones which plays important role in GVT reaction. In first CLL patient who had undergone HSCT, six antileukemic T cell clones were identified, four of them are considered to be immunodominant. In second CLL patient after HSCT, just one highly immunodominat autileukemic T cell clone was observed. *Conclusions*. Clonotypic assay is highly sensitive method for characterization of immunodominant tumor reactive T cells and can be used as a marker for evaluation of adoptive cell immunotherapy by potential further monitoring of individual T cell clones in patients peripherial blood or tissue.

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0150

RETROSPECTIVE SINGLE CENTER ANALYSIS OF PENTOSTATIN FOR THE TREATMENT OF STEROID-REFRACTORY ACUTE GRAFT-VERSUS-HOST DISEASE

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Background. Clinically significant acute graft-versus-host disease (aGVHD) remains a major cause of morbidity and mortality in patients undergoing allogeneic stem cell transplantation. In particular, the outcome of those patients who have failed first line therapy with glucocorticosteroids is still poor. Preliminary reports suggested that pentostatin, a purine analogue inhibiting T-cell numbers and function, might be clinically effective with a mild and manageable spectrum of side effects. *Patients and Methods.* Here we report on our single center experience using pentostatin for patients with acute GVHD or overlap syndrome after failure of first line therapy with glucocorticosteroids. From 2005 to 2008, a total number of n=25 patients (out of 301 at risk) who had been treated with pentostatin for GVHD were identified and retrospectively analyzed. Diagnoses included acute lymphoblastic leukemia (ALL, n=2), acute myeloid leukemia/myelodysplastic syndrome (AML/MDS, n=14) and lymphoma/myeloma (n=9). Gender was well balanced (male n=13, female n=12) with a median age of 47 (23-64). All transplants were peripheral blood stem cells from matched siblings (n=8), matched unrelated (n=4) or mismatched donors (n=13). Predominantly, aGVHD affected the gastrointestinal tract (24/25) with grades being III (n=11) or IV (n=13). Subjects received 1 (n=16) to 2 (n=9) cycles of pentostatin (1 mg/m^2, days 1-3). *Results.* Patients with resolved diarrhoea and patients discharged from inpatient care were classified as complete responders (CR). Using this definition, a CR rate of 46% (11/24) was observed with a median time to response of 7 days (3-29). Toxicity was assessed by comparing WBC, platelets, serum bilirubin, ALAT, creatinine and CRP values immediate prior pentostatin and 7 days after the first dose. Whereas significant changes in WBC (median -1.9 G/L), platelets (median -35 G/L), creatinine (median +0.03 mg/dL) and CRP (median +2.9 mg/dL) did not occur, moderate increases in total bilirubin (median +2 mg/dl, $p=0.083$) and ALAT (median +38 U/L, $p=0.016$) were observed. The toxicity data is summarized in Table 1.

Table 1. Toxicity data.

Parameter	d0	d7	p
Leukocytes (G/l)	7.1 (1 - 43.6)	5.2 (0.5 - 42.1)	ns
Platelets (G/l)	64 (11 - 210)	29 (18 - 203)	ns
ALAT (U/l)	*50 (5 - 402)*	*88 (14 - 936)*	*.016*
Bilirubin (mg/dl)	*1.4 (0.4 - 13.9)*	*3.4 (0.3 - 20.6)*	*.083*
Creatinine (mg/dl)	0.78 (0.52 - 4.3)	0.81 (0.31 - 3.7)	ns
CRP (mg/l)	5.3 (0 - 185)	8.2 (0 - 274)	ns

Cytomegalovirus (CMV) reactivation after pentostatin was observed in 44% of patients at risk (8/18) after a median time of 8 days (3-11). 22% of subjects (4/18) suffered from CMV reactivation before pentostatin administration within a median time of 4 days (2-6). Patients failing to respond to pentostatin also failed to respond to further salvage therapies including MSCs, tacrolimus, basiliximab, rituximab and infliximab, resulting in an overall non-relapse mortality of 64%. 16% of subjects (4/25) relapsed and died because of their underlying malignant hemato-

logical disease, translating in an overall survival rate of 18% at 2 years. 3 out of 4 long-term survivors living disease-free (with a median of 886 [820-959] days after transplantation) suffer from extensive chronic GVHD. *Conclusions.* Pentostatin is a safe and simple to use therapy for glucocorticosteroid-refractory aGVHD showing response and outcome rates similar to other clinically used aGVHD salvage regimens. Given the high rate of non-relapse mortality and failure to respond to further second line therapies, we conclude that new therapeutic strategies are still needed.

0151

GENETIC MISMATCH IN GSTT1 IS ASSOCIATED WITH HEPATIC ACUTE GRAFT VERSUS HOST DISEASE (GVHD) IN ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-SCT)

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Background. Glutathione S-transferase T1 (GSTT1) is a hepatic enzyme involved in drug metabolic phase II pathway. Twenty percent of Caucasian population and 60% of Asiatic population has a genetic variant of GSTT1, by which GSTT1 is deleted, the so called "null genotype", resulting in the absence of the enzyme. Our group has previously found that a specific antibody-mediated alloimmune response is triggered when a null GSTT1 recipient receives a GSTT1 positive liver graft. The potential influence of GSTT1 genotype on immunobiologic reactions in the liver after allo-SCT has not been studied yet. *Aims.*We analyzed the association of GSTT1 genetic variants with the incidence of hepatic acute GVHD in 40 patients undergoing allo-SCT. *Methods and Results.* Transplants were performed in a single institution, donors were HLA identical siblings and all patients received myeloablative-conditioning regimen. All donor and recipient pairs were Caucasians. Median follow-up was of 75 months (range, 2-171 months). GSTT1 variants were analyzed, by PCR method, in 40 patients and donors. Of them, 3 pairs were null GSTT1 donor (donor -) and positive GSTT1 recipient (recipient +) (Group 1), 8 pairs were positive GSTT1 donor and null GSTT1 recipient (Group 2), 18 pairs were positive GSTT1 donor and positive GSTT1 recipient (Group 3), and 9 pairs were null GSTT1 donor and null GSTT1 recipient (Group 4). Results of the association of GSTT1 genotype and hepatic acute GVHD was as shown in Table 1 (attached). *Conclusions.* Genetic mismatch in GSTT1 between donors and recipients has a strong association with hepatic acute GVHD. This could help in the differential diagnosis of hepatic alterations after allo-SCT.

Table 1.

	Donor - Recipient + (group 1)	Donor + Recipient – (group 2)	Donor + Recipient + (group 3)	Donor – Recipient – (group 4)	Comparison between groups	P*
Hepatic acute GVHD	3/3 (100%)	4/9 (44.4%)	0 /18 (0%)	1/9 (11.11%)	1 vs 3 2 vs 3 1+2 vs 3 1+2 vs 3+4	0.0001 0.00006 0.0003 0.0002

* Fisher test (two tail).

0152

SECONDARY PROPHYLAXIS OF INVASIVE FUNGAL INFECTIONS IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS: A RETROSPECTIVE STUDY OF 73 CASES AT A SINGLE INSTITUTION

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Background. Invasive fungal infections (IFI) remain a major cause of infection-related mortality following hematopoietic stem cell transplantation (HSCT). Transplanted recipients with a history of IFI are at a high risk for developing recurrent fungal infection. *Aims.*In the present retrospective clinical study, we evaluated the efficacy of secondary prophylaxis (SP) with different antifungal agents in allogeneic HSCT patients with a previous history of IFI. *Methods.* Seventy-three patients with hematological malignancies were enrolled in this study: the median age was 27(5-59) years. There were 50 males and 23 females. Many of them had high risk diseases, such as the second/third remission in 15 cases and refractory/relapse leukemia in 16 patients. Donor origins were from haploidentical (n=36), unrelated (n=19) or identical sibling (n=18). The previous diagnosis of IFI was made according to European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria. Median time from diagnosis of IFI to HSCT was 85 (14-575) days. All patients received SP from the beginning of conditioning. Patients were divided into two groups according to different prophylaxis agents: In narrow-spectrum group, patients received fluconazole (n=24); in broad-spectrum group, patients received voriconazole/itraconazole/caspofungin or micafungin (n=49). Broad-spectrum agents were changed to fluconazole at a median 58 (14~120) days after stem cell transfusion when patients were without any signs of active fungal infections. Prophylaxis was continued until the withdrawal of immunosuppressants. Recurrent fungal infection was evaluated at 30 and 100 days after stem cell transfusion. *Results.* Log-rank analysis revealed that at day +30 the recurrent IFI rates were 25%, 12.4% in narrow-spectrum group and broad-spectrum group respectively, without significant difference (p=0.161). At day +100, however, patients in narrow-spectrum group developed much higher proportion of IFI recurrence compared with those in broad-spectrum group (41.7% versus 18.3%, p=0.035). In the broad-spectrum group, the day +100 recurrent IFI rates were 0/10 (0%) in voriconazole group, 6/24 (25%) in itraconazole group, 2/7 (28.5%) in caspofungin or micafungin group and 1/8 (12.5%) in the group who received caspofungin or micafungin during conditioning and then switched to voriconazole there after. Recurrent IFI developed in only one patient within 7 days after the change from broad-spectrum agents to fluconazole. At a median follow-up of 285 (18-1426) days, there were 53 patients alive with disease free, one alive with tumor and 19 died, but only 5 of them were died of severe fungal infection. The overall mortality rate was 26% and the fungus-related mortality was only 6.8%. Other reasons of death were relapse of original disease (n=8), severe GVHD (n=4), virus infection (n=1) and acute heart failure (n=1). Better overall survival rate (79.2%) was seen in broad-spectrum group compared with that in narrow-spectrum group (69.4%) without significant difference (p=0.285). *Conclusions.* History of IFI is not a contraindication to HSCT, SP by broad-spectrum antifungal agents can significantly reduce the incidence of recurrent IFI in allogeneic HSCT recipients.

0153

NATURAL KILLER CELL NUMBERS POST STEM CELL TRANSPLANT ARE STRONGLY ASSOCIATED WITH DONOR CHIMERISM AND ARE INCREASED FOLLOWING UMBILICAL CORD STEM CELL TRANSPLANT

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Background. Full donor chimerism (FDC) with disease eradication is achieved following haemopoietic stem cell transplantation (HSCT). However the utility of HSCT is limited by loss of FDC with returning host haempoiesis - mixed donor chimerism (MDC) - and disease relapse. The use of umbilical cord blood (UCB) as a donor cell source in HSCT is associated with higher rates of FDC and potentially with improved disease control compared with bone marrow (BM) or peripheral blood (PB) as donor cell sources. *Aims.* We proposed that these differences in rates of FDC reflect natural killer (NK) activity of engrafting tissue. We compared NK cell numbers following SCT in patients with FDC and MDC transplant recipients and NK cell numbers in UCB recipients compared with BM and PB recipients. *Methods.* Peripheral blood from 50 paediatric HSCT patients was characterised via flow cytometric analysis of CD16 and CD56. Chimerism levels were assigned following short tandem repeat (STR) analysis of fluorescently labelled STR alleles; D3S1358, D5S818, THO1, D7S820, D12S391, FGA, D18S51 and Penta E. Analysis was of NK cell numbers in UCB vs. BM/PB recipients and FDC vs. MDC patients. *Results.* NK cells were significantly increased in the peripheral blood of patients with FDC compared to MDC (p=0.036). There was also a highly significant elevation of NK cells in patients transplanted with UCB in comparison to BM (p<0.001). *Conclusions.* The highly significant elevation of NK cells in patients with FDC and those transplanted with CB may indicate a role for them in successful donor engraftment. The use of NK cells to achieve FDC and eradication of pathological host cells may be an avenue for increasing the success of HSCT, without requiring intensity of conditioning or use of Graft versus Host Disease to eradicate host cells. The success of UCB in establishing FDC might reflect cord NK cell activity.

0154

ASSOCIATION BETWEEN PLASMA LEVEL CHANGES OF ENDOTHELIAL INJURY MARKERS AND GRAFT VERSUS HOST DISEASE IN PATIENTS TREATED WITH ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The endothelial injury caused by conditioning regimen is thought to play a central role in the activation of immune system and to precede non-infectious complications of vascular origin and graft versus host disease (GVHD). The aim of the study was to evaluate a possible correlation between the changes of plasma endothelial injury markers and the occurrence of GVHD after alloHSCT. Patients and mthods: Plasma levels of von Willebrand factor antigen (vWF Ag), trombomodulin (TM) and vascular endothelial growth factor (VEGF) were measured by immunoassay tests in 31 pts transplanted with allogeneic stem cells after myeloblative (18 pts) or reduced-toxicity (13 pts) conditioning regimen for AML (15 pts), ALL (9 pts), CML (6 pts) and AA (1 patient). Endothelial injury markers were measured before conditioning regimen (day -10), on the day of stem cells infusion (day 0) and on the day +10 and +30 after alloHSCT. *Results.* The concentration of vWF Ag increased significantly on the day +10 and +30 in comparison to the day -10 in all patients, whether or not GVHD occurred. The TM levels increased on the day +30 in comparison to the pretransplantation level (p=0.043) in the group with chronic GVHD (cGVHD), and no changes of TM levels were observed in the group of pts that did not develop cGVHD. The TM levels increased also on the day +30 in comparison to the pretransplantation level (p=0.016) in the group with acute GVHD (aGVHD) II-IV. No changes of TM levels were observed in the group of pts with aGVHD 0-I. VEGF level did not change significantly after transplantation in any group of pts. The concentration of plasma endothelial injury markers levels however did not differ significantly between group of pts with and without cGVHD, nor between group of pts with aGVHD II-IV and with aGVHD 0-I. *Conclusions.* vWF Ag is a sensitive endothelial injury marker, but not specific enough to assist in the early detection of GVHD. Our findings on a limited number of pts suggest that TM level increase may predict the occurrence of chronic GVHD after allogeneic HSCT, but it increases not early enough to precede aGVHD symptoms. Further studies with a larger group of patients and measurement of a broader panel of endothelial injury markers are necessary to evaluate the potential role of these markers as GVHD predictors after allogeneic HSCT.

0155

HUMAN MESENCHYMAL STROMAL CELLS EXPANDED WITH HUMAN PLATELET LYSATE ARE SAFE AND EFFECTIVE FOR THE TREATMENT OF STEROID-REFRACTORY GRAFT VERSUS HOST DISEASE

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Background. Very recently, encouraging results indicate that third party human Mesenchymal Stromal Cells (hMSCs) are a therapeutic tool for the treatment of severe steroid resistant acute graft versus host disease (aGVHD). We have established a highly efficient protocol for *in vitro* expansion, under strict GMP compliance, of bone marrow derived hMSCs using human platelet lysate (hPL) in place of FBS. In this study, upon Ethical Committee approval and patient's informed consent, hMSCs were administered on a compassionate basis for the treatment of refractory GVHD. *Methods.* hMSCs were prepared from third party HLA-mismatched healthy donors using washouts of bags and filters of normally discarded bone marrow collection sets left over at the end of the filtration of the bone marrow explants performed for hematopoietic stem cell (HSC) transplantation. Cells were grown in the presence of α-MEM with 5% hPL. In a short period of time (10-33 days), low density seeding of unmanipulated cells (100-200/cm^2), obtained from 7 bone marrow harvests, allowed to prepare large quantities of hMSCs, with only one *in vitro* passage. *Results.* 4 adults and 1 pediatric patients were treated for aGVHD (grade III-IV) and 1 adult and 2 pediatric patients for extensive chronic GVHD (cGVHD), using 18 hMSCs certified bags. Before hMSCs, second or third line treatments had been given to patients with aGVHD, including Etanercept (n= 4), Mycophenolate Mofetil (MMF, n= 3) and Extracorporeal Photopheresis (ECP, n= 2). Patients with cGVHD were previously treated with ECP (n=3), MMF (n=3), Imatinib (n=1), Rituximab (n=1) and Etanercept (n=2). Each infusion contained a median dose of 1x10^6/kg (range 0.7-1.2x10^6) hMSCs. As far as concerns patients with aGVHD, a single infusion was performed in the pediatric patient, while from 1 to 4 infusions were performed in 4 adult patients. The 3 patients with cGVHD received from 1 to 5 infusions. All infusions were very well tolerated with no immediate or late adverse events according to WHO common criteria. Among pediatric patients, 3/3 complete responses were registered. A complete response was observed in 1 adult with grade III cutaneous aGVHD, although the patient rapidly relapsed and died of leukemia progression. Three partial responses were observed in 3 adults, while only one adult showed no response and died of progressive grade IV gut and liver aGVHD. *Conclusions.* These data show that large numbers of third party hMSCs can be expanded *in vitro* with hPL-containing medium. Moreover, the clinical results and the toxicity profile confirm those reported with hMSCs expanded in FBS containing media.

Table 1.

Patient n° (gender,age)	*GVHD Type and Grade*	*Organ involvement*	*Previous treatment besides steroids*	*N° of hMSCs infusions*	*Dose hMSCs (x10^6/kg)*	*Time of infusion (months after HCT)*	*Clinical response +30 days after infusion*	*GVHD re-flare after response*	*GVHD status at last follow-up*	*Status (cause of death)*
1 (F,48)	Acute, III	Skin		1	0.7	+3	Complete	yes	Active	Death (relapse)
2 (M,37)	Acute, IV	Skin, gut, liver	Etanercept	3	1.2	+1	None	NA	Active	Death (GVHD)
3 (F,57)	Chronic Extensive	Skin, oral, eye mucosa	ECP, MMF, Imatinib	5	1.0	+41, +43, +44, +45	Partial	NA	Active	Alive in CR
4 (M,22)	Acute, persistent, III	Skin, liver	CSA, Etanercept, MMF, ECP, Budesonide	4	1.0, 0.9, 1.2, 1.0	+6, +11, +15, +16	Partial	yes	Active	Alive in CR
6 (F,5)	Chronic, overlap, III	Skin, gut, lungs	CSA, Etanercept, MMF, Budesonide, ECP, Rituximab, Azatioprina	1	1.0	+5	Complete	yes	Absent	Alive in CR
7 (M,11)	Chronic, overlap, III	Skin, liver, gut	CSA, Etanercept, MMF, ECP, Budesonide, Cyclofosfamide, FK506	2	1.0	+7, +10	Complete	yes	Active	Alive in CR
8 (M,7)	Acute, III	Skin	CSA, Etanercept, MMF	1	1.0	+2	Complete	no	Absent	Alive in CR
9 (F,21)	Acute, persistent. III	Skin, gut	CSA, MMF, Etanercept, Budesonide, ECP	1	1.0	+2	Partial	yes	Active	Alive in CR

0156

HIGH CYTOMEGALOVIRUS -SPECIFIC CD8+ TEMRA CONTENT IN DONOR GRAFT PREDICTS A LOW RISK OF CMV REACTIVATION FOLLOWING UNMANIPULATED ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Cytomegalovirus (CMV)-specific CD8$^+$ T cells (CTLCMV) are instrumental to protect against HCMV relapse and disease in human. *Aims.*To determine whether CTLCMV and its subsets in allogeneic stem cell transplantation (allo-HSCT) ameliorate CMV reactivation after transplantation. *Methods.* We phenotypically quantitated the absolute numbers of CTLCMV in 64 related donor unmanipulated allografts infused into HLA-matched/HLA-mismatched recipients and examined the incidence of CMV reactivation in respective recipients. High CTLCMV with TEMRA (CD45RO$^-$ CD62L$^-$) phenotype in the donor graft was associated with a reduced risk of CMV reactivation. We monitored CTLCMV during immune reconstitution in 44 patients with malignant hematologic disorders. *Results.* Early after SCT, there was a significant expansion in CTLCMV TCM (CD45RO$^+$ CD62L$^+$) compart-

ment when CMV reactivated. The frequency of CTLCMV TCM and CTLCMV TEMRA early post SCT (day 90, day 60, respectively) was higher in transplant recipients with more CTLCMV TEMRA infused, suggesting protective immunity transferred by infusion of CTLCMV within allografts. Our findings suggest that CTLCMV TEMRA content in donor graft may predict for risk of CMV reactivation early after SCT, which is closedly correlated with the immune reconstitution and differentiation of CTLCMV subsets. *Conclusions.* Our data suggest that determining the CTLCMV TEMRA levels in the donor and manipulating CTLCMV TEMRA cells at or early after transplantation may provide a new approach to controlling CMV reactivation. Address for Correspondence.

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Table 1. Proportional hazards modeling of risk of CMV reactivation after allogeneic HSCT.

Characteristic	CMV reactivation Multivariate HR (95% CI)*	P	persistent CMV-DNA positivity Multivariate HR (95% CI)*	P
Donor/recipient CMV serostatus				
CMV D+/R-	1.909(0.448-8.140)	0.382	2.656(0.440-16.038)	0.287
CMV D+/R+	1		1	
CMV D-/R+ (*)				
Donor weight	0.994(0.969-1.019)	0.634	0.992(0.958-1.028)	0.668
HLA matching: matched donor	0.195(0.067-0.566)	0.003	0.083(0.010-0.660)	0.019
Donor HBV serostatus	1.402(0.346-5.681)	0.636	3.116(0.554-17.527)	0.197
Recipient age, ?35 years	0.854(0.356-2.045)	0.723	0.293(0.098-0.877)	0.028
Stem cell source: Peripheral blood	0.485(0.051-4.632)	0.530	0.863(0.065-11.478)	0.911
Acute GVHD: grades II-IV	0.999(0.988-1.009)	0.778	1.005(0.991-1.019)	0.507
Graft composition				
CD34+ ?1.965×10^6/kg	1.744(0.797-3.814)	0.164	1.671(0.663-4.215)	0.276
CD3+ ?1.544×10^8/kg	0.997(0.388-2.562)	0.994	1.278(0.302-5.405)	0.739
CD8+ ?0.558×10^{11}/kg	0.961(0.328-2.817)	0.943	1.802(0.395-8.222)	0.447
CMV-CTL?0.576×10^5/kg (**)				
CMV_{CTL} T_{EM} CD45RO+CD62L-?0.282×10^6/kg	4.163(1.161-14.926)	0.029	2.887(0.844-9.875)	0.091
CMV_{CTL} T_{Naive} CD45RO-CD62L+?0.029×10^6/kg	1.018(0.350-2.962)	0.974	1.061(0.229-4.915)	0.940
CMV_{CTL} T_{CM} CD45RO+CD62L+?0.008×10^6/kg	0.645(0.233-1.782)	0.397	0.918(0.246-3.433)	0.899
CMV_{CTL} T_{EMRA} CD45RO-CD62L-?0.008×10^6/kg	0.187(0.052-0.665)	0.010	0.215(0.060-0.769)	0.018

* Only one patient was in CMV D-/R+ pairs

** CMV_{CTL} excluded due to marked colinearity of CMV_{CTL} and CMV_{CTL} TEMRA

0157

PROPHYLAXIS WITH MYCOPHENOLATE MOFETIL AND CYCLOSPORINE CAN DECREASE THE INCIDENCE OF SEVERE ACUTE GRAFT-VERSUS-HOST DISEASE FOLLOWING REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-SCT) FROM MATCHED UNRELATED DONORS

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The use of reduced intensity conditioning (RIC) regimens for allo-SCT can result in a significant decrease in early procedure-related toxicity in patients not eligible for standard myeloablative regimens. However, acute graft-versus-host disease (GVHD) remains a matter of concern after RIC allo-SCT, especially in the context of matched unrelated donors (MUD). Indeed, the rapidly increasing use in elderly and high risk patients and evolving nature of RIC allo-SCT, emphasize the need for renewed clinical research of GVHD prophylaxis. This pilot report investigated mycophenolate mofetil (MMF) and cyclosporine (CsA) combination in comparison to CsA alone for GVHD prophylaxis in 35 consecutive patients with hematological malignancies receiving RIC allogeneic stem cell transplantation (allo-SCT) from a MUD, and treated within the same period in a single institution. In this series, the median age was 52 (range, 6-64; 20 males). The RIC regimen included fludarabine, busulfan and ATG in all patients (with minor adjustments in 3 patients). The first patients from this series (n=19; group I) received CsA alone for GVHD prophylaxis. The next patients (n=16; group II) received CsA and MMF. Mycophenolate mofetil (MMF) was given at a fixed oral dose of 1 g x 2/day without any treatment adjustment. The two groups had no significant differences as for demographic and disease features, GVHD risk factors, and allo-SCT procedure characteristics (except for GVHD prophylaxis). 31 patients (89%) received a 10/10 HLA matched graft at the allelic level. One allelic mismatch was observed in 4 cases, with this being comparable between both groups. 3 patients from this series did not engraft (early disease recurrence) and were not evaluable for acute GVHD. ANC >500/µL was achieved at a median of 19 (range, 0-27) days. The cumulative incidence of grade 2-4 acute GVHD at day 100 was 67% (95%CI, 45-89%) in patients receiving CsA alone as compared to 53% (95%CI, 28-78%) in those receiving the CsA and MMF combination (p=NS). However, the cumulative incidences of grade 3-4 acute GVHD was significantly lower in the "CsA+MMF" group (13%) as compared to 56% in the "CsA" group (p=0.03). After a median follow-up of 19.5 (range, 9-93) months for surviving patients, the one year overall survival was significantly higher in the "CsA+MMF" group (81% vs. 47%; p=0.03). Overall, 4 deaths were attributed to refractory acute GVHD in the "CsA" group versus none in the "CsA+MMF" group. Though a randomized trial is needed before drawing final conclusions, this pilot study suggests that adjunction of MMF to CsA can result in a significant reduction of the incidence of severe acute GVHD following ATG-based RIC allo-SCT using MUD, and this may have a significant impact on the probability of a favorable outcome.

Stem cell transplantation – autologous, DLI, miscellaneous

0158

DONOR LYMPHOCYTE INFUSIONS FOR CHRONIC MYELOID LEUKAEMIA RELAPSING AFTER ALLOGENEIC STEM CELL TRANSPLANT: PROGNOSTIC FACTORS FOR A 'PURE' GVL EFFECT ARE DIFFERENT ACCORDING TO THE STAGE OF RELAPSE

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Patients (pts) with chronic myelogenous leukemia (CML) relapsing after allogeneic stem cell transplant (SCT) may achieve a molecular remission (MR) with transfusions of donor lymphocytes (DLI), whenever relapse occurs in stable chimera. CML relapse may be diagnosed at molecular, cytogenetic and clinico-hematological level, bringing to an extreme heterogeneity of patient/disease status when treatment with DLI is applied. This is even more complex nowadays with the availability of TK inhibitors, capable to restore MR in some pts without the risk of secondary graft versus host disease (GvHD2), but with unknown effects on responsiveness to subsequent DLI. Infact CML response to DLI is frequently, but not always, accompanied by GvHD2. Thus physicians and/or pts may initially prefer to use pills rather than donor cells. We aim to provide information that may be used to select when treatment of CML relapse with DLI can be *optimal* (i.e. achievement of a prolonged survival and a durable MR without GvHD2). Five-hundred pts treated with DLI for CML relapse (not in blast crisis) before 2004 at 68 EBMT centres were retrospectively analyzed: 73% had an HLA-identical sibling donor, 27% unrelated. DLI was given at time of cytogenetic and/or molecular relapse (MoCyRel) (n=230), or at haematological relapse (HemRel) (n=270). DLI was given either in single (58%) or multiple (42%) infusions. Median initial cell dose (ICD)(T cells/Kg given at 1st DLI) was 1×10^7 for MoCyRel (range 0.001-31) and 4×10^7 for HemRel (range 0.01-36). 340 pts (68%) achieved a MR in a median of 7.5 months, 44% had GvHD2 in a median of 3 months, 16 pts recurred at a median of 19 months. Actuarial probability of being alive in MR without GvHD2 was 29% at 5 years: 40% in MoCyRel, 20% in HemRel ($p<0.001$). Thus prognostic factors for survival in MR without GvHD2 were analyzed separately according to the type of relapse. Factors into analysis (i.e. logrank test and logistic regression) were: patient age at DLI, donor type, donor sex, sex mismatch with the donor, phase at SCT, stem cell source, T-depletion, TBI in conditioning, GvHD prior to DLI, interval from SCT to DLI, ICD, and the stage of relapse at time of DLI (i.e. molecular, cytogenetic, chronic phase, accelerated phase). Results in MoCyRel: interval from SCT to DLI <1yr (HR 2.2, 95CI: 1.5-3.2, $p<0.001$) and a history of chronic GvHD prior to DLI (HR 1.8, 95CI: 1.3-2.5, $p=0.001$) were independent adverse prognostic factors; 48%, 41%, and 11% had 0, 1,and 2 adverse features, respectively. Survival in MR without GvHD2 at 5 years improved from 0%, 31%, to 65% in pts with 2, 1, and 0 adverse features, respectively. Results in HemRel: sex mismatch with the donor (HR 1.6, 95CI: 1.1-2.4, $p=0.01$), history of chronic GvHD prior to DLI (HR 1.7, 95CI 1.2-2.5, $p=0.006$), and ICD $>5\times10^7$/Kg (HR 1.8, 95CI: 1.2-2.7, $p=0.002$) were independent adverse prognostic factors; 17%, 40%, 33%, and 9% had 0, 1, 2, and 3 adverse features, respectively. Survival in MR without GvHD2 at 6 years improved from 0%, 17%, 33%, to 37% in pts with 3, 2, 1, and 0 adverse features, respectively. We conclude that: [a] the chance of durable remission without GvHD2 was higher when DLI is given at MoCyRel than at HemRel; [b] the chance of exploiting the *pure GvL effect* was as high as 65% when DLI were given beyond 1 year from SCT for a MoCyRel that was not preceded by cGvHD.

0159

EFFICACY OF RITUXIMAB AND DONOR LYMPHOCYTE INFUSIONS AS SALVAGE TREATMENT IN LYMPHOMA PATIENTS RELAPSED AFTER REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Patients with lymphoma who relapse after allogeneic stem cell transplantation (alloSCT) have a dismal prognosis. Salvage treatment is not standardized and the role of Rituximab and donor lymphocyte infusions (DLI) has not been clearly assessed yet. *Aims.* The aim of the study was to evaluate whether DLI may improve progression free survival (PFS) and overall survival (OS) compared to other salvage regimens in patients with non-Hodgkin's (NHL) and Hodgkin's (HL) lymphoma relapsed after alloSCT. We also aimed to assess whether Rituximab associated with DLI could further improve survival outcomes. *Methods.* Seventy-eight consecutive patients allografted between 2000 and 2007 were reviewed. Patients were affected by indolent NHL (23, 29%), aggressive NHL (38, 49%) or HL (17, 22%). All the patients were allografted after a reduced-intensity conditioning (RIC) from HLA-matched siblings (49, 63%) or matched unrelated donors (29, 37%). Thirty-five patients relapsed after allograft (45%). Median age of relapsed patients was 45 years (range 19-63). Eleven patients had indolent NHL (31%), 16 aggressive NHL (46%), 8 HL (23%). All relapsed patients underwent salvage treatment: 17 (49%) received chemotherapy and DLI (median of 2 DLI, range 1-4), 18 (51%) chemotherapy alone. Nine patients could not receive DLI because of GVHD. Fourteen patients (40%) received Rituximab as part of the salvage regimen, 11 (31%) were treated with Rituximab and DLI. Survival was calculated from relapse to death (OS) or to second relapse or death (PFS) with Kaplan-Meier method applying log-rank test. Cumulative incidence (CI) of relapse was estimated with CI method with competing risks and Gray's test. Multivariate analysis was done with Cox models. *Results.* Median follow-up from relapse after alloSCT was 15 months (range 1-104). One- and 2-year PFS was 34% and 31%, respectively. One- and 2-year OS was 54% and 45%, respectively. Patients who received DLI had a better PFS ($p=0.04$, HR=0.44 [CI95%=0.1-1.0]) compared to patients who did not. The combination of Rituximab and DLI improved both PFS ($p=0.007$, HR=0.25 [CI95%=0.08-0.75]) and OS ($p=0.001$, HR=0.07 [CI95%=0.01-0.5]). CI of relapse was 38% at 1 and 2 years. Rituximab and DLI significantly reduced CI of relapse ($p=0.03$) compared to all the other salvage treatments. Seven out of 17 patients (41%) who received DLI developed aGVHD (grade>=2 n=5), while 5 patients (29%) developed cGVHD (extensive n=1). GVHD after DLI did not affect PFS and OS ($p=0.9$ and $p=0.8$ respectively). In patients who received DLI, the association with Rituximab significantly improved both PFS ($p=0.008$, HR=0.20 [CI95%=0.05-0.74]) and OS ($p<0.001$, HR=0.06 [CI95%=0.01-0.52]) compared to DLI alone. Multivariate analysis, which included the salvage treatment (DLI+Rituximab vs other) and disease (HL vs aggressive NHL vs indolent NHL), confirmed that DLI and Rituximab significantly improve both PFS ($p=0.03$, HR=0.28 [CI95%=0.09-0.88]) and OS ($p=0.01$, HR=0.06 [CI95%=0.01-0.54]) regardless of lymphoma subtype. *Conclusions.* The results show that DLI can improve PFS of lymphoma patients relapsed after RIC alloSCT. The use of Rituximab prior to DLI can further improve PFS and OS, suggesting that this strategy may be the optimal salvage treatment in this setting.

0160

ACUTE LUNG INJURY AFTER ALLOGENEIC HEMTOPOIETIC STEM CELL TRANSPLANTATION IS ASSOCIATED WITH INFERIOR SURVIVAL - A RANDOMIZED TRIAL ON THE EFFECTS OF EARLY NON-INVASIVE VENTILATION

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Background and Aims. The prognosis of patients suffering from respiratory failure after allogeneic stem cell transplantation is still poor. Recent data suggested beneficial effects of non-invasive ventilation (NIV) in this setting. We therefore initiated a randomized trial to prove the impact of early NIV in patients in the early posttransplant period. *Patients and Methods.* 526 patients undergoing allogeneic hematopoietic stem cell transplantation in a single center were investigated for signs of acute lung injury (ALI) as defined by either an oxygenation index <300, a breath-

ing rate >25/min or a continuous decrease in oxygen saturation below 92%. Patients with the diagnosis were randomly assigned to either a conservative treatment arm A (oxygen supply only) or an intensified treatment arm B (oxygen + intermittent NIV). *Results.* ALI had to be diagnosed in 86 patients after transplantation (16%). Determination of oxygenation index using capillary blood gas analysis was found to be early predictor of ALI even in the absence of clinical signs of respiratory distress. In a multivariate analysis higher age at transplant and the use total-body-irradiation (TBI) of 8 Gy or more were indentified as risk factors for ALI. Irrespectively of randomization to a treatment arm in multivariate analyses ALI turned out to be an independent risk factor for both 100 day mortality (OR 2.76; $p<0.001$) and long term survival (OR 1.57; $p<0.01$). Although early NIV tended to be associated with a lower incidence of treatment failure (39% in arm A vs. 24% in arm B, $p=0.17$), neither ICU admission rate, nor need for intubation or survival parameters were affected by the treatment strategy. *Conclusions.* The occurence of ALI in the early postransplantation period is an important negative prognostic factor for patients undergoing hematopoietic stem cell transplantation. Oxygenation index is an early and sensitive indicator of respiratory distress. Early intervention by NIV failed to improve prognosis of the patients. The limited impact of NIV may be related to the design of the study allowing for switching of the treatment arm in case of unsatisfactory efficacy.

0161

THERAPEUTIC DRUG MONITORING IS AN IMPORTANT FACTOR DURING IV BUSULFAN TREATMENT

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Hematopoietic stem cell transplantation (HSCT) is increasing rapidly as a curative treatment for several malignant and non malignant disorders. More than 40% of patients undergoing HSCT are conditioned using chemotherapy including busulfan. Intravenous busulfan is a myeloablative chemotherapeutic drug which is increasingly used in numerous pediatric conditioning protocols for pediatric hematopoietic stem cell transplantation (HSCT). Between 2006 and 2008, we performed therapeutic drug monitoring of intravenous busulfan in 26 pediatric HSCT patients (age 5 mo - 23 yrs; medium 7 yrs), with n = 8/18 malignant/non-malignant diseases and n = 10/11/2/3 MSD/MUD-MMUD/MMRD/ autologous transplants. In order to optimize the targeted drug exposure and to minimize drug-related toxicity, a modified administration protocol was used. Intravenous busulfan was administered twice daily in a 4-hour infusion according to company-recommended weight-based doses and busulfan drug level measurements were performed after ‘O, ‘30, ‘60, ‘120, ‘240 and ‘360 minutes post infusion. Pharmacokinetic analysis was performed and the kinetic parameters were calculated for eventual dose adjustments. The targeted busulfan exposure was aimed to range between areas under the curve (AUC) of 9’000 - 14’000 ng/mLxh. In 18/26 patients (69%) the busulfan dose had to be adjusted at least once. In 13/18 patients (72%), the dose had to be increased in a range of +7 to +20% while in 5/18 patients (28%), the dose had to be lowered by -8 to -20%. The need of dose adjustment was not related to weight, age or underlying disease. Non-engraftment was observed after one CD34-positively selected haploidentical HSCT in an infant with T-,B-,NK+ SCID with no busulfan adjustment despite a low AUC of 6676 ng/mLxh. Hepatic veno-occlusive-disease (HVOD) was not observed in patients with >12 kg body weight, while 5/8 infants <12 kg developed mild to moderate VOD responding positively to defibrotide therapy. All HVOD patients had received a conditioning comprising cyclophosphamide. Two patients with Wiskott Aldrich syndrome (9 mo and 18 yrs) died before day +100 due to CMV infection/pulmonary hypertension and fulminant cryptosporidium-induced pancreatitis, respectively, with no signs for pulmonary or hepatic veno-occlusive disease or other busulfan-associated toxicity at autopsy. In 23/26 patients (88%) receiving our protocol, no events of transplant related mortality or transplant rejection were observed. Combined pharmacokinetic assessments and dose-adjustments of i.v. busulfan are essential for further improvement of efficacy and safety of busulfan-based myeloablative conditioning protocols in pediatric HSCT recipients.

0162

TOTAL BODY IRRADIATION AND THIOTEPA AS CONDITIONING TO AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH FOLLICULAR LYMPHOMA

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Background. Patients with follicular lymphoma can potentially be cured by supralethal chemo- radio- therapy followed by autologous stem cell transplantation (ASCT). This approach is however associated with an increased risk of transplant related mortality (TRM) and second neoplasms. *Aims.* To assess TRM and the incidence of late complications after a total body irradiation (TBI)-based conditioning regimen for ASCT in patients with follicular lymphoma. *Methods.* Between February 1992 and December 2005, 63 patients were recruited to the study (38 males, 25 females, age range 28-60 years; median age 47 years). Disease stage at diagnosis was advanced in 53 patients (clinical stage III in 12, clinical stage IV in 41); B symptoms were present in 15; 47 patients had undergone first-line therapy and 14 two lines of therapy before ASCT; 19 patients had received also rituximab. At the time of transplant 34 patients were in complete remission (CR), 20 were in partial remission (PR), 4 had achieved very good partial remission, 3 were refractory to chemotherapy and 2 were in relapse. Conditioning to ASCT included single fraction 8 Gy TBI at high dose rate with lung shielding followed by Thiotepa 5 mg/Kg/day for two days. Graft content was as follows: $CD34^+$ cells: 9.1×10^6/Kg (range 2.0 -23.5×10^6); $CD34^+$ cells were purged by positive selection in 23/63 patients. *Results.* All patients engrafted with good haematological reconstitution. Short term complications included 2 fatal cases of interstitial pneumonia, 1 case of VOD which quickly resolved, 1 case of renal failure which became chronic. TRM was 3.1%. Major long term complications included one bladder cancer after 2 years and one breast cancer after 6 years with both patients surviving after surgery and chemotherapy. At a median follow-up of 6.7 years (range 0.21-16.7 years) 52/63 patients survive and 44/63 were in complete remission. Three year overall survival (OS) and event free survival (EFS) rates were respectively 90% and 79%; projected 5-year OS and EFS rates were respectively 87% and 73%. Disease status at transplant was a significant prognostic factor for EFS ($p=0.007$ CR vs PR; Cox multivariate analysis: $p=0.047$), as was time from diagnosis to transplant. Patients who received rituximab before ASCT tended to have better EFS. *Summary and Conclusions.* Single fraction 8 Gy TBI at high dose rate plus thiotepa as conditioning to autologous transplantation for patients with follicular lymphoma is feasible with low TRM and low incidence of second neoplasms. OS and EFS rates were similar or even better in respect of other reportes of autologous transplantations.

In memory of Prof Antonio Tabilio, close friend and colleague, eminent physician and scientist.

0163

RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR SIGNIFICANTLY DECREASES THE EXPRESSION OF CXCR3 AND CCR6 ON T CELLS AND PREFERENTIALLY INDUCES TH CELLS TO A TH17 PHENOTYPE IN PERIPHERAL BLOOD HARVESTS

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Backgroud. Neither the incidence nor the severity of acute graft-versus-host disease (GVHD) is higher than with marrow transplantations, although recombinant human granulocyte colony-stimulating factor (rhG-CSF)-mobilized peripheral blood harvests contain many more mature T cells. However, these mechanisms are not fully understood. *Aims.* The aim of this study was to investigate the expression of chemokine receptors on T cells and functional changes of T helper cells (Th cells) in peripheral blood stem cell harvests after treating healthy donors with rhG-CSF. *Methods.* Using multiparameter flow cytometry, we analyzed the expression of CXCR3 and CCR6 on T cells and the production of interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-17 by $CD4^+$ Th cells in peripheral blood stem cell (PBSC) grafts of healthy donors after *in vivo* rhG-CSF application. Alterations in the relative expression levels of TCRBV family members were determined using real-time PCR. All sample collections had the approval of the Peking University First Hospital Ethical Committee. *Results.* Our data demonstrated that rhG-CSF mobilization significantly decreased the expression of CXCR3 and CCR6 on T cells (Figure 1). The expression of CXCR3 on $CD4^+$ and $CD8^+$ T cells was significantly decreased after rhG-CSF mobilization ($p<0.001$). There were significant differences between PBSC grafts and steady-state peripheral blood (SS-PB) in the per-

centage of CCR6 expression on CD4+ and CD8+ T cells (p<0.001 and p=0.007, respectively). Treating donors with rhG-CSF resulted in decreased IFN-γ production and dramatically increased IL-4 and IL-17 secretion by CD4+ Th cells, leading to T cell polarization from the Th1 to the Th2 phenotype and a preferential increase in IL-17-producing CD4+ Th cells. We did not observe any differences in the relative expression levels of TCRBV family members before and after *in vivo* rhG-CSF application. *Conclusions.* Our results suggest that the expression of CXCR3 and CCR6 on donor T cells was dramatically downregulated and an IL-17 phenotype of CD4+ Th cells was preferentially induced in PBSC grafts after treating healthy donors with rhG-CSF. The observed effects of rhG-CSF on T cells may be independent of the relative expression levels of TCRBV family members. *Key Words.* recombinant human granulocyte colony-stimulating factor mobilization, chemokine receptors, IL-17, TCRBV.

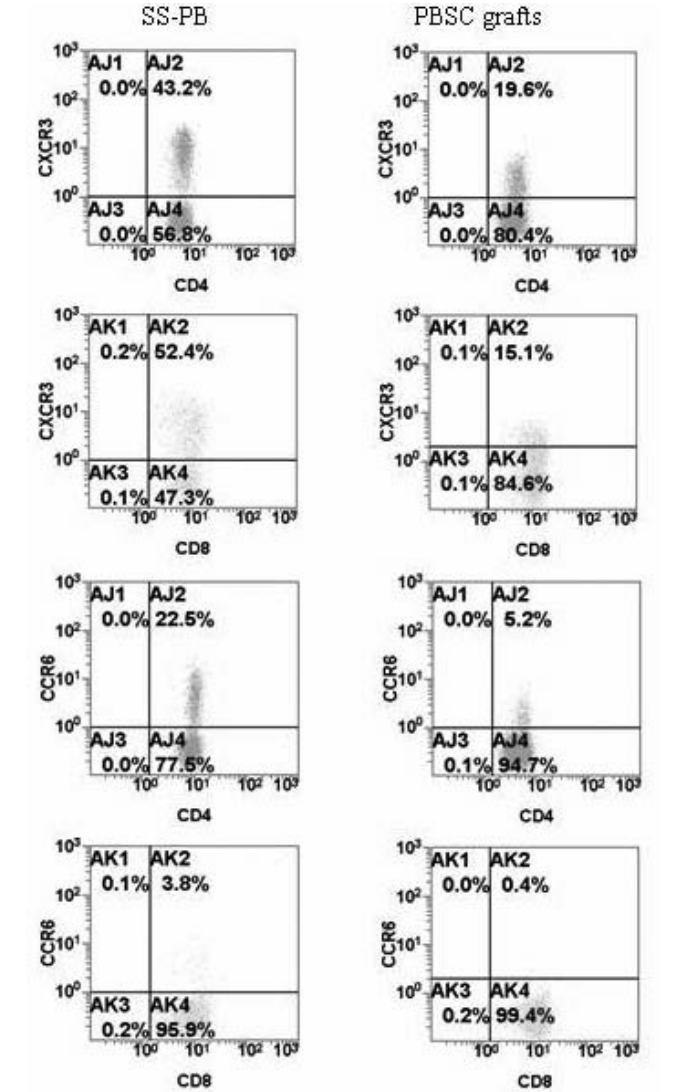

Figure 1. Representative example of CXCR3 and CCR6 expression on CD4+ and CD8+ T cells in SS-PB and PBSC grafts.

0164

REDUCED INTENSITY CONDITIONING UMBILICAL CORD BLOOD TRANSPLANTATION (RIC-UCBT) IN ADULTS: AGE, DISEASE STATUS AND INFECTIOUS COMPLICATIONS ARE IMPORTANT RISK FACTORS FOR OUTCOME

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We report the outcome of 35 patients (pt) who received a RIC UCBT in a single centre between 2005 and 2008. All pt had high risk hematological malignancies (AML=19; ALL=9; NHL=5; CML=1; and Hodgkin disease=1). 27 pt (77%) were in CR (CR1=18; CR2=8; CR3=1), whereas 8 had a more advanced disease (PR=2; refractory=6) at time of UCBT. The median age was 44 (range, 17-62) years. For age we distinguished 2 groups: group 1 (n=13, age > 50 years) and group 2 (n=22, age ″ 50 years). 13 pt (37%) received a single CB unit, whereas 22 (63%) received 2 CB units in order to achieve a minimum required cryopreserved cell dose of 3.0×10^7 TNC/kg. For the entire group the median infused cell dose was 3.7×10^7/Kg (range, 1.9-5.5). Neutrophil engraftment occurred in 33 pt (94%) at a median of 20 (range, 6-45) days and a sustained platelet recovery was observed in 25 pt (71%) at a median of 36 (range, 23-136) days.The overall incidence of grade II-IV acute GVHD was 54% (95%CI=[37-71]; 7 grade II, 10 grade III and 2 grade IV) and 37% for chronic GVHD (95%CI=[21-55]; 8 limited and 5 extensive cases) with no difference between the 2 groups. 21 pt (60%; 95%CI=[37-71] experienced at least one episode of a severe infectious complication (SINC) (virus, n=11; bacteria, n=10; fungal, n=4), requiring long-term hospitalization. With a median follow-up of 468 (95%CI=[201-681]; range, 50-1170) days, 10 pt (28%) had relapsed or progressed with this being significantly lower in younger pt (p=0.045) and in those transplanted in CR (p=0.007). Regarding the 2 groups, 7 pt (54%) in group 1 died (infection=3; GVHD=2; disease=2; TRM=38%) whereas 7 (32%) in group 2 (infection=2; GVHD=1; disease=4; TRM=14%).The KM estimate of OS and DFS was 61% (95%CI=[42-75]) and 52% (95%CI=34-67) at 2 years respectively for the entire population with significant better outcome in pt in CR (OS, p=0.004; DFS, p=0.007) and pt in group1 (OS, p=0.052; DFS, p=0.041). The multivariate analysis showed a significant impact of 2 factors on DFS : age (group 1 vs group 2: HR 3.05, 95%CI=[1.07-8.73], p=0.037) and disease status at transplant (No CR vs CR: HR 6.33, 95%CI=[1.77-22.6], p=0.005). Age and disease status at transplant are crucial for patients outcome and further efforts are needed to define risk factor specific transplant procedures. Infectious complications and GVHD are still a matter of concern warranting better strategies to provide optimal prophylactic and therapeutic approaches.

0165

CLINICAL IMPLICATIONS OF PULMONARY FUNCTION TESTS IN ALLOGENEIC STEM CELL RECIPIENTS

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Pulmonary dysfunction is one of major causes of morbidity and mortality in the allogeneic stem cell recipients. From Jun 2002 to Oct 2008, we retrospectively analyzed pulmonary functions (PF) in 116 consecutive patients who received allogeneic stem cell transplantation (SCT) and survived at least 3 months after transplantation. Pulmonary function test was performed at baseline, 3 months, 6 months, 1 year, and annually thereafter. Ventilatory capacity was measured by forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), and FEV1/FVC ratio. Lung volume measurements included total lung capacity (TLC), residual volume (RV), and RV/TLC ratio. Diffusion capacity for carbon monoxide (DLCO) was determined with correction for hemoglobin concentration. Of the 116 patients, 63 patients were followed more than 1 year. The patients with acute graft-versus-host disease (GVHD; p=0.011), chronic GVHD (p=0.009), or CMV infection (p=0.410) showed a decline of FEV1 and FEV1/FVC after 1 year of transplantation. TLC and RV/TLC also showed an increasing tendency after 1 year of transplantation which reflected abnormal ventilatory pulmonary function. Conditioning intensity, unrelated donor type, stem cell dose did not adversely affect the pulmonary function. We calculated the lung function score (defined as FEV1 score plus DLCO score; NIH chronic GVHD consensus Project, 2003) at 1 year of transplant and compared it from that of baseline to identify the factors affecting late pulmonary dysfunction. In the multivariate analysis, extensive cGVHD (OR=3.692, 95% CI=1.154-11.818; p=0.028) and previous CMV infection (OR=5.356, 95% CI=1.311-21.882; p=0.019) negatively affected on late pulmonary function. In addition, the patients with early decline of FEV1 more than 10% at 3 months compared to baseline showed a higher incidence of cGVHD during follow-up (p=0.016). In conclusion, patients who had extensive cGVHD or CMV infection had a higher possibility of late pulmonary dysfunction and decline of FEV1 at 3 months of transplantation reflected the development of cGVHD.

0166

HUMAN BONE MARROW AS SOURCE OF STEM CELLS FOR INTRAPANCREATIC TRANSPLANTATION IN IMMUNODEFICIENT MICE: EVIDENCE OF PANCREATIC-CELL LINEAGE COMMITMENT BY GENE EXPRESSION ANALYSIS

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Background. A major goal of diabetes therapy is to look for new sources of insulin-producing cells, preferably of autologous origin, and to explore other sites of implants different to liver (which provides a microenvironment that probably does not generate the correct signals to maintain the differentiation of such a fragile cellular system). The human bone marrow contains different types of stem cells, including mesenchymal stromal cell (MSC) and multipotent adult progenitor cell (MAPC), which have a large proliferative potential, the ability to differentiate into different cell types and to produce a variety of cytokines and growth factors. Pancreatic-cell differentiation follows a specific cascade or sequential pattern of gene expression that may help to recognize the cellular lineage. *Aims.* To analyze if human bone marrow-derived mononucleated cells are capable of nesting in the pancreas of immunodeficient mice, and to investigate the appearance of gene expression changes that indicate a commitment of the transplanted cells to pancreatic differentiation. *Methods.* Human bone marrow was obtained by aspiration from healthy voluntary donors. Mononucleated cells were separated by Ficoll gradient procedure, and then MSC and MAPC were isolated (the latter by negative immunoselection using magnetic cell sorting, MACS) and biologically characterized (by morphologic and immunophenotypic analysis). An intrapancreatic transplantation of this purified cellular fraction was performed in immunodeficient mice (SCID beige, a B and T deficient animals used for xenotransplantations). Among others, analyzed variables included the survival of mice, the development of pancreatitis by measurement of pancreatic enzymes in blood, the viability of the transplanted cells assessed by immunohistochemistry (presence or not of human vimentin and human ERV-3), and the expression of pancreatic genes (GATA4, HNF1β, PDX-1, Ngn3, Neuro D, Pax4, Nkx2.2, Nkx6.1, Isl-1, Glut-2, SUR1, insulin, glucagon, and somatostatin), by quantitative real-time PCR. Animals were sacrificed four and eight weeks after transplantation to perform all the above mentioned analysis. *Results.* The global survival of the mice was 94%, and the animals showed a healthy appearance along the whole experiment. An increment in serum levels of pancreatic enzymes, suggestive of pancreatitis, was never observed. Immunohistochemical studies demonstrated the presence of human vimentin and ERV-3 in the pancreas both at four and eight weeks after transplant. An increase in the expression of HNF1β (specific of endoderm-cell phase) and NKx6.1 (pancreatic) was detected; any other change in gene expression cascade of pancreatic differentiation was observed. *Conclusions.* Our results support that human bone marrow-derived stem cells may survive for a significant period of time after intrapancreatic transplantation in an animal experimentation model. Although further studies are certainly needed, MSC and MAPC might constitute a source of autologous stem cells for pancreatic regeneration. The constant and specific increase in the expression of some genes involved in pancreatic differentiation (HNF1β and NKx6.1), indicate that the microenvironment may be responsible, at least partially, of these cellular changes. Our group is currently carrying out a study with the same methodology but in diabetic mice, to analyze if the natural course of the disease may be influenced by this intrapancreatic transplantation.

0167

RESULT OF DOUBLE UNITS CORD BLOOD TRANSPLANTATION IN CHILDREN AND ASSOCIATION OF CFU-GM WITH THE DETERMINATION OF DOMINANCY

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Background. Cord blood transplantation (CBT) is an alternative means of allogeneic stem cell transplantation. To enhance engraftment and to improve transplantation results, CBT has been conducted using two units with promising results in adults but results in pediatric patients is not known yet. Our preliminary results showed the dominance of one unit which occurred early after CBT (Bone Marrow Transplant. 38:197). However, little is known about the mechanism of engraftment and factors influencing the dominancy. *Aims.* To improve the outcomes, CBT was performed with 2 units in pediatric patients with the analysis of factors that affect the determination of dominancy. *Methods.* In total 61 patients including 25 AML, 19 ALL, and 17 other hematologic diseases received double units CBT, the numbers of total nucleated cells, $CD34^+$ cells, $CD3^+$ cells, CFU-GM, order of infusion, and HLA disparities were viewed as factors that potentially influence dominancy. *Results.* The median age and body weight of patients (M:43, F:18) were 9 (1-18) years and 35 (9-72)kg, respectively. The median number of the infused nucleated cells and $CD34^+$ cells by the sum of 2 units were 5.4 (0.7-18.4)$\times10^7$/kg and 2.1 (0.6-7.2)$\times10^6$/kg, respectively. Donor type engraftment achieved in 50/61 patients (5 engraftment failure, 5 autologous recovery, and 1 early death). Engraftment failure rate in hematologic disease (35%) was significantly higher than in leukemia (9%) (p=0.02). Except one patient with persistent mixed chimerism of two units, other 49 patients showed dominancy of one unit. Among the factors influencing dominancy, only the CFU-GM was significant (p=0.02). The event free survival (EFS) of all 61 patients was 57.4%. The EFS of leukemia and other hematologic disease were 59% and 53%, respectively. The transplantation related mortality (TRM) rate of all patients was 28%. The EFS (22%) of 9 high risk leukemia with more than third complete remission (CR), second transplantation, or refractory disease was significantly worse than that (69%) of other patients received double units CBT in first or second CR (25%-should omit) (p=0.01). The relapse rate of high risk leukemia (40%) was significantly higher than others (13%) (p=0.02), but the TRM rate was not different. Among leukemia patients in first or second CR, the relapse rate was significantly lower (0%) in patients with higher $CD3^+$ cells (>0.25$\times4\times10^7$/kg) in winner unit than that (30.8%) of others (p=0.03), but the EFS was not different owing to high TRM rate in patient with higher $CD3^+$ cells. *Summary and Conclusions.* Double units CBT was a way to overcome the limitation of cell dose in single unit. However engraftment and TRM were not so satisfactory yet. Multiple factors associated with outcomes of CBT but optimal unit selection guidelines that take into account influencing factors are not available yet. CFU-GM was associated with the dominancy in our preliminary result but not associated with survival and $CD3^+$ cells seems to be associated with relapse. To improve the outcomes of double units CBT, development of new selection guideline and technology to enhance engraftment and reduce TRM were warranted.

0168

A PHASE I STUDY OF CLOFARABINE PLUS HIGH DOSE MELPHALAN AS A CONDITIONING REGIMEN FOR ALLOGENEIC TRANSPLANTATION

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Background. Allogeneic Stem Cell transplantation remains the only curative treatment modality for hematologic malignancies such as AML, ALL, and MDS. Reduced intensity regimens were designed which replaced the alkylating agent cyclophosphamide with the purine nucleoside antimetabolite, fludarabine, a potent immunosuppressive with a substantially milder toxicity profile. The success of these reduced intensity, less toxic regimens has allowed the benefits of transplantation to be extended to groups previously excluded from transplantation, such as the older population. Clofarabine is a purine nucleoside analogue designed to exploit a double halogen strategy which confers resistance to adenosine deaminase, increases stability and bioavailability and makes the drug more efficient than fludarabine at inhibiting Ribonucleotide Reductase (RR) and disrupting mitochondrial function, leading to apoptosis. This latter activity may explain the increased killing of non-dividing lymphocytes, an advantage which would theoretically reduce the likelihood of rejection in the allogeneic setting, and perhaps augment the potential graft versus tumor effect of the donor graft. *Aims.* To evaluate a novel clofarabine containing regimen as conditioning for allogeneic stem cell transplant. *Methods.* phase I dose escalation: clofarabine (dose level one= 30 mg/m^2, dose level two and three=40 mg/m^2) IV daily days -7 to day -3 infused over 30 minutes IV, plus Melphalan (dose level one and two, 100 mg/m^2 , dose level three, 140 mg/m^2) administered over 30 minutes IV on day -2. Related or unrelated allogeneic stem cells were infused on day 0. GVHD prophylaxis: initially CSP plus mycophenelate, then tacrolimus plus sirolimus was adopted

as per COH standard of care. Patients age ≥18 years with AML, ALL, MDS in either CR1, CR2 or in relapse (up to 50% marrow blasts), not deemed eligible for standard transplant regimens by the attending physician, or at high risk for relapse, are eligible. *Results.* We report on the first 2 dose levels. 8 eligible patients, all with AML, have been treated thus far, 4 Males, 4 Females, with a median age of 62.5 years (57-65). Four patients were in CR1, 2 patients were in CR2, and 2 patients were transplanted in relapse. Grade 3 non-hematologic toxicities included elevation of AST and LFT, diarrhea, and hyponatremia. No dose limiting toxicities (DLT) were seen in level one. One patient in dose level 2 died prior to engraftment due to hepatic, renal, and infectious toxicities; that dose level has been expanded thus far to five patients and no further DLT have been seen. One patient in relapse received an unrelated donor graft and had complete engraftment and obtained remission. Engraftment was rapid, full data is presented in the Table 1. Mild acute skin graft versus host disease (GvHD) was seen in one patient, mild chronic GvHD in one patient. No relapses have occurred with median followup of 366 days. *Conclusions.* The combination of clofarabine and melphalan is adequate as a conditioning regimen leading to rapid complete engraftment of related and unrelated allogeneic stem cells.

Table 1. Engraftment and survival data.

Dose Level	Pt	Days* to WBC≥1	Days* to PLT≥100	Follow-up**	Status
1	1	16	16	458	Remission
1	2	14	14	451	Remission
1	3	23	23	402	Remission
2	4***			23	Expired
2	5	11	13	330	Remission
2	6	15	12	413	Remission
2	7	10	13	94	Remission
2	8	12	13	44	Remission
	Median	14	13	366	

* - From Transplant
** - Days from transplant to relapse/death or last contact.
*** - Patient expired prior to engraftment.

0169

PROGNOSTIC VALUE OF POSITRON EMISSION TOMOGRAPHY/ COMPUTED TOMOGRAPHY IN PATIENTS WITH LYMPHOMA TREATED WITH AUTOLOGOUS STEM CELL TRANSPLANTATION FOR RELAPSED/REFRACTORY OR HIGH RISK DISEASE

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Background. Positron emission tomography/computed tomography (PET/CT) imaging has increasingly been used for management of malignant diseases including lymphoma. This emerging modality combines the advantages of both PET and CT imaging especially improving specificity. Few prospective studies addressed its prognostic value in the setting of high dose therapy with autologous stem cell transplantation (ASCT) so far. *Aim.*We evaluated the predictive role of PET/CT before ASCT (PET/CT1) and after ASCT (PET/CT2) in lymphoma patients in terms of overall survival (OS) and disease-free-survival (DFS). *Methods.* OS and PFS have been evaluated using Kaplan-Meier estimates; OS and DFS curves have been assessed using Log-rank test. *Results.* We conducted a prospective study including 12 patients with Hodgkin Lymphoma (6 in relapse, 6 refractory diseases at early PET/CT in induction), 38 patients with non Hodgkin Lymphoma (13 in relapse, 9 in PR after induction therapy, 10 high-risk patients while in CR and 6 patients with Mantle cell Lymphoma treated with R-HDS regimen). All patients presented a positive PET/CT before starting treatment. 35 patients underwent both PET/CT1 and PET/CT2. After ASCT 5 patients never achieved a CR and all died because of progressive disease; 41 patients after ASCT were in CR (including 30 patients in CR before ASCT); 4 were in PR and they obtained CR after radiotherapy. No patients died of treatment-related-mortality. Of note 3 patients presented a PET/CT positivity after ASCT with a decreasing uptake at subsequent controls and without a clear correlation with any lesion detectable by CT: none of them relapsed. Median follow up is 28 months (5-77); OS of whole cohort of patients was 85.7% and DFS 67.1%. On the basis of PET/CT1 we did not observe statistically significant differences in both OS (92.3% negative vs. 78% positive) and DFS (74% negative vs. 60% positive). Conversely, on the basis of PET/CT2, OS was 100% negative vs. 46.7% positive (p<0.01), while DFS was 81.8% negative vs. 54.1% positive (p<0.01) (Figure 1). All relapses have been documented in PET/CT2 positive group within 12 months from ASCT. In PET/CT2 negative group 3 patients died, one of TRM after allogeneic stem cell transplantation, one of secondary leukaemia after radioimmunotherapy and the last because of progressive disease. Seven PET/CT2 positive patients died: 6 because of progressive disease and one of TRM after allogeneic stem cell transplantation. *Conclusions.* In this cohort of lymphoma patients, the role of PET/CT1 in predicting relapse after ASCT is less clear compared to previous published studies, while the role of PET/CT2 seems to be more reliable, especially for early relapses.

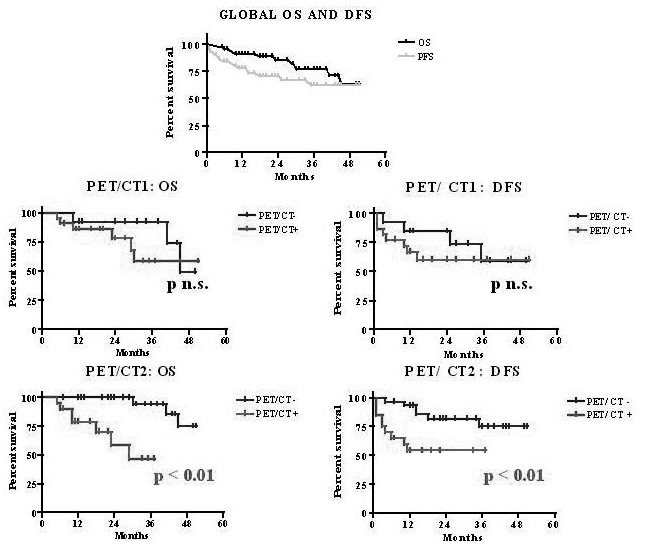

Figure 1.

0170

BOTH IL-3 AND IL-6 ARE NECESSARY FOR BETTER *EX VIVO* EXPANSION OF CD133+ CELLS FROM UMBILICAL CORD BLOOD

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Background. CD133$^+$ cells, a subpopulation of CD34$^+$ cells, constitute a group of earlier, less-differentiated hematopoietic stem cells (HSC) with a potentially higher capacity for engraftment. Umbilical cord blood (UCB) is the ideal source for HSC for transplantation; readily available, rich in progenitor cells with lower risk for severe acute graft-versus-host disease. However, UCB contains fewer transplantable cells, compared to bone marrow aspirate or mobilized from peripheral blood. The identification of conditions favoring UCB-HSC *ex vivo* expansion and their repopulating potential remains the major challenge of experimental and clinical hematology. SCF, FLT-3l and TPO have been proved crucial growth factors for promoting this expansion. However the role of IL-3 and IL-6 in CD133$^+$ and/or CD34$^+$ cells *ex vivo* expansion has not been clearly established. *Aim.* Investigate the influence of IL-3 and IL-6 or both on CD133$^+$ from UCB *ex vivo* expansion for 8 days and the effect of these cytokines upon cell phenotype. *Methods.* After mothers informed consent, UCB samples were collected from normal full-terms by standard procedures. CD133$^+$ cells were isolated by immunomagnetic method, and cultured as follows: basal medium (BM): IMDM with 10% FBS, SCF (25 ng/mL), TPO (10 ng/mL), FLT-3l (10 ng/mL); BM+IL-3: BM +IL-3 (10 ng/mL); BM+ IL-6: BM + IL-6 (10 ng/mL); BM+IL-3+IL-6: BM+ IL-3 (10 ng/mL) + IL-6 (10 ng/mL). Colony Formation Unit (CFU). Cells were seeded in methylcellulose medium with recombinant cytokines without EPO for 14 days. Real time PCR. RNA was extracted from CD133$^+$ cells to quantify relative SOX-2 and NANOG expression using ABL gene as endogenous control. *Results.* Flow cytometry analysis revealed 85% of purity of CD133$^+$ cells, immediatly after isolation, but this percentage diminished after 4 and 8 days of expansion as follows: BM alone (65.55% and 71.39%, respectively); BM+IL-3 (56.64%, p<0.01; and 50.50%, p<0.001); BM+IL-6 (66.53% and 70.22%) and BM+IL-3+IL-6 (61.67%, p<0.05; and 61.95% p<0.05) respectively. Fold-increase (calculated as

CD133+ number on days 4 or 8 divided by CD133+ number on day 0) of BM+IL-3 and BM+IL-3+IL-6 was higher on day 8 (13.83 and 17.47 fold increase, respectively, $p<0.001$). CD133+ cells cultured with BM+IL-6 for 4 and 8 days presented no significant difference compared with BM alone. CFU number (CFU-mix) doubled in BM+IL-3+IL-6 after 8 days of incubation compared with BM-group ($p<0.05$). Cell cycle analysis revealed a quiescent cell after isolation, 95.5% of CD133+ cells in the G0/G1-phase, 1.33% S-phase and 3.27% G2-M. Regardless culture period or cytokine incubation, approximately 70% of CD133+ cells remained in G0/G1-phase, 30% CD133+ cells in S-phase and 3% in G2-M phase ($p<0.001$). A higher relative gene expression of SOX-2 and NANOG on day 0 after isolation was detected. BM+IL-6 prevented decrease in NANOG and SOX-2 gene expression levels (no significant difference compared on day 0), when compared to cells incubated with BM, BM+IL-3 or BM+IL-3+IL-6 ($p<0.05$). *Conclusions.* CD133+ cells isolated from UCB are better ex vivo expanded in the presence of SCF, Flt3-L, TPO, IL-6 + IL-3. IL-3 probably promotes higher expansion of CD133+ cells and IL-6 maintains the immature phenotype.

0171

TRACKING OF DONOR TYPE GREN FLUORESCENT AND LUCIFERASE POSITIVE MARROW MONONUCLEAR OR MESENCHYMAL STEM CELLS IN POSTMORTEM SPINAL CORD HISTOPATHOLOGICAL SECTIONS FOR DETERMINATION OF REGENERATION IN A SPINAL CORD INJURY MODEL

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Background. Spinal cord trauma induces disability as a consequence of different mechanisms. Studies on reversal of injury are focused on two areas: 1. the use of neuroprotective agents 2. cellular therapy to induce axonal regeneration. *Aim.*In this experimental study, the effects of neuroglial tissue regeneration and functional recovery of intralesionally implanted bone marrow mononuclear (MNC) or mesenchymal stem cells (MSC) are examined by *in vivo* bioluminesence imaging and postmortem immunohistochemical techniques. *Method*. The study design included four groups: 1. non traumatized control group, 2. the cord were traumatized with 3 gram Tator klips but not followed by cellular implants , groups 3 and 4:Following similar trauma luciferase (+) bone marrow (BMMNC) (1×10^6/100 microliters) or MSC (1×10^5) obtained from CMV-Luc or β-Actin Luc transgenic mice were given intralesionally using fine needles. Following surgery, before the effects of seboflorein anesthesia ends, 150 mg/kg luciferine was enjected intraperitoneally to obtain *in vivo* bioluminescence imaging (Xenogen IVIS 50). All mice were neurologically examined weekly by oblique plane, BBB scores and *in vivo* imaging of the surgery area. On day 60, mice were decapitated and immunohistochemical evaluation was performed. Neural stem cells, neurons, myelin were detected by antibodies: nestin, MAPO and MOSP. *Results.*All mice of the groups 3 and 4 showed various degrees of improvement in the BBB scores. However there were no changes in groups 1 and 2. The improvement was significantly better in the group 4 compared to group 3 ($p=0.002$). The immunohistochemical staining demonstrated tracking of donor type gren fluorescent (GFP)+Luc+ neuronal cells that were also positive with any of these markers: nestin, MAG, MOP or MOPB which supplies evidence for the donor type neuronal regeneration. Neuronal cells expressing variable levels of neuronal differentiation and GFP/Luc were present in 13/25 mice(group3) and 17/25 mice(group 4). *Conclusions.* Current findings demonstrate, compared to a control group which did not receive cellular implants, that donor marrow cells contribute to neuronal regeneration and functional improvement.

0172

TEAM (THIOTEPA, ETOPOSIDE, CYTARABINE, MELPHALAN) AS CONDITIONING REGIMEN FOR LYMPHOMA TREATMENT WITH AUTOLOGOUS HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Standard conditioning regimen before an autologous haematopoietic stem cell transplantation (AHSCT) for chemosensitive relapsed Hodgkin Lymphoma (HL) and Non Hodgkin Limphoma (NHL) patients (pts) is still represented by a scheduled combination of carmustine, etoposide, cytarabine and Melphalan (BEAM). Despite the efficacy of this regimen, it is disadvantaged by the presence of carmustine, which is responsible of a high pulmonar toxicity with fibrosis and progressive reduced diffusion capacity. In order to prevent pulmonar toxicity, we replaced carmustine with Thiotepa in 26 chemosensitive relapsed HL and NHL pts. We have also evaluated the efficacy and tolerability of this new regimen. *Patients*. At day -7 27 pts received 10 mg/kg thiotepa (5 mg twice every 12 hours) followed by cytarabine 200 mg/m^2 die (-5 to -3) etoposide 200 mg/m^2 die (-5 to -3) and melphalan 140 mg/m2 (day -2). Out of these pts, 16 were male and 11 female. Sixteen pts were affected by da NHL and 11 by HL. At the moment of transplant only 8 pts were in CR, 15 were in PR and 4 were in progression. All patients were in second or sebsequent line of therapy. The median time of neutrophil >500 and platelets >20000 recovery was of 10 (range 8-12) and 12 (range 10-20) days respectively. No severe infectious event has been observed. Eight pts maintain the CR, 13 have reached CR, 3 have a stable disease and 3 pts did not respond to the treatment. Three pts that were in CR have subsequently relapsed. *Conclusions.* Our experience with TEAM protocol has shown a significant efficacy not lower to our historical experience with BEAM protocol. The tollerability was significantly accetable and no serious adverse event has been documented during the treatment or during aplastic phase, therefore the TRM at 100 days has been equal to zero. In no case, oral mucositis was higher than grade 2, even if most of the pts underwent total parenteral nutrition. Finally no cases of serious organ toxicity was observed (pulmonar, hepatic, cardiac and renal). Hematopoietic recovery, stimulated with G-CSF, has been reached as scheduled and no cases of prolonged neutropenia were observed.

Novel therapeutics, targeted therapies and gene therapy - Preclinical and Clinical

0173

ACE-041, A SOLUBLE ACTIVIN RECEPTOR-LIKE KINASE FUSION PROTEIN, IS A NOVEL ANTI-ANGIOGENIC AGENT

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Background. Activin receptor-like kinase 1 (ALK-1) is an endothelium-specific type I receptor of TGF-beta receptor family, which is believed to play an essential role in modulating angiogenesis. Mutations in ALK-1 result in hereditary hemorrhagic telangiectasia (HHT2). HHT2 patients have chronic hemorrhaging and arteriovenous malformation, indicating a key role of this receptor in the regulation of angiogenesis. The homozygous inactivation of the ALK-1 gene in mice leads to embryonic lethality resulting from severe vascular anomalies, while heterozygous (ALK-1 +/-) mice exhibit phenotypes that are reminiscent of HHT2. However, overexpression of ALK-1 has been associated with inhibition of angiogenesis, and ligands of ALK-1 (BMP-9 and BMP-10) appear to inhibit various angiogenic activities *in vitro*. Thus, while the available data demonstrate that ALK-1 participates in the regulation of blood vessel formation and maintenance, the data do not clarify whether an antagonist of the ALK-1 signaling pathway is likely to have a pro- or anti-angiogenic effect when administered to an adult animal in a therapeutic modality. *Aims.*Here we describe an ALK-1 pathway inhibitor, a soluble form of ALK-1 receptor fused to the Fc region of human IgG1 (ACE-041), and we demonstrate that ACE-041 inhibits angiogenesis *in vitro* as well in tumor models. *Methods.* ACE-041 was expressed in CHO cells and purified from conditioned medium by immunoaffinity, ion-exchange and hydrophobic interaction chromatography to greater than 95% purity. Biacore T100 was used to study selectivity and kinetics of ACE-041 binding to target ligands. Chick Chorioallantoic Membrane (CAM) Assay was used to assess anti-angiogenic potential of ACE-041 *in vivo*. *Results.* We demonstrate that ACE-041 binds to the circulating ligands BMP9 and BMP10 and prevents them from signaling through the endogenous ALK-1 receptor. By using Surface Plasmon Resonance (SPR) analysis we studied the selectivity of ACE-041, and demonstrated that ACE-041 binds with high affinity to its target ligands BMP9 and BMP10. We also showed that receptor binding to BMP9 and BMP10 is blocked by ALK-1 mutations found in HHT2. Treatment with ACE-041 reduced vessel formation and branching induced by multiple pro-angiogenic factors in chick CAM assays. Melanoma explant CAM assays showed similar anti-angiogenic effects after treatment, exhibiting reduced size, weight and vascularization of tumors. Anti-tumor activity was additionally evaluated in an orthotopic breast cancer tumor model using MDA-MB-231 cells. ACE-041 treatment decreased tumor volume, tumor weight and reduced blood vessel density. *Conclusions.* These data demonstrate the efficacy of ACE-041 in abrogating vessel formation in *in vitro* and *in vivo* assays, suggesting that it is a powerful anti-angiogenic agent with anti-tumor activity.

0174

PHARMACOLOGY OF CPX-351: A NANO-SCALE LIPOSOMAL FIXED MOLAR RATIO CYTARABINE-DAUNORUBICIN FOR PATIENTS WITH ADVANCED LEUKEMIA

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Background. Antitumor activity of cytarabine and daunorubicin chemotherapy exhibits maximum synergy at a 5:1 molar ratio *in vitro*, implying a drug-drug interaction that can be exploited to maximize clinical efficacy by maintaining this molar ratio *in vivo*. CPX-351, a nano-scale liposomal formulation of cytarabine and daunorubicin encapsulated at the 5:1 molar ratio has marked synergy in murine leukemia models, and has undergone Phase I clinical testing (see Blood (ASH Annual Meeting Abstracts), Nov 2008; 112: 2984.). That study demonstrated a high degree of clinical efficacy in relapsed/refractory acute leukemia, with complete responses observed at doses ranging from 32-134 u/m2 (1 unit represents 1 mg of cytarabine plus 0.44mg of daunorubicin). Analyses of plasma drug levels from that study are presented here to examine the pharmacokinetic (PK) features associated with this anti-leukemic activity. *Aims.*To characterize how encapsulation of cytarabine and daunorubicin within CPX-351 affects drug bioavailability, elimination kinetics, and delivery of the 5:1 molar ratio. *Methods.* Plasma specimens following CPX-351 administration by 90-minute infusion on Days 1, 3, and 5 were collected and assayed for the following dose levels: 24, 32, 43, 57, 76, 101, and 134 u/m^2. Validated LC/MS/MS assays were developed for measuring total cytarabine, daunorubicin, Ara-U, and daunorubicinol. WinNonlin (v5.2) was used for analysis of noncompartmental PK data. *Results.* Both Ara-U and daunorubicinol were present in the plasma indicating that encapsulation did not impair bioavailability. Cytarabine and daunorubicin exhibited linear multiple-dose PK with mono-exponential plasma elimination of both drugs and a very limited distribution phase. The Cmax and AUC(0-tau) of cytarabine and daunorubicin were linearly correlated with dose and there was evidence of accumulation observed between Day 1 and Day 5 for some dose groups. Mean cytarabine:daunorubicin molar ratios for 24 hours following Day 1 and Day 5 administration of CPX-351 were generally very close to the intended 5:1 ratio (3.33-5.85 after Day 1 and 5.38-6.89 after Day 5). The Table 1 presents PK data at the MTD (101 u/m^2). When compared to the literature, liposome encapsulation increased the daunorubicin exposure (~200 fold for Cmax and ~1200 fold for AUC) and multiplied the exposure to cytarabine (up to 900 fold for AUC). Inter-patient variability was low. *Summary and Conclusions.* Encapsulation of cytarabine and daunorubicin using this methodology markedly increases and prolongs the systemic exposure of the two drugs while maintaining their bioavailability. Plasma elimination kinetics for both cytarabine and daunorubicin were predictable and dose independent. The ability of CPX-351 to deliver synergistic cytarabine:daunorubicin ratios at high absolute concentrations for extended times may underlie clinical efficacy observed in the Phase 1 study and support further evaluation of CPX-351 in ongoing Phase II trials in untreated and relapsed acute myeloid leukemia.

Table 1.

Mean (SD) Cytarabine, Daunorubicin, Arabinofuranosyuracil, and Daunorubicinol Pharmacokinetic Parameters on Day 5 Following Intravenous Administration of 101u/m^2 CPX-351 over 90 minutes on Days 1, 3 and 5

Analyte	C_{max} (ng/mL)	T_{max} (hr)	$AUC_{(0\text{-}tau)}$ (ng*hr/mL)	$t_{1/2}$ (hr)	C_{max}/Dose [(ng/mL) /(u/m^2)]	$AUC_{(0\text{-}tau)}$/Dose [(ng*hr/mL) /(u/m^2)]
Cytarabine	64608 (23230)	3.02 (2.25)	1851089 (934523)	36.9 (24.5)	640 (230)	18328 (9253)
Daunorubicin	30185 (6198)	1.87 (0.74)	666640 (209198)	25.2 (11.6)	680 (140)	15014 (4712)
Ara-U	2119 (789)	13.4 (12)	78411 (31709)	. .	21 (7.81)	776 (314)
Daunorubicinol	180 (103)	27.7 (15.6)	7308 (4030)	. .	4.05 (2.31)	165 (90.8)

0175

UTILIZING CELL-BASED SCREENING TO IDENTIFY NOVEL USES FOR EXISTING DRUGS: INDUCING SPECIFIC APOPTOSIS OF B-CELL LYMPHOCYTES

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Background. We have developed a cell-based screening platform that incorporates both automated sample preparation and automated evaluation by flow cytometry, in conjunction with proprietary analytical software and a database structure, that is geared for rapid data acquisition, analysis and reporting of results. This platform, we call ExviTech, has been used to screen a compound library comprised of 2,000 drugs already approved for human use. Vivia's research is based on this reprofiling type of screening, searching for new indications for existing drugs. Additionally, all screening is done directly on patient samples, either peripheral blood or bone marrow, extracted from persons diagnosed with haematological malignancies. The complete patient sample is diluted and plated, retaining the erythrocyte population and serum proteins,

screening targets in an approximation of their biological context. Using these patient samples, we have identified a series of related compounds that induce apoptosis specifically in B-cell lymphocytes through a mechanism that appears to be fully independent of the known primary mode of action for these drugs. *Methods.* Working in collaboration with four hospitals in Spain, samples are extracted from patients diagnosed with haematological malignancies, having first obtained informed consent. The experimental assay is setup within 2-6 hours of obtaining the sample. The sample is diluted to achieve a leukemic cell concentration of approximately 3,000 cells/µL, then 45 µL of the suspension is added to each well of 96-well plates that contain the pharmacological agents (final concentration of 30 µM). Sample extraction from the patients and the experimental setup are all done under sterile conditions. The compound plates are then incubated for 24 hours at 37°C with 5% CO2 for screening, and additionally for 48 and 72 hours in subsequent tests. After incubation, the erythrocytes are lysed and Annexin V-FITC, monoclonal antibodies anti-CD45-APC and anti-CD19-PE, are added to each well. The plates are then transferred to an automated flow cytometry system where the contents of each well is aspirated and analyzed by a CyAn flow cytometer. *Results and Conclusions.* Analyzing primary screens from over 60 patients, a series of three related compounds (Vivia007, Vivia008 and Vivia009) were consistently able induce apoptosis in patients with B-cell lymphoproliferative disease, at levels equal to or greater than known cytotoxic agents. Of these, Vivia007 is the most effective. Dose-response analysis was done on 20 additional patients diagnosed with B-cell chronic lymphocytic leukaemia (B-CLL) and Vivia007 displays an average EC50 of 6.8 µM. The effect is specific to B-cell lymphocyte populations, with only a minor level of apoptosis being induced in the T-cell lymphocytes. Additionally, these compounds do not appear to be effective in chronic myelomonocytic leukemia or in T-cell lymphoproliferative disease. The kinetics of the induction of apoptosis is faster for the Vivia compounds than it is for Fludarabine, Cyclophosphamide or Mitoxantrone, cytotoxics used in the treatment of B-CLL. These results demonstrate the potential of the ExviTech technology platform as a successful model for the systematic search of new uses for existing drugs, with testing being done directly on patient samples.

0176

HUMAN MESENCHYMAL STROMAL CELLS DISPLAY SKEWED ADHESION RECEPTOR USAGE FOR ARREST ON ENDOTHELIAL CELLS AND TRANSENDOTHELIAL MIGRATION, FAVOURING E-SELECTIN, BETA1 INTEGRINS, AND A PLEIOTROPIC CHEMOKINE RESPONSE: IMPLICATIONS FOR CELLULAR THERAPY WITH MSC

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Background. Mesenchymal Stromal Cells (MSCs) have been used in various preclinical and clinical studies. In proof-of-concept experiments, MSCs were found to suppress immune reactions such as Graft versus Host Disease in children, to target the tumor microenvironment, and to improve outcome in patients with severe sepsis. We have previously shown that these fibroblast-type cells can roll on endothelial cells and can co-ordinately extravasate into tissues (Blood 108:3938, 2006). However, the repertoire of adhesion receptors active on MSCs has not been fully characterized. *Aims.*We asked which of the known key receptors used by leukocytes to roll and arrest on endothelial cells, to polarize, and to transmigrate across endothelial cells can be activated on MSCs under conditions of shear stress. *Methods.* Human MSCs were isolated from normal healthy donors and characterized using differentiation assays, RT-PCR, flow cytometry, and in parallel plate flow chambers preloaded with endothelial cells or recombinant adhesion ligands. *Results.* Of the main selectin-type rolling receptors, MSCs did not or hardly express P-selectin glycoprotein ligand (PSGL)-1, L-selectin and CD24, whereas CD44 which encodes an E-selectin ligand was highly expressed. β1 integrins were expressed by MSCs and comprised Very Late Antigens (VLA)-4 and -5, whereas β2 integrins LFA-1 and Mac1 were present only in low amounts. Chemokine receptors of the CCR and the CXCR families were expressed both intracellularly and, at lower levels, on the cell surface of MSCs. In shear stress assays, MSCs showed comparable frequencies of rolling and arresting cells on activated endothelial cells as human $CD34^+$ cells or blood lymphocytes. Function-blocking antibody experiments and experiments on coated recombinant single ligands showed that E- and also P-selectin were the main mediators of MSC rolling. In addition, MSCs rolled on VLA-4. Arrest of MSCs was efficiently induced by co-coated chemokines, and was dependent on VLA-4 but not β2 integrins. Once arrested, MSCs could transmigrate through endothelial cells. Blockade of MSC arrest and of transmigration was seen after pretreatment of MSCs with pertussis toxin, an inhibitor of G α subunits, and NSC23766, a specific inhibitor of the small GTPase Rac, confirming involvement of chemokine initiated G protein coupled receptor signalling in this response. We next dissected potential chemokine responses and found that integrin-mediated MSC adhesion can be efficiently elucidated through an array of individual chemokine receptors including CCR 5 (used by neutrophils, monocytes), CCR6 and 7 (used by B- and T-lymphocytes as lymph node entry receptors), CXCR4 (used by many cell types) and CXCR5 (B cell lymph node entry receptor). *Summary and Conclusions.* MSCs show a unique profile of adhesion receptor usage when binding endothelial cells under shear stress. They roll and arrest very similarly as hematopoietic cells, but show little response to L-selectin and β 2 integrins. Moreover, they display a highly pleiotropic chemokine receptor usage. These data provide a basis for MSC graft engineering by modulating expression and activation of homing receptors.

0177

A NOVEL RECOMBINANT BISPECIFIC ANTIBODY TARGETING HM1.24 ON MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a malignant plasma cell disorder that accounts for about 10% of all hematologic cancers. Although continuous progress has been made by adding novel treatment modalities (e.g. stem cell transplantation, proteasome inhibitors such as bortezomib, and IMiDs) to conventional chemotherapy regimes, for a substantial number of patients MM is still a fatal disease. Besides small molecule inhibitors that interfere with various signalling pathways, antibody-based therapeutic strategies are urgently needed. HM1.24 (CD317) has been identified as a type II transmembrane protein that is preferentially expressed on terminally differentiated B cells and its overexpression has been observed on malignant plasma cells. Although the HM1.24 antigen has been identified as a possible target for immunotherapy, this has not led to translation into the clinic yet. Besides target antigen selection, efficient recruitment of immune effector cells via activating Fc'gamma' receptors is important for the anti-tumor activity of therapeutic antibodies. Enhancing antibody-dependent cellular cytotoxicity (ADCC) - the major effector mechanism triggered by conventional monoclonal HM1.24-directed antibodies - may represent a promising strategy to enhance the cytolytic potency of HM1.24-directed antibody therapy. *Aims.* Here, we tested the hypothesis whether specific engagement of FcγRIII by HM1.24-directed bispecific antibodies could further enhance the major effector mechanism reported for HM1.24-directed IgG1 antibodies. *Methods.* A novel bispecific scFv targeting HM1.24 on multiple myeloma and Fc'gamma'RIII (CD16) on immune effector cells was generated. HM1.24xdsCD16 was expressed in 293T cells and purified by two-step purification. Specific binding to HM1.24 and CD16 was analyzed by flow cytometry with antigen positive and negative cells. The lytic capacity of the bispecific scFv was evaluated in ADCC experiments (chromium-release assay) with mononuclear cells as effector cells and different myeloma cell lines (RPMI8226; U266; JK6-L; INA-6) as well as primary tumor cells from a patient with plasma cell leukemia as target cells. *Results.* HM1.24xdsCD16 was successfully expressed and purified to homogeneity. The bispecific scFv specifically bound to HM1.24 expressing myeloma cell lines and CD16 positive effector cells. In ADCC experiments using human peripheral blood mononuclear cells as effectors, HM1.24xdsCD16 mediated specific lysis of both myeloma cell lines and primary patient-derived plasma cell leukemia cells. In these experiments HM1.24xdsCD16 demonstrated superior activity in comparison to a humanized HM1.24 IgG1 antibody. Summary / *Conclusions.* A single-chain bispecific antibody, HM1.24xdsCD16 was designed and produced for targeting human myeloma cells and recruitment of Fc'gamma'RIII-positive immune effector cells. In antibody-dependent cellular cytotoxicity experiments HM1.24xdsCD16 demonstrated superior activity in comparison to a humanized HM1.24 IgG1 antibody. Thus, HM1.24xdsCD16 bispecific molecules represent a novel tool for efficient effector-cell mediated tumor lysis in multiple myeloma.

0178

D EPITOPE EVALUATION OF THE 25 RHESUS D SPECIFIC IGG1 ANTIBODIES IN ROZROLIMUPAB (SYM001), THE FIRST RECOMBINANT POLYCLONAL ANTIBODY PRODUCT

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Rozrolimupab is the first in class of recombinant polyclonal antibody products and is in clinical development for the prevention of hemolytic disease of the newborn by anti-D prophylaxis (ADP) and for the treatment of Immune Thrombocytopenic Purpura (ITP). Rozrolimupab is composed of 25 genetically unique IgG1 antibodies all specific for the Rhesus D erythrocyte protein. The 25 Sym001 anti-D antibodies are derived by phage display from the antibody repertoires of 8 female human anti-RhD plasma donors, with high antibody titers against RhD, enrolled in a Danish anti-RhD collection program. The antibodies of the rozrolimupab composition have been selected to ensure that the final product, apart from the complete RhD phenotype commonly expressed in the human population (>99%), also recognizes the rare Rhesus D variants DIII, DIV, DVI and DVII that account for <1% of the overall RhD+ population. The RhD epitope (epD) specificities of the individual rozrolimupab antibodies have been determined following the principle applied at the Paris 2001 workshop on epitope mapping of monoclonal anti-D antibodies. The reactivity of the individual rozrolimupab antibodies with an extensive panel (n=19) of genetically defined RhD erythrocyte variants, present in populations of different ethnic origin, was determined applying the low-ionic strength saline (LISS) indirect antiglobulin test (IAT). The results show that 7 of the currently defined D epitopes (epD1, epD2, epD3, epD4, epD5, epD6 and epD16, including several subspecificities thereof) were recognized by 22 of the 25 antibodies. Some of the epitopes were recognized by more than one of the antibodies. The epitope specificities of the remaining 3 antibodies could not be defined by this approach due to their broad reactivity profiles. The 7 epD recognized are found to constitute epitopes present, in different combinations, on all RhD variants of clinical importance in a situation of RhD incompatibility between mother and foetus that may lead to isoimmunisation of the mother. Thus, the selected polyclonal antibody composition of rozrolimupab would ensure adequate recognition of foetal erythrocytes with potential clinical impact, which is a basic requirement for use in anti-D prophylaxis.

0179

IBRITUMOMAB TIUXETAN IN LYMPHOMA- A SINGLE CENTRE EXPERIENCE FROM KUWAIT

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Background. Ibritumomab is a murine monoclonal antibody, conjugated to tiuxetan that chelates yttrium or indium, and is directed against CD 20 molecules of B lymphocytes. Ibritumomab tiuxetan is FDA approved for treatment of low grade lymphomas that have failed prior therapy. Emerging trials show promising results in indolent as well as aggressive lymphomas, in both upfront and relapsed settings. *Aim.* To see the outcome of radio-immunotherapy (ibritumomab tiuxetan) in lymphoma patients. *Methods.* We treated 20 lymphoma patients from April 2005 until June 2008 with ibritumomab tiuxetan. Median age of the patients was 62yrs. 14 cases were indolent and 6 cases were aggressive lymphomas. 3 patients received ibritumomab tiuxetan as part of first line chemotherapy and the remaining 17, in relapsed setting. A bone marrow examination was done in all patients before administration of the drug, and those having bone marrow involvement of lymphoma or hypocellular marrow were excluded. During administration the patients were closely monitored for infusion related complications. Serial complete blood counts were done on follow up. All patients were reevaluated after 3 months by CT scan and gallium scan. They were periodically reviewed in the out-patient clinic to look for long term complications. *Results.* 12 patients achieved complete remission (60%) and 5 achieved partial remission (25%), constituting an ORR of 85%. 3 patients developed long standing myelosuppression (lasted more than 2 months), out of which, 1 patient died due to related complications. 3 patients (15%) developed treatment related acute leukemia. All the three patients who developed leukemia had received ibritumomab tiuxetan in relapsed setting and two of them had received radiotherapy to the pelvic area, as part of the first line treatment. Mean time of development of leukemia from the diagnosis of lymphoma was 6 years and from radio-immunotherapy was 2 years. Multiple cytogenetic aberrations were found in all the three patients but, interestingly, chromosome 5 or 7 anomalies were not found. *Conclusions.* Although ibritumomab tiuxetan produced high response rate in lymphomas, high incidence of fatal complications, especially secondary leukemias is worrisome and warrants long term follow up. Previous radiation exposure to pelvic area might be an additional risk factor for the development of radio-immunotherapy induced secondary leukemias.

0180

THE CXCR4 ANTAGONIST 4F-BENZOYL-TN14003 STIMULATES THE EXIT OF HEMATOPIETIC CELLS AND THE REGENERATION OF THE BONE MARROW FOLLOWING TRANSPLANTATION

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Background. Cytopenia represents a significant complication following chemotherapy, irradiation prior to bone marrow (BM) transplantation or as a therapy for cancer. The mechanisms that determine the pace of BM recovery are not fully understood. *Aim.* Studying the role of CXCR4 in the regeneration of the BM following chemotherapy or irradiation. *Methods.* The role of CXCR4 in the recovery of the bone marrow following irradiation was tested using transplantation model and the role of CXCR4 in the recovery of the bone marrow following chemotherapy was done using Cyclophosphamide model of cytopenia. The role of CXCR4 in the proliferation and differentiation of hematopoietic progenitors and mature cells was tested *in vitro* using BM stromal culture. In all experimental models the CXCR4 antagonist 4F-benzoyl-TN14003 (T-140) and AMD3100 were used to modify CXCR4 activity. *Results.* During the recovery phase following chemotherapy or irradiation, the signals for retention of white blood cells (WBC) within the BM increase significantly. This leads to a delay in the release of WBC. The CXCR4 antagonist, known as T-140, can induce mobilization of hematopoietic stem cells and progenitors within a few hours post-treatment in a dose-dependent manner. Furthermore, T-140 can also increase the number of WBC in blood including monocytes, B and T cells. T-140 was found to efficiently synergize with G-CSF in its ability to mobilize WBC and progenitors including erythroblasts. We found that by targeting the CXCR4 axis with T-140 the delay in the release of WBC can be overcome. The delay in the WBC release of WBC is also accompanied by suppression in the production of progenitor cells and mature cells by the BM stroma. Interestingly, *in vivo* administration of T-140 to mice transplanted with BM cells stimulates the production of all types of progenitors and mature cells and increases the exit of mature cells to the periphery. Moreover, addition of T-140 to *in vitro* BM stromal cultures stimulates the production of mature cells and progenitors from all lineages. *Summary.* The unique ability of the CXCR4 antagonist, T-140 to stimulate the production and exit of WBC cells may be used as a novel therapeutic approach to overcome cytopenia associated with treatments for cancer and BM transplantation.

0181

DEXAMETHASONE AND DASATINIB INDUCE SYNERGISTIC INHIBITION OF MAJOR T CELL EFFECTOR FUNCTIONS

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Background. In addition to their antitumor activity tyrosine kinase inhibitors (TKIs) exert immuno-modulatory effects on T cells. We (Weichsel *et al.* 2008) and others (Blake *et al.* 2008; Fei *et al.* 2008; Schade *et al.* 2008) have shown that the multitargeting TKI dasatinib may lead to a complete inhibition of T cell effector functions via the SRC kinases LCK and FYN. *Aims.* We investigated the impact of combining clinically relevant doses of dasatinib [1-100nM] with the immunosuppressant dexamethasone [1-1000nM] on T cells. *Methods.* Purified human $CD3^+$ cells from healthy blood donors were studied *ex vivo.* Functional outcomes assessed included IL-2 production, CD69 upregulation, proliferation (CFSE dilution) and apoptosis/necrosis induction. EBV or CMV antigen specific $CD8^+$ T cell proliferation were evaluated applying tetramers. Results. Complete inhibition of proliferation and activation occurred with dasatinib at 50 nM, whereas application of dexamethasone did not lead to complete inhibition even at 1000 nM. Dose-dependent inhibition of OKT3-induced T cell activation and proliferation was observed with the combination of dasatinib and dexamethasone.

Strongest synergistic inhibitory effects of the drug combination were observed for OKT3-induced cytotoxic $CD8^+$ T cell proliferation (mean±SEM given: OKT3 induced 63±5% proliferating T cells after 4 days, OKT3 + 10nM dexamethasone 44±5%, OKT3 + 10nM dasatinib 39±10%, OKT3 + combination 16±4%; n=5; $p<0.05$). A significant inhibition of OKT3 induced CD69 up-regulation was demonstrated with the combination but not for dexamethasone alone (n=5). Our previously published data on dasatinib alone showed inhibition of OKT3-induced up-regulation of CD69 expression, and this was potentiated in combination with dexamethasone. The pre-treatment time did not influence the dexamethasone effect except for increased reduction of IL-2 production after 24h vs. 1h pre-incubation. Overall, helper $CD4^+$ T cells were more sensitive to the inhibitory effects of the drug combination regarding activation and proliferation than cytotoxic $CD8^+$ T cells. Of note, synergistic effects occurred primarily in the different memory CD4 and CD8 subsets but not in naïve T cells (e.g. for $CD8^+CD45RO^+CD27^+$ memory T cells mean±SEM given: OKT3 92±1%, OKT3 + 100nM dexamethasone 77±2%, OKT3 + 10nM dasatinib 69±15%, and OKT3 + combination 27±11%; n=5; $p<0.05$). IL-2 production in purified T cells was significantly reduced ($p<0.05$) in a dose dependent nature for both dasatinib and dexamethasone compared to the OKT3-stimulated condition, either alone or in combination (n=5). Similarly, activation induced cell death (AICD) was significant reduced when the two drugs were combined, whereas no synergistic effects were observed regarding necrosis inhibition (n=5; $p<0.05$). In contrast, initial results suggested that dexamethasone did not inhibit clinically relevant EBV or CMV antigen specific $CD8^+$ memory T cells proliferation when used alone and did not show synergistic effects with dasatinib (n=3). This may be due to a reduced sensitivity of the specific viral memory subset composition towards dexamethasone. *Summary and conclusion.* With an indication that each drug, when combined, could be used at reduced dose, this research may pave the way for synergistic uses of TKIs and glucocorticosteroids in graft versus host disease or autoimmune diseases potentially without increasing the risk of infections.

0182

EX VIVO ACTIVITY OF SGI-1776, A POTENT SMALL MOLECULE PIM KINASE INHIBITOR, IN PRIMARY HUMAN LEUKEMIA AND LYMPHOMA CELLS

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Background. The serine-threonine kinases Pim-1, Pim-2 and Pim-3 are downstream effectors and potent inhibitors of apoptosis involved in various signaling pathways. Pim-1 and Pim-2 are commonly over-expressed in hematological malignancies, while Pim-3 is over-expressed in many tumors of epithelial origin. Pim-1 over-expression has been also associated with a poor prognosis in mantle cell lymphoma. SGI-1776 is a novel small molecule identified through our proprietary CLIMB' process and characterized to be a selective pan-Pim kinase inhibitor (6.7 nM, 69 nM and 363 nM biochemical IC50s for Pim-1, Pim-3 and Pim-2, respectively) with potent effects on cellular signaling pathways and cancer cell proliferation *in vitro* and *in vivo*. SGI-1776 is entering into phase 1 clinical studies in patients with relapsed and refractory non-Hodgkin's lymphoma, as well as patients with relapsed/refractory leukemias. *Aims.* Evaluate the sensitivity to different concentrations of SGI-1776 on fresh or viably preserved cancer cells from 3 Acute Lymphocytic Leukemia (ALL), 5 Acute Myeloid Leukemia (AML) , 5 Chronic Myeloid Leukemia (CML) and 5 non-Hodgkin's Lymphoma (NHL) patients. By Immunohistochemistry (IHC), the expression of Pim kinases in formalin-fixed, paraffin embedded (FFPE) human leukemia and lymphoma samples were evaluated. *Methods.* De-identified clinical specimens were obtained by Mosaic Laboratories (Lake Forest, CA) in accordance with an IRB-approved protocol. Drug sensitivity was evaluated by incubating cells for 96 hours at 37°C in the presence of different concentrations of SGI-1776 (range, 0.005-2 μM) and assessed by a differential staining cytotoxicity (DiSC) assay. ALL and AML cells were also incubated with vincristine (VCR), NHL cells with 4-hydroperoxycyclophosphamide (4HC) and CML cells with imatinib. *Results.* Viability of 14 specimens (out of 18 tested) was affected by SGI-1776. Overall, the IC50 for NHL samples was the most sensitive to SGI-1776 (0.43 μM), followed by CML (0.6 μM), AML (0.92 μM) and ALL (1.51 μM). Specifically, the effects of SGI-1776 was further characterized by a low or sub-μM IC50 in MDR-1 ALL (2.01 μM), FLT-3 mutated AML (4.15 μM) and 4 BCR-ABL t(9;22) CML (range, 0.39-0.66 μM) specimens. In NHL, 2 μM of SGI-1776 was significantly more active than 4HC at all concentrations tested (up to 50 μM); SGI-1776 also demonstrated a lower IC50 than Imatinib in 4 of the 5 CML specimens and this difference was significant at the equimolar concentration of 2 μM. The highest expression of Pim-1,-2 and -3 was observed in NHL cells consistently with their sensitivity to drug effects. CML cells displayed a relatively low expression of the 3 Pim kinases with the exception of Pim-3. *Conclusions*. The pan-Pim kinase inhibitor SGI-1776 potently reduces ex-vivo survival of primary human leukemia and lymphoma cells and retains its activity in cells otherwise resistant to standard chemotherapy. Pim kinases expression is evident in these primary cells and, in NHL, may correlate with sensitivity to SGI-1776.

0183

IN VITRO ANALYSIS OF NOVEL ANTI-CD20 ANTIBODY GA101 AND RITUXIMAB IN MANTLE CELL LYMPHOMA

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Background. Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin-lymphoma characterized by a poor prognosis with a median survival of 3 years. The type I monoclonal antibody Rituximab has shown a marked anti-proliferative effect in MCL and achieves increased response rates in combination with chemotherapy. The new third generation, glycoengineered type II IgG1 anti-CD20 monoclonal antibody GA101 is considered having enhanced antibody dependent cellular cytotoxicity (ADCC) and superior caspase-independent cell death induction by targeting a type II epitope on CD20. *Aims.* In this study GA101 was analyzed for its anti-proliferative effects on a panel of MCL cell lines both as single agent and in combination therapies in direct comparison to Rituximab. *Methods.* Using an established panel of MCL cell lines (Rec-1, HBL-2, Jeko-1, Granta-519, JVM-2 and Z-138) we determined the effect of GA101 alone as well as in combination with Rituximab on cell proliferation and viability. MCL cells were treated with GA101 at concentrations of 1,25-20 mg/mL and Rituximab. Cell viability was analyzed by trypan-blue exclusion tests at 0h, 24h, 48h and 72h. The panel of MCL cell lines was treated with GA101 and Rituximab each at 10 mg/mL to determine potential synergism of antibody combinations. Accordingly, fractional product was calculated: synergism > 0.1; antagonism < -0,1. *Results.* After monotherapy with GA101 (1 mg/mL), Granta-519 and Rec-1 showed the highest sensitivity (65-75% cell reduction in Granta-519 and 25-30% in Rec-1). Intermediate results were achieved for Z-138, HBL-2, Jeko-1 and JVM-2 (15-20%). Rituximab mono-exposure at 12,5 mg/mL showed a 25% reduction of cell count in Granta-519, 20% in HBL-2 and < 5% in Rec-1, Jeko-1 and Z-138. Combination experiments suggested the competitive binding of the two antibodies. *Conclusions.* Even at a >10-fold lower concentration, GA101 demonstrates higher efficacy in MCL cell lines compared to Rituximab. Combination experiments suggested the competitive binding of the two antibodies. In addition, further experiments suggested an additive effect of GA101 in combination with various cytostatic compounds.

0184

EFFECTS OF THE PI3 KINASE/MTOR INHIBITOR NVP-BEZ235 ON GROWTH AND IGE-DEPENDENT MEDIATOR RELEASE IN HUMAN BASOPHILS AND MAST CELLS

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Growth and function of human basophils (BA) and mast cells (MC) are triggered by various cytokines and other ligands, and a complex network of signal transduction pathways. In allergic reactions, cross-linking of high affinity IgE-binding sites leads to activation and mediator secretion. In MC, the KIT ligand stem cell factor (SCF) promotes IgE-dependent mediator secretion. In mastocytosis, the SCF receptor KIT is mutated at codon 816 and expressed as a constitutively activated target in neoplastic MC. Recent data suggest that IgE-receptor downstream signalling molecules and KIT downstream signalling molecules represent potential therapeutic targets. The phosphoinositide 3-kinase (PI3 kinase) is a key signalling molecule in IgE receptor-dependent activation and KIT D816V-dependent activation in MC and has been implicated in growth and survival of normal and neoplastic cells. In the present study, we examined the effects of the dual PI3 kinase/mTOR inhibitor NVP-BEZ235 (Novartis, Basel, Switzerland) on IgE-dependent mediator release in human BA and cultured cord blood cell-derived MC as well as on growth of neoplastic BA (KU812) and neoplastic MC (HMC-1). We found that NVP-BEZ235 inhibits IgE-dependent histamine release in BA

in healthy individuals in a dose-dependent manner (IC_{50} 0.5-1 μM). In addition, NVB-BEZ235 inhibited anti-IgE-induced upregulation of CD63 and CD203c on the surface of BA, although responses were not seen with both CD molecules in all donors. In cultured MC, NVP-BEZ235 decreased IgE-dependent upregulation of surface CD63 as well as the release of β-hexosaminidase, but did not block upregulation of CD203c. Moreover, as assessed by 3H-thymidine uptake, NVP-BEZ235 was found to inhibit the growth of the BA leukemia cell line KU812 (IC_{50}: 0.01-0.05 μM) and the KIT D816V+ MC leukemia cell line HMC-1 (IC50: 0.005-0.01 μM). Apoptosis-inducing effects of NVP-BEZ235 were examined by light microscopy and AnnexinV-staining and were also observed in both cell lines, although the concentrations required to induce clear effects were substantially higher compared to effects on proliferation (effective range: >0.5 μM). In summary, NVP-BEZ235 inhibits spontaneous growth of neoplastic BA and MC as well as IgE-dependent activation and mediator secretion in normal mature BA and MC. Whether these effects of NVP-BEZ235 have clinical implications remains at present unknown.

0185

EFFECT OF THE NON-PEPTIDE THROMBOPOIETIN RECEPTOR AGONIST ELTROMBOPAG (PROMACTA® /REVOLADETM) ON BONE MARROW CELLS FROM PATIENTS WITH ACUTE MYELOID LEUKEMIA AND MYELODYSPLASTIC SYNDROMES

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Disease-related or chemotherapy-induced thrombocytopenia is a frequent symptom and clinical challenge in patients with myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML). Recently, the novel non-peptide Thrombopoietin receptor agonist Eltrombopag (Promacta®/ Revolade™) was approved for treatment of patients with chronic idiopathic thrombocytopenic purpura (ITP) in the US. Eltrombopag has been shown to effectively elevate platelet numbers and to reduce thrombocytopenia-related complications. Therefore, Eltrombopag might be a new option to treat thrombocytopenia in patients with MDS or AML, provided that it does not stimulate malignant hematopoiesis in these diseases. We therefore studied the effects of Eltrombopag on bone marrow mononuclear cells (BM-MNC) of patients with AML and MDS. BM-MNC of 10 patients with AML/MDS (5 AML, 3 AML with previous history of MDS, 2 MDS (RAEB-II) and 5 healthy controls were exposed to different concentrations of Eltrombopag (0.1-30 ug/mL). We assessed cell growth, colony formation (suspension culture, colony-forming assays), proliferation (Bromodeoxyuridine (BrdU) incorporation assays), apoptosis (Annexin-V assays), and differentiation (cytomorphology, FACS analysis of differentiation markers). Long-term malignant self-renewal was tested *in vitro* by serial replating assays in semi-solid methylcellulose medium. Eltrombopag effectively stimulated megakaryocytic colony formation of BM-MNC in semisolid methylcellulose assays, resulting in 80% increased colony numbers (compared to 100 ng/mL TPO as a positive control [100%]) at concentrations of 1-3 ug/mL. A preferential effect on early megakaryocyte-erythrocyte progenitors (MEP) and immature megakaryocytic (Mk) progenitors was seen, while more mature MK progenitors were less affected. In contrast, malignant BM-MNC of patients with AML/MDS did not show increased proliferation, neither in suspension culture, nor in methylcellulose assays at any of the examined concentrations of Eltrombopag. In fact, while cell numbers of two patients were unchanged we observed a decrease of numbers of malignant cells in 8 of 10 patients after two weeks Eltrombopag treatment. However, due to interindividual variation, this inhibition (mean: 56%, SD: 28%, at 3 ug/mL Eltrombopag) was statistically not significant. In line with these observations we could not detect increased proliferation utilizing BrdU incorporation assays, nor significantly decreased apoptosis (Annexin-V assays) in the malignant cells of any of the patients upon Eltrombopag treatment. We neither found evidence for an increase of more immature cells or blasts when we examined cells morphologically and by FACS for surface marker expression (CD34, CD33). Utilizing serial replating assays at a concentration of 3 ug/mL Eltrombopag, we did not observe long-term malignant self-renewal of AML/MDS BM-MNC in any of the samples (4 replatings). *in vivo* studies of the effect of Eltrombopag on AML/MDS BM-MNC in a murine xenotransplantation model (NOD-SCID IL2Rgamma null) are currently ongoing. In conclusion, utilizing a variety of assays, we did not find any evidence that Eltrombopag stimulates malignant growth from BM-MNC from patients with AML/MDS. These results provide a preclinical rationale for the use of Eltrombopag in future clinical trials in AML/MDS.

0186

FETAL HEMOGLOBIN MODULATION BY NOVEL ALKYLATING AGENTS IN HUMAN ERYTHROID CELLS; SYNERGISTIC EFFECTS WITH ESTABLISHED HBF-INDUCING AGENTS

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Pharmacological induction of fetal hemoglobin (HbF) is beneficial for some patients with β-thalassemia and ameliorates the severity of pain episodes in sickle-cell anemia, mainly by hydroxyurea (HU). However, refractoriness or poor response of some patients treated with HU triggered research for other drugs. In the present study, we evaluated the effects of novel steroidal alkylating agents EA80(0.1-1 μM), XK4(0.1-30 μM) and CS-211(0.1-30 μM) alone or in combination with HU, hemin (HE) and butyric acid (BU) on HbF induction in $CD34^+$ cell cultures from normal donors. HbF cells were determined by Flow-cytometry. α/β/γ-globin mRNA transcripts were evaluated by Real-Time RT-PCR. Continuous exposure of $CD34^+$ to EA80 had a dose(up to 0.4 μM)- and time-dependent effect on cell number (measured by trypan blue) and on gamma-globin mRNA level; EA80(0.4 μM) caused a time-dependent increase in γ-globin mRNA (1-3 days:1.5 to 2.0-fold) as well as 1.5- and 2.4-fold increase in benzidine-positive and HbF-cells, respectively. The addition of HU, HE and BU combined with EA80 in $CD34^+$ cell cultures led to a 1.3, 3.0 and 2.0-fold increase in cell number at day 3, respectively. Furthermore, the combination of EA80 with either HU or HE was accompanied by an increase in γ-mRNA content (2.5 and 3.0-fold, respectively), whereas the addition of BU in combination with EA80 caused the predominant increase in γ-mRNA levels (5.0-fold at day 3); the addition of HE and BU increased HbF-cell number (3.0 and 2.0-fold, respectively). XK4 at any concentration and duration proved toxic. The third drug, CS-211, had a dose(up to 5 μM)- and time-dependent effect on cell number and γ-globin mRNA transcripts (highest effect at day 3, 3.8-fold at 5 μM). The addition of HU, HE and BU together with CS-211(5 μM) led to a 2.9, 3.7 and 2.9-fold increase in cell number at day 3, respectively. The combination of HU and CS-211 caused a 3.2-fold increase in γ-mRNA transcripts, whereas CS-211 combined with BU resulted in 3.0-fold decrease in the γ-mRNA levels. In the contrary, the combined effect of HE and CS-211 led to an extraordinary-(13.3-fold)-increase in γ-mRNA content and a 4.2-fold increase in HbF-cells. The level of both adult globin mRNAs was not affected by any of the tested drugs. The simultaneous addition of EA80 and CS-211(5 and 10 μM) in the $CD34^+$ cell cultures showed their synergistic effect in cell proliferation. As for their effect in γ-mRNA levels, it arises that CS-211(5 μM) enhances EA80 effect in increasing γ-mRNA transcription, whereas CS-211(10 μM) has a more synergistic effect with EA80 promoting γ-mRNA in quite higher levels; this is support by the high number of HbF-cells. Our findings suggest that the beneficial effect of EA80 and CS-211 might be threefold: i)increasing cell number and Hb-F cells ii)affecting preferentially the rate of transcription of γ-globin mRNA, iii)acting synergistically with HE, HU and BU, most likely through transcriptional and posttranscriptional mechanisms. These results indicate that novel alkylating agents EA80 and CS-211, either alone or in combination with other HbF-augmenting drugs, might provide a potentially useful treatment for patients with β-hemoglobinopathies with poor or no response to established Hb-F inducing agents.

0187

LENALIDOMIDE IN ELDERLY PATIENTS WITH MANTLE CELL LYMPHOMA

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Background. Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin's lymphoma characterized by a low response rate and a short progression-free survival (PFS) after conventional therapy. In refractory or relapsed MCL, and for elderly patients, non-eligible for intensive treatments, the prognosis is even worsen. Several novel therapeutic approaches have been proposed, including monoclonal antibodies, radioimmunotherapy, proteasome inhibitors, m-TOR inhibitors and immunomodulatory drugs (Thalidomide and Lenalidomide). It has been recognized that stromal cells provide important stimuli for the survival of malignant B-cells. Immunomodulatory drugs interact with the microenvironment, altering cytokines secretion and expression of adhe-

sion molecules, which can result in apoptosis of malignant B-cells. *Aims.* The aim of this study was to evaluate the efficacy and the adverse events of Lenalidomide, as treatment of mantle cell lymphoma within elderly patients, non-eligible for intensive treatments. *Methods.* Seven patients, with median age of 78 years (72-86 years), non-eligible for intensive chemotherapies, were treated with Lenalidomide. It was administrated, as single agent in 5 patients, associated with Dexamethasone or Rituximab in one patient each. Five of seven patients were in relapsed disease and two were in first line of treatment. We evaluated the response to Lenalidomide, the time to response and the presence of adverse events. *Results.* All seven patients presented a response to the treatment with Lenalidomide. We obtained one complete remission (CR), one unconfirmed complete remission (uCR) and five partial remissions (PR), with two minor responses. The median time to response was 5 months (1-12 months). The tolerance of the treatment was good. We noticed moderate hematological toxicity, essentially neutropenia and thrombopenia. Only two patients, with partial remission, presented an evolution of the lymphoma. *Conclusions.* MCL represents a distinct clinico-pathologic entity among the non-Hodgkin's lymphomas, characterized by a short PFS. For the elderly patients, non-eligible for intensive treatments, Lenalidomide as a single agent, or in combination with Rituximab or Dexamethasone, proves to be an important therapeutically agent. We obtained one complete remission, one uCR and 3 partial remissions and two minor responses. The tolerance of the treatment was good, with only moderate hematological toxicity.

0188

BINDING OF THE RECOMBINANT POLYCLONAL ANTI-D PRODUCT ROZROLIMUPAB (SYM001) AND PLASMA DERIVED ANTI-D TO RHD VARIANTS

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Rozrolimupab is the first in a new class of recombinant polyclonal antibody products and is under clinical development for the prevention of hemolytic disease of the newborn by anti-D prophylaxis (ADP) and for the treatment of Immune Thrombocytopenic Purpura (ITP). Rozrolimupab is composed of 25 genetically unique IgG1 antibodies all specific for the Rhesus D erythrocyte protein and derived by phage display from the antibody repertoires of 8 female human anti-RhD plasma donors enrolled in a Danish anti-RhD collection program. The 25 antibodies have been selected with the aim to obtain a recombinant anti-D product that mimics the D-specific polyclonal antibody repertoire of the plasma derived anti-D products but with the advantage of being devoid of antibodies irrelevant for the believed mechanism of action of anti-D products in ADP and ITP. Also, a recombinant product does not carry the risk of transmission of blood borne pathogens and can be produced independent of plasma donor availability. The individual antibodies in rozrolimupab have been shown to collectively recognize a broad panel of RhD variants present in populations of different ethnic origin and that may be of clinical importance in ADP. In order to confirm that this polyclonality has been adequately maintained in the final product, the binding of 7 batches of rozrolimupab to a similar panel of genetically defined RhD erythrocyte variants was analyzed and compared to that of two marketed plasma derived products. For this purpose the low-ionic strength saline (LISS) indirect antiglobulin test (IAT) was applied with the approach to give an estimate of titers of antibodies needed in order to induce agglutination with an exhaustive panel of D variants including 3 weak D types, DIV, DV, 3 DVI types, DVII, DFR, R0Har, DOL, DBT and DHMi. Also the binding under more physiological buffer conditions, mimicking the *in vivo* situation, to 4 RhD variants (DIII, DIV, DVI and DVII) was compared applying flow cytometry. The results show that polyclonality is maintained in rozrolimupab in a reproducible manner and that rozrolimupab and two commercially available plasma derived products are comparable in terms of binding to RhD variants when tested both at non-physiological and physiological buffer conditions. Thus, in regards to the anti-D antibody repertoire and coverage of RhD variants rozrolimupab constitutes a potential alternative to plasma derived anti-D in prevention of hemolytic disease of the newborn and treatment of ITP.

0189

CD200, A CELL SURFACE PROTEIN WITH IMMUNOREGULATORY FUNCTION, IS A NEW POTENTIAL TARGET FOR HEMATOLOGIC MALIGNANCIES IMMUNOTHERAPY

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Background. CD200 is a transmembrane glycoprotein with immunoregulatory function expressed on several tissues in rats and humans. Through its interaction with CD200 receptor, CD200 seems to switch cytokine production from a TH1 to a TH2 pattern, thus reducing T-cell mediated cytotoxic response and promoting tumor escape and growth. CD200 is also a target for a novel blocking humanized monoclonal antibody (Anti-CD200 MAb) and interesting results have been obtained in *in vivo* assays testing Anti-CD200 MAb with CD200 positive Burkitt lymphoma cell lines. Expression of CD200 has been already described in chronic lymphocytic leukemia/small lymphocyte lymphoma (CLL/SLL) and CD200 positivity is known as an unfavorable prognostic factor in multiple myeloma (MM) and in acute myeloid leukemia (AML). *Aim.* Starting from this data we performed a comprehensive cytometric study on a large number of hematologic neoplasms to identify specific diseases in witch Anti-CD200 MAb could be considered as a therapeutic option and to explore the possibility to use CD200 as a diagnostic tool in clinical cytometry. *Methods.* Between February 2007 and December 2008, using six-color flow cytometry, we analyzed CD200 expression in 269 samples: 185 bone marrow aspirates (BM), 58 peripheral blood specimens (PB) and 26 fine needle aspiration cytology samples (FNAC). 160 were lymphoproliferative disorders, 21 MM, 20 myelodysplastic syndromes (MDS) and 68 acute leukemias. CD200 expression was evaluated by using CD200-PE-conjugated antibody. CD200 positivity was assigned to every single case when CD200 mean fluorescence intensity (MFI) was higher then 256 arbitrary units, a channel close to the cut-off point between positive and negative cells, in our experience. *Results.* All results concerning our analysis are showed in the Table 1.

Table 1.

Disease	N	Positive N (%)	PPC (mean)	PPC (25°-75° percentile)	MFI (median)	MFI (25°-75° percentile)
AML	50	29 (58)	32.9	4-51	290	134-591
APL	4	0 (0)	1.7	1-3	35	11-126
MDS	20	13 (65)	28.3	2-44	458	140-1003
AHL	5	4 (80)	60.2	27-87	420	238-1194
ALL	9	8 (88)	64.2	57-84	1099	369-1532
CLL/SLL	73	73 (100)	98.4	98-99	3004	2051-3590
LPL	8	8 (100)	58.2	40-66	536	326-839
MCL	13	6 (46)	33.5	2-71	69	20-751
FL	15	1 (6)	8.3	2-8	46	30-83
DLBCL	19	7 (37)	19.7	1-15	68	30-279
MZL	14	9 (64)	39.4	2-74	344	35-1017
HCL	10	6 (100)	95.6	92-99	3016	1382-5430
MM	21	20 (95)	61.3	31-88	2491	1227-5764
T-NHL	8	3 (37)	26	1-63	39	23-964

CLL/SLL and hairy cell leukemia (HCL) displayed a constant positivity for CD200 with a high level of MFI. Lymphoplasmacytic lymphoma (LPL) was positive in 100% of cases but with lower MFI and lower percent of positive cells (PPC) as compared to CLL/SLL and HCL ($p<0.001$). MM/MGUS and B-cell acute lymphoblastic leukemia (B-ALL) showed CD200 positivity in, respectively, 96% and 86% of cases. CD200 was also expressed in cases of marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), T-non Hodgkin lymphoma (T-NHL), AML, acute hybrid leukemia (AHL) and T-cell acute lymphoblastic leukemia (T-ALL). Only one case of follicular lymphoma (FL) was found to be CD200 positive. In AML we also found a significant inverse correlation with myeloperoxidase (MPO-7) (Spearman's r=-0.5; $p<0.001$). No case of acute promyelocytic leukemia (APL) was positive for CD200. *Conclusions.* CD200 is candidate as a new specific target for immunotherapy with anti-CD200 in all cases of CLL/SLL, B-ALL and HCL as well as in selected cases of MZL, DLBCL, MCL, LPL, MM, T-NHL, AML, AHL and T-ALL.

0190

SUCCESSFUL TREATMENT WITH BEVACIZUMAB IN POEMS-SYNDROME

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Background. POEMS syndrome (acronym for polyneuropathy, organomegaly, endocrinopathy, M protein, skin changes) is a rare, paraneoplastic disorder associated with an underlying plasma cell disorder. In addition to other symptoms that are not included in the acronym, a markedly elevated level of vascular endothelial growth factor (VEGF) is usually confirmatory and might account for some of the symptoms (A Dispenzieri, Blood Reviews, 2007, 21:285-299). Since therapeutic VEGF targeting is available, 7 cases of bevacizumab treated patients have been reported describing controversial results (4 patients had a successful and 3 a fatal outcome). *Aim.* To assess response and tolerance of treatment with bevacizumab following radiotherapy and cyclophosphamide in 2 newly diagnosed patients with POEMS- syndrome. *Methods.* After local irradiation of the solitary bone lesions, high dose cyclophosphamide (5 g/m^2) prior to stem cell collection was administered, followed by 5 (patient 1) and 4 (patient 2) cycles respectively of bevacizumab (5 mg/kg BW). *Results.* Patient 1 is a 44 years old woman who was diagnosed with POEMS syndrome in May 2005. Beside other typical symptoms, she was affected by a rapid progressive, peripheral sensory-motor neuropathy on both legs and arms which rendered her wheelchair bound. VEGF level was 7 times the normal range at diagnosis. Patient 2 is a 57 year old man that first experienced symptoms of chronic progressive, distal, sensory-motor neuropathy in November 2007 making him unable to walk longer distances. In February 2008 diagnosis of POEMS syndrome was established upon other, charactersitic symptoms. VEGF level was 9 times the normal range at diagnosis. Treatment was tolerated very well in both patients without any severe side effects. In patient 1 physical condition improved dramatically after bevacizumab with fully recover of mobility 1 year after end of treatment. In patient 2, already after the first bevacizumab course, nerve conduction studies improved significantly. A few months after end of treatment, the patient reports less pain and better muscular strength with continuous improvement. *Summary and Conclusions.* According to a very limited experience with bevacizumab treatment in POEMS patients it seems that bevacizumab is poorly tolerated at a stage when VEGF levels are extremely high. Taking the published knowledge in account, we compiled a treatment sequence for both patients: after local irradiation, one cycle of high dose cyclophosphamide as conditioning therapy prior to prophylactic stem cell collection was administered. Subsequently the anti-VEGF antibody bevacizumab could be administered safely, since VEGF levels have dropped already significantly prior to anti-VEGF treatment initiation. Even if some, including ours, of the available experiences with bevacizumab in POEMS syndrome are encouraging, we need to learn more about the interactive role of VEGF in this uncommon syndrome in order to select the appropriate patients and to place it proper into sequence with other treatment options.

0191

SLUG AND ERK (P42-44): NEW TARGETS IN CHRONIC MYELOID LEUKEMIA THERAPY

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Background. SLUG (SNAI2) is a Snail-related zinc-finger transcription factor involved in the regulated functions of hematopoietic stem cells downstream of stem cell factor (SCF)/c-KIT ligand. Its aberrant expression has been found in t(17;19) leukemic cells, rhabdomyosarcoma expressing the PAX3-FKHR translocation and breast cancer (where it correlates with the loss of E-cadherin). Besides promoting the transition to a less differentiated phenotype, SLUG provides protection against apoptotic death in response to DNA damage and has been thereby involved in tumor progression towards drug resistance (Kajita M *et al.*, Mol Cell Biol 2004;24:7559-66). Notably, SLUG is required for BCR-ABL leukemogenesis *in vivo*, as it promotes leukemic cell survival and migration into different environments (Pérez_Mancera PA *et al.*, Oncogene 2005;24:3073-92). *Aim.* We investigated the role of SLUG in Imatinib (IM) resistance of BCR-ABL-expressing cells. *Methods.* The critical role of SLUG in BCR-ABL-induced transformation and drug resistance came from experiments with a specific silencing of SLUG through the following siRNA duplex oligoribonucleotides: 1) AUCAGAAUGGGUCUGCAGAUGAGCC; 2) GGCUCAUCUGCAGACCCAUUCUGAU. Fourty eight hrs exposure to this siRNA promoted, in fact, PUMA induction followed by massive apoptosis induction in BCR-ABL-transduced 32D and Ba/F3 cells either IM-sensitive and IM-resistant. Apoptotic cell death was measured by Annexin V and PI labelling of cells in control condition and after treatment with siRNA, IM and PD9805. SLUG overexpression in CML cells, investigated by semiquantitative PCR, may arise from the constitutive activation of mitogen-activated protein kinase (MAPK) pathway. Protein expression and post-translational modifications were investigated by western blotting. *Results.* We found that SLUG is over-expressed in Ba/F3 murine B cell progenitor and 32D cell lines stably transduced with a wild type (wt) p210 BCR-ABL construct. SLUG over-expression was revoked by anti-SLUG oligonucleotides, by PD9805, a specific inhibitor of ERK 1/2 (p42/44 MAPK) and by IM (1 microM for 24 h), supporting its dependence upon the constitutive activity of p210 BCR-ABL and MAPK pathway. SLUG reduction in response to IM had a significant impact on its downstream targets, the pro-apoptotic PUMA (which was reduced) and the anti-apoptotic BCL-xL (which was conversely reduced). Accordingly, it addressed BCR-ABL-expressing cells towards apoptotic death. Ba/F3 and 32D cells expressing BCR-ABL mutations T315I and E255K associated with IM resistance *in vivo* exhibited a further and significant increase of SLUG expression compared to cells expressing the wt BCR-ABL construct. Such further increase arises from transcriptional events. Notably, anti-SLUG oligonucleotides and PD9805 revoked SLUG over-expression and induced apoptotic death also in this drug-resistant context. *Conclusion*. Our results support a role of SLUG in the development of IM resistance in chronic myeloid leukemia (CML), likely contingent upon MAPK activation. SLUG induction might be prevented by dual SRC/ABL inhibitors, that efficiently target BCR-ABL downstream intracellular signals, including p42/44 MAPK (Konig H *et al.*, Cancer Res 2008;68:962433). MEK or MAPK inhibitors might further potentiate the cytotoxic effects of TK inhibitors and be considered for the therapy of drug-resistant CML (La Rosée P *et al.*, Clin Cancer Res 2006;12:6540-6; Nguyen TK *et al.*, Blood 2007;109:4006-15).

0192

CMC-544, AN ANTI-CD22 IMMUNO-CONJUGATE OF CALICHEAMICIN, DECREASES THE LEVEL OF COMPLEMENT INHIBITORY FACTORS ON MALIGNANT B-CELL LYMPHOMA CELLS

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Background. Investigation of complement inhibitory factors (CIF) demonstrated that they play an important role as a regulator of complement-dependent cytotoxicity (CDC) in the treatment of malignant B-cell lymphoma, and that their expression correlated with rituximab resistance. CMC-544, a conjugate of N-acetyl gamma-calicheamicin dimethyl hydrazide and a recombinant humanized antibody (IgG4) directed against the CD22 antigen, has been introduced as a promising agent to treat refractory or resistant B-cell lymphoma (BCL). The additive effects of the CMC-544 have been reported in combination with rituximab recently. However, the mechanism has not been well elucidated. We analyzed the quantitative alteration of CIFs in cell lines as well as on cells obtained from patients with BCL to clarify the underlying mechanisms of the positive concomitant effect of CMC-544 and rituximab. *Methods.* $CD22^+$ cell lines used were Daudi, Raji and Ramos cells, as well as malignant cells obtained from 15 patients with BCL after informed consents. Humanized IgG4 anti-CD22 mAb (G5/44) and NAc-gamma-calicheamicin DMH conjugated antibody (CMC-544) as well as unconjugated NAc-gamma-calicheamicin DMH were kindly provided by Wyeth Research (PA, USA). Rituximab was purchased from Zenyaku Co (Tokyo, Japan). To assess the effect of CMC-544 on CIF, cells were cultured for 2 hours in a medium containing CMC-544, switched to an antibody-free medium, and then examined at various time points thereafter. The levels of CD55 was analyzed by RT-PCR, laser microscopy and flow cytometry. The CDC effect of rituximab was studied in the presence or absence of CMC-544. To determine if CDC is enhanced by the sequential incubation of rituximab and CMC-544, cells were first incubated in a medium containing CMC-544 before the incubation for 12 hours in an antibody-free medium, and then CDC by rituximab was analyzed. *Results.* The level of CD55 significantly reduced ($p<0.001$) after incubation with CMC-544 in the three different methods. It did not change after the incubation with G5/44. The viable cell count significantly decreased after incubation with rituximab via CDC. However, significant effect on CDC was not observed with CMC-544 or G5/44 alone. Furthermore, the CDC caused by rituximab was not enhanced by simultaneous incubation with CMC-544. The CDC effect of rituximab was significantly increased within 12 hours following incubation with CMC-544 ($p<0.001$). The effect was not observed after the incubation with G5/44. *Conclusions.* CIFs are important regulators of CDC in the treatment of BCL. The increase of these molecules has been reportedly related to their resistance to rituximab, and the decrease to their susceptibility to rituximab. In our study, the reduction of CD55 and the enhancement of CDC were shown on BCL cells after incubation with CMC-544. These results support the rationale for the combined use of CMC-544 and rituximab.

Red blood cells and Iron I

0193

EFFICACY AND SAFETY OF DEFERASIROX IN REDUCING MYOCARDIAL SIDEROSIS IN PATIENTS WITH B-THALASSAEMIA MAJOR

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Background. Despite the availability of iron chelation therapy, heart failure secondary to myocardial siderosis remains the main cause of death in regularly transfused patients with β-thalassaemia. Once-daily oral iron chelation therapy with deferasirox effectively reduces body iron burden in patients with transfusion-dependent anaemias, and the removal of myocardial iron has been demonstrated in preclinical and small clinical studies. A substudy of the EPIC trial has also allowed assessment of the efficacy of deferasirox in reducing cardiac iron. *Aim.* To evaluate the efficacy of deferasirox in removing myocardial iron in heavily transfused β-thalassaemia patients with myocardial siderosis. *Methods.* Patients aged ≥10 years with cardiovascular magnetic resonance (CMR) imaging of myocardial T2* >5-<20 ms, 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 were included. Deferasirox was initiated at 30 mg/kg/day and subsequent dose adjustments of 5-10 mg/kg/day were based on changes in SF, month-6 cardiac T2* and safety parameters. The primary endpoint was change in myocardial T2* from baseline to 1 year. *Results.* 114 patients (54 male:60 female; mean age 20.9±7.3 years) were enrolled. Baseline myocardial T2* was <10ms (severe cardiac siderosis) in 47 patients (41%), and 10-20ms (mild-to-moderate cardiac siderosis) in 67 (59%). Patients had received a mean of 185 mL/kg of blood in the previous year, had a mean baseline LIC of 28.2±10.0 mg Fe/g dw and a median SF of 5235ng/mL. 68% had received deferoxamine and 32% deferoxamine/deferiprone combination therapy. Mean deferasirox dose over 1 year was 32.6 mg/kg/day. At 1 year, myocardial T2* improved significantly from baseline (Figure 1); improvement in T2* (>4% increase) was observed in 69.5%; no change in 14.3%; and worsening (>4% decrease) in 16.2%.

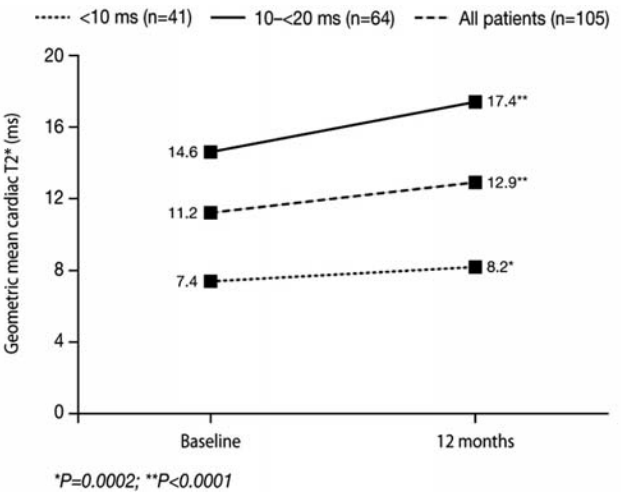

Figure 1. Geometric mean change in cardiac T2* over 12 mths.

LVEF remained stable. Both mean LIC and absolute change of median SF were reduced significantly from baseline by -6.6±9.9 mg Fe/g dw and -1257ng/mL, respectively (p<0.0001). Change from baseline in T2* was inversely correlated with change in LIC (r=-0.2, p=0.03). 105 patients (92.1%) completed treatment; four discontinuations (3.5%) were due to adverse events (AEs) and five (4.4%) for other reasons. No deaths were reported. Investigator-assessed drug-related AEs were reported in 56 patients (49.1%); rash was the most common (n=15; 13.2%). Most drug-related AEs (78.6%) were of mild-to-moderate severity. Two drug-related serious AEs (one nephritis leading to acute renal failure, one renal tubular disorder) were reported that resolved following drug discontinuation. Five patients (4.4%) had a non-progressive increase in serum creatinine >33% above baseline and upper limit of normal (ULN) on two consecutive visits. Two patients (1.8%) had an increase in alanine aminotransferase >10xULN on two consecutive visits; levels were already elevated at baseline in both patients. *Summary and Conclusions.* Deferasirox was effective in removing iron from the heart in β-thalassaemia patients with cardiac siderosis. The statistically significant improvement in myocardial T2* was associated with maintained normal cardiac function, and a concomitant significant decrease in hepatic and total body iron burden.

0194

LABILE PLASMA IRON LEVELS IN HEAVILY IRON-OVERLOADED PATIENTS WITH TRANSFUSION-DEPENDENT ANAEMIAS IN RESPONSE TO DEFERASIROX THERAPY: RESULTS FROM THE LARGE-SCALE, PROSPECTIVE 1-YEAR EPIC TRIAL

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Background. Labile plasma iron (LPI) is a toxic, directly chelatable form of non-transferrin-bound iron produced in conditions of iron overload when levels surpass the binding capacity of transferrin. LPI is taken up in hepatocytes, myocardium and endocrine tissues leading to oxidative tissue damage. Continuous presence of adequate levels of an effective iron chelator in the plasma may bind LPI, ameliorating tissue damage and minimizing iron-related morbidity and mortality. *Aim.* To evaluate the effect of deferasirox on LPI levels in patients with a variety of transfusion-dependent anaemias enrolled in the prospective, 1-year EPIC trial. *Methods.* Patients aged ≥2 years with transfusion-dependent anaemia and serum ferritin (SF) ≥1000 ng/mL, or <1000 ng/mL with a history of multiple transfusions (>20 transfusion episodes or >100 mL/kg of red blood cells) and an R2 MRI-confirmed liver iron concentration >2 mg Fe/g dry weight were enrolled. Deferasirox starting dose was determined based on blood transfusion frequency (10-30 mg/kg/day). Dose adjustments in steps of 5-10 mg/kg/day (in the range 0-40 mg/kg/day) were based on SF trends and safety markers. Blood samples to measure LPI and assess the deferasirox pharmacokinetic profile were taken pre-administration and 2 hours post-administration at baseline, weeks 12, 28 and 52. *Results.* LPI data are available from 1602 patients (825 male, 777 female; mean age 30.4±23.0 years). Underlying anaemias were: β-thalassaemia (n=1029), myelodysplastic syndromes (MDS; n=305), aplastic anaemia (AA; n=104), sickle cell disease (SCD; n=79), rare anaemias (mostly hemolytic in nature) (RA; n=42), and other transfusion-dependent anaemias (n=43). Mean LPI, pre- and post-deferasirox administration, at baseline, weeks 12, 28 and 52 are shown in the Table. Overall mean deferasirox dose during the 1-year treatment period was 22.4±6.0 mg/kg/day (β-thalassaemia, MDS, AA, SCD and RA: 24.3±5.5, 19.3±5.7, 17.8±4.7, 20.2±3.8 and 18.6±5.6 mg/kg/day, respectively). At each time point, peak LPI levels observed just before deferasirox dosing were decreased compared with baseline and were mostly within the normal range from week 12 onwards in each underlying anaemia. A slight increase at 52 weeks was seen in the thalassaemia sub-group: this could be associated with decreased compliance or other reasons, to be investigated further. There was a significant correlation between change from baseline in pre-dose LPI and changes from baseline in SF (data previously presented) in patients with β-thalassaemia and SCD (p=0.03 for both).[1] In all patients, mean steady-state deferasirox plasma concentration (ie pre-administration value) at weeks 12, 28 and 52 was 32.9, 35.9 and 39.4 μmol/L, respectively. At each time point this was approximately half the concentration value observed at 2 hours post-administration (84.4, 89.3 and 97.0 μmmol/L, respectively). *Summary and Conclusions.* These results confirm the ability of once-daily deferasirox to achieve sustained reductions in toxic LPI levels to within the normal range across various transfusion-dependent anaemias, supporting previous data in β-thalassaemia and MDS patients.[2,3] This is likely due to trough levels of deferasirox being within the therapeutic range, thereby preventing LPI levels rebounding between doses. These data support the proposal that deferasirox therapy may prevent end-organ damage through ablation of toxic LPI in these patients.

Table 1. Mean LPI, pre and post deferasirox administration, at baseline and after repeat doses.

	Baseline		Week 12		Week 28		Week 52	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
All patients	0.89 ±1.84	0.33 ±1.59	0.30 ±0.90	0.11 ±0.56	0.27 ±0.87	0.11 ±0.62	0.47 ±1.35	0.30 ±1.33
β-thalassemia	1.25 ±2.34	0.58 ±2.10	0.40 ±1.07	0.15 ±0.67	0.34 ±0.94	0.13 ±0.72	0.60 ±1.55	0.39 ±1.52
MDS	0.53 ±0.63	0.02 ±0.11	0.08 ±0.28	0.02 ±0.10	0.15 ±0.88	0.03 ±0.18	0.14 ±0.32	0.02 ±0.07
AA	0.23 ±0.34	0.01 ±0.02	0.08 ±0.20	0.01 ±0.04	0.06 ±0.20	0.01 ±0.03	0.04 ±0.10	0.03 ±0.10
SCD	0.11 ±0.54	0.04 ±0.29	0.09 ±0.42	0.06 ±0.31	0.11 ±0.28	0.14 ±0.46	0.10 ±0.21	0.07 ±0.19
RA	0.50 ±0.55	0.01 ±0.04	0.05 ±0.13	0.01 ±0.03	0.06 ±0.18	0.01 ±0.03	0.29 ±0.52	0.02 ±0.03

NOTE: Data are mean LPI ± SD (μmol/L); normal levels are 0–0.40 μmol/L

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0195

THE EFFICACY OF IRON CHELATOR REGIMES IN REDUCING CARDIAC AND HEPATIC IRON IN PATIENTS WITH THALASSAEMIA MAJOR: A CLINICAL OBSERVATIONAL STUDY

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Background. Available iron chelation regimes in thalassaemia may achieve different changes in cardiac and hepatic iron as assessed by MRI. *Aims.* To assess the four available iron chelator regimes with respect to their efficacy in reducing cardiac and hepatic iron. *Methods.* Patients attending the centre are transfused at approximately 2 weekly intervals and have access to iron chelation either with desferrioxamine (DFO), deferiprone (DFP) since the year 2000, combination of desferrioxamine and deferiprone (Comb) and since 2006 deferasirox (DFX). Since the start of 2001, patients have had MRI of the heart and liver to assess the degree of iron load in those organs. Patients who had at least two MRI were included in the study. The rate of change of cardiac and hepatic MRI were assessed according to the chelation regime that they received for the greatest period of time between each set of two MRI measurements. Only the first two MRIs that were at least 12 months apart while on each prescribed regime were included in the analysis. The MRI T2* results were converted to both Cardiac Iron Concentration (CIC) and Liver Iron Concentration (LIC), because of the lack of linearity between tissue iron and T2* (both with methods of Wood *et al.*). Compliance and dose of medications were not taken into account for the purposes of this study as the aim was to observe expected clinical outcomes according to prescription of the chelation regime. *Results.* 232 patients had more than one MRI with a total of 464 studies. Table 1 shows the mean estimated change in CIC mg/gdw (Δ CIC) according to severity of cardiac siderosis. Similarly, Table 2 shows the mean estimated change in LIC mg/gdw (Δ LIC). In summary, for the heart, Comb significantly reduced cardiac iron at all levels of iron loading while DFP did so in the mild and moderate groups. DFO and DFX did not show a significant reduction in cardiac iron in patients with cardiac iron load. In the liver, Comb resulted in the most rapid decline at all levels of iron load and DFX achieved

significant falls in the moderate and heavily loaded patients. DFO and DFP maintained hepatic iron levels. *Discussion and Conclusions.* The outcomes of this retrospective study conform to expected outcomes based on other studies but that were usually not comparative between regimes. The advantages of this study were that it was from a large clinic with homogeneous care and does compare outcomes of the different chelation regimes. With the knowledge of the efficacy of the different available regimes and the specific iron load in the heart and the liver, appropriate tailoring of chelation therapy should allow clearance of iron. These outcomes may be useful to physicians as to the chelation they should prescribe according to the levels of iron load found in the heart and liver by MRI and should contribute to the reduction in morbidity and mortality associated with iron load.

Table 1. Mean estimated+ change in CIC mg/gdw (ΔCIC) according to severity of cardiac siderosis between first two MRIs (Initial interval analysis - minimum interval 12 months).

		Overall			Stratified								
					Heavy			Moderate			Mild		
Regime	Time* (years)	n	ΔCIC	p	n	ΔCIC	p	n	ΔCIC	p	n	ΔCIC	p
DFO	1.83	32	-0.23	0.43	9	-1.3	0.028	16	0.16	0.71	7	+0.27	0.95
DFP	1.80	26	-0.64	0.001	7	-0.57	0.49	13	-0.82	0.002	6	-0.36	0.027
Comb	1.80	88	-0.78	<0.001	36	-1.12	0.002	30	-0.69	<0.001	22	-0.41	<0.001
DFX	1.33	16	+0.66	0.10	6	+1.43	0.24	4	+0.73	0.46	6	-0.15	0.17

*Time= mean time (in years) between MRI studies.
†Wilcoxon matched-pairs signed rank test

Table 2. Mean estimated+ change in LIC mg/gdw (ΔLIC) according to severity of hepatic siderosis between first two MRIs (minimum interval 12 months).

		Overall			Stratified								
					Heavy			Moderate			Mild		
Regime	Time* (years)	n	ΔLIC	P	n	ΔLIC	p	n	ΔLIC	p	n	ΔLIC	p
DFO	2.0	36	+1.34	0.095	5	+2.31	0.68	11	+1.64	0.21	20	+0.94	0.27
DFP	1.9	14	-6.2	0.068	3	n.a.‡	n.a.	6	-0.97	0.3	5	+2.68	0.5
Comb	1.8	99	-4.19	<0.001	25	-9.18	0.003	45	-3.27	<0.001	29	-1.33	0.004
DFX	1.3	53	-2.80	0.005	21	-5.38	0.042	16	-2.08	0.042	16	-0.14	0.35

*Time= mean time (in years) between MRI studies.
†Wilcoxon matched-pairs signed rank test
‡Numbers too small for analysis

0196

INCREASED PLASMA LEVELS OF NGAL AND IL-18 CORRELATE WITH REDUCED GFR PROVIDING EVIDENCE OF TUBULOGLOMERULAR DEFECT IN PATIENTS WITH HBS/BETA-THALASSEMIA

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Background. Promising novel biomarkers for acute kidney injury (AKI) or chronic kidney injury (CKI) include a plasma levels of cystatin C, Neutrophil Gelatinase-associated Lipocalin (NGAL) and Interleukin-18 (IL-18) and urinary levels of NGAL, IL-18 and Kidney Injury Molecule-1 (KIM-1). These biochemical indices have been reported to be useful for evaluating the duration and severity of kidney disease and for predicting progression and adverse clinical outcomes. Furthermore, these biomarkers have given promising results in differentiating the various causes of AKI or CKI. Progressive renal failure is one of the main complications in HbS/β-thalassemia (HbS/β-thal). Early identification of patients at high risk of developing renal failure is of great importance as it may allow specific measures to delay the progression of renal damage and thus to reduce the incidence of end-stage renal failure and mortality. *Aims.*To explored the activation of tubuloglomerular feedback in patients with HbS/β-thal. *Methods.* Fifty-eight adult patients with HbS/beta-thal (age 42±17years, males/females 20/58) were included in the study. We determined the above mentioned parameters, along with the more classical ones such us β2-microglobulin and N-acetyl-β-D-glucosaminidase (NAG) in blood and urine using standard methodology. GFR values were calculated according to the recently proposed cystatin C-based prediction equation using only each concentration in mg/L: GFR [mL/min/1.73m(exp)2] = 76.7 x Cystatin C(exp)-1.18. *Results.* We found that: a) impairment of GFR in 21/58 patients (36%); b) increased plasma concentrations of NGAL and IL-18 in 34/58 (58%) and 58/58 (100%) patients, respectively; c) increased and/or detectable urine concentrations of NGAL and IL-18 in 54/58 (93%) and 46/58 (79%) patients, respectively; d) significant negative correlations between GFR and plasma NGAL and IL-18 (r=-0.735, $p<0.0001$ and r=-0.350, $p<0.007$, respectively) and e) significant positive correlations between cystatin C and urine NGAL and IL-18 (binomial, $p<0.005$ and r=0.412, $p<0.03$, respectively). *Conclusions.* These findings suggest that tubuloglomerular feedback is activated in almost all studied patients with HbS/β-thalassemia. Measurements of plasma and urine NGAL and IL-18 contribute significantly to the early detection and risk stratification of renal disease in these patients. However, prior to their clinical usefulness, these biomarkers must undergo through rigorous validation in multiple cohorts.

0197

ANALYSIS OF IRON HOMEOSTASIS AND ERYTHROID ACTIVITY IN A COHORT OF LOW-RISK MYELODYSPLASTIC PATIENTS

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Background. The small liver peptide hepcidin is the principal regulator of iron homeostasis in humans through the binding and the subsequent degradation of ferroportin both in enterocytes and macrophages. A broad range of different molecules are known to influence hepcidin levels in different conditions. Inflammation, through Interleukin-6 production and iron overload through BMP activation increase hepcidin expression while iron deficiency and hypoxia have opposite effects. Growth differentiation factor 15 (GDF15), a member of the transforming growth factor-β family, has been described as a potential hepcidin inhibitor in clinical conditions characterized by ineffective erythropoiesis such as thalasssemia and congenital dyserythropoietic anemia type 1. Little is known about iron regulation in myelodysplastic syndromes (MDS) in which anemia is a common clinical feature and a high degree of ineffective erythropoiesis coexist with chronic inflammation or iron overload leading to opposite effects on hepcidin production. *Aims.*to evaluate iron parameters and markers of erythroid activity in a cohort of low-risk MDS patients at diagnosis or never transfused. *Methods.* 47 serum samples were collected from 40 low-risk MDS patients after written informed consent. WHO diagnosis was as follows: 17 refractory anaemia, 6 unclassifiable MDS, 10 refractory anaemia with blast excess, 2 refractory anaemia with ringed sideroblasts (RARS), 5 refractory cytopenia with multilineage dysplasia. In 7 out of 40 patients, samples were analyzed before and after 4 months of EPO treatment. Serum GFD15 evaluation was performed by ELISA. Serum hepcidin evalution was performed by SELDI-TOF-MS technique. The results were compared to 30 matched healthy controls and 30 transfusion-dependent thalassemic patients. *Results.* Median transferrin saturation was 28% (range 4-76) , median serum ferritin levels were 558,06 ng/mL (range 58-1959), median haemoglobin value was 11,7 g/dL (range 7.5-14.6). Serum GDF15 values in MDS patients with erythroid dysplasia were significantly increased compared to healthy controls (median value 2537 pg/mL versus 206, $p<0.001$ by Mann-Whitney test) and significantly lower than thalassemic patients. No difference was found between GDF15 values of MDS patients without erythroid dysplasia and healthy subjects. Hepcidin median value was 7,045 nM/L (range 0.55-41.54) compared to a range of 3-7 nM/L in controls. In patients with erythroid dysplasia hepcidin median value was 10.44 nM/L but was very low in RARS patients. Regression analysis showed a statistically significant correlation between sTFR and either GDF15 or hepcidin levels. We failed to found any significant correlation between GDF15 and hepcidin values and also between hepcidin and ferritin, haemoglobin and EPO values. In patients who reached a positive erythroid response after EPO treatment we detected a significant reduction in GDF15 values and a parallel increase in hepcidin levels. *Summary and Conclusions.* Serum GDF15 and hepcidin are both increased in a cohort of low-risk MDS patients with erythroid dysplasia but not in cases with only megakaryocytic or myeloid dysplasia. GDF15 is confirmed a marker of ineffective erythropoiesis also in MDS patients but in our series did not show significant correlation

with hepcidin levels probably due to the heterogeneity of the cohort. Furthermore both variables appear to be modulated by a successful EPO treatment.

0198

MYOCARDIAL LESIONS AND IRON DEPOSITS AFTER TREATMENT WITH ANTHRACYCLINES

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The involvement of iron in anthracycline-induced cardiotoxicity is suggested by several experimental models but there is no available data of iron role in clinical studies. Only a few postmortem series have attempted to characterize the cardiac injury induced by anthracyclines. However, these studies are previous to those supporting the involvement of iron in the pathogenesis of anthracycline cardiotoxicity, and therefore they did not evaluate the relationship between deposits of iron and myocardial damage. *Aim.* The aim of this work was to describe the myocardial lesions induced by anthracyclines in necropsies obtained from cancer patients and determine the relationship between myocardial lesions and cardiovascular risk factors, clinical cardiac events, and cardiac iron deposition. *Methods.* We retrospectively studied 97 clinical necropsies from patients with a solid or haematological tumour treated in the Hematology and Oncology Department between 1996 and 2005. Inclusion criteria were histologically confirmed diagnosis of cancer, availability of complete clinical record, and archived heart and liver histological tissue. Clinical, histopathology, and iron deposition studies were performed. All cardiac and liver samples were examined by light microscopy. Heart and liver iron concentration (iron mg/g of dry tissue) was determined in all cases in which formalin fixed tissue was available for atomic absorption spectroscopy. Statistical analysis was performed using SPSS program version 15.0 and SNPStats software. *Results.* 97 patients were included in the study. The median age for all patients was 56 years (range, 5-83 years). 48 (49%) patients had received treatment with anthracyclines, 24 (25%) with chemotherapy without anthracyclines, and the other 25 (26%) had not been treated with chemotherapy. The median dose of anthracycline was 281 mg/m^2 (range 36-639). We found a statistical association of anthracycline treatment with myocytolysis (29/48 vs. 10/49, p=0.001), with interstitial fibrosis (30/48 vs. 19/49, p=0.09), and with patches of necrosis (9/4 vs 0/49, p=0.001). Nuclear hypertrophy was associated with higher doses of anthracyclines (301 vs 90.8 mg/m^2, p=0.016). Conversely, myocardial lesions were not correlated either with cardiovascular risk factors (sex, age, hypertension, diabetes, dyslipemia, alcohol, tobacco, thoracic radiotherapy), or previous cardiac disease. Congestive heart failure was significantly more frequent in patients with anthracycline treatment (7/48 vs. 2/49, p=0.03), whereas no relationship was found between clinical cardiac events and histopathologic lesions. A cumulative anthracycline dose greater than 200 mg/m^2 was associated with a higher iron concentration in heart tissue (0.49 vs. 0.24 mg/gr; T-Student, p=0.01). Myocytolysis, interstitial fibrosis and patches of necrosis were associated with higher iron stores, but the differences were not significant. History of transfusions (p=0.32) and systemic iron deposits were not related to heart iron load. *Conclusions.* Our data shows that anthracycline treatment is associated with myocitolysis, interstitial fibrosis, and patches of necrosis. Also, anthracycline treatment induces a specific heart iron overload independent of systemic iron load which is in accordance with previously published experimental data

0199

ASSESSMENT OF SAFETY IN PATIENTS RECEIVING LONGER-TERM IRON CHELATION THERAPY WITH DEFERASIROX WHO HAD ACHIEVED SERUM FERRITIN LEVELS OF <1000 NG/ML DURING THE STUDY COURSE

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Background. A target serum ferritin (SF) value was not specified in study protocols in the deferasirox clinical trial program, but was determined by the individual investigators. A large proportion of patients achieved SF levels below 1000 ng/mL, a threshold that is known to be associated with improved survival and a reduced risk of iron-overload-related complications (eg heart failure) in patients with thalassaemia. As the use of deferoxamine (DFO) has been associated with increased toxicity at low SF, assessment of the adverse event (AE) profile of patients enrolled in deferasirox studies has been performed in the subgroup of patients that achieved low SF levels. *Aim.* To evaluate the safety of deferasirox in a cohort of adult and paediatric patients with transfusion-dependent anaemias and iron overload from two large clinical trials (0107 and 0108) whose SF levels fell to <1000 ng/mL during treatment with deferasirox. *Methods.* In the 1-year core studies, frequently-transfused patients ≥2 years of age with a range of chronic anaemias received deferasirox 5-30 mg/kg/day. Eligible patients were subsequently enrolled in 4-year extension trials, where dose was adjusted based on SF levels. Patients eligible for this analysis had baseline SF of ≥1000 ng/mL but achieved a SF level of <1000 ng/mL on ≥2 consecutive visits any time after starting deferasirox therapy. The number of days when SF was <1000 ng/mL was calculated for each patient. AEs in these patients were determined for the entire treatment period and for the period following the first SF measurement of <1000 ng/mL, irrespective of future SF levels. *Results.* 474 patients were included in this analysis: underlying anaemias were β-thalassaemia (n=379), myelodysplastic syndromes (n=43), Diamond-Blackfan anaemia (n=30) and other anaemias (n=22). The percentage of patients achieving a SF <1000 ng/mL by year of treatment were as follows: 13.5% in year 1, 18.6% in year 2, 25.7% in year 3, 32.5% in year 4 and 36.7% in year 5 to date. Overall 174 patients (36.7%) reached a SF level of <1000 ng/mL at ≥2 consecutive visits, while SF levels remained ≥1000 ng/mL in 300 patients. The median duration of SF levels <1000 ng/mL was 149 days (range 18-1726). Patient demographics, baseline characteristics and safety profiles are shown in the Table 1, demonstrating a comparable safety profile in the two groups. In addition, the incidence of drug-related AEs (gastrointestinal, renal and liver) did not appear to increase during the periods after SF levels first decreased below 1000 ng/mL. *Summary and Conclusions.* The safety profile of patients achieving low SF levels during the core and extension study phases was comparable to those who did not, suggesting that when appropriately dosed, iron-overloaded patients can be safely chelated to SF levels of <1000 ng/mL. The observed lack of increase in the proportion of patients with creatinine increases >33% above baseline and ULN or with ALTs >10xULN is of particular note.

Table 1. Demographics, baseline characteristics and safety profile of patients who achieved SF levels <1000 ng/mL and patients who did not.

	Patients who achieved SF <1000 ng/mL	Patients who did not achieve SF <1000 ng/mL
n	174	300
Male:female	85:89	145:155
Mean age ± SD, years	23.8 ± 16.7	23.5 ± 18.2
<16, n (%)	65 (37.4)	123 (41.0)
≥16, n (%)	109 (62.6)	177 (59.0)
Enrolled from study 107:108	120:54	175:125
Median exposure to deferasirox, months	56.3	45.2
Mean actual deferasirox dose, mg/kg/day	20.3	22.9
Median baseline SF (range), ng/mL	1791 (321–11453)	2883 (762–13943)
Drug-related AEs* (≥5% in either group), n (%)		
Nausea	26 (14.9)	38 (12.7)
Diarrhoea	17 (9.8)	42 (14.0)
Vomiting	14 (8.0)	25 (8.3)
Abdominal pain	12 (6.9)	32 (10.7)
Upper abdominal pain	6 (3.4)	20 (6.7)
Rash	9 (5.2)	16 (5.3)
Audiological abnormalities	7 (4.0)	4 (1.3)
Ophthalmological abnormalities	4 (2.3)	5 (1.7)
Two consecutive SCr increases >33% above baseline and above ULN	26 (14.9)	36 (12.0)
Increase in ALT >10xULN on at least one visit	12 (6.9)	20 (6.7)
Baseline levels elevated	6 (3.4)	16 (5.3)

*Investigator-assessed; SCr, serum creatinine; ULN, upper limit of normal; ALT, alanine aminotransferase

0200

SAFETY AND EFFICACY OF IRON CHELATION THERAPY WITH DEFERASIROX IN PATIENTS WITH SICKLE CELL DISEASE: 3.5-YEAR FOLLOW-UP

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Background. Patients with sickle cell disease (SCD) require repeated blood transfusions to manage complications, inevitably resulting in the development of chronic iron overload and the need for lifelong iron chelation therapy. Controlled studies of the long-term effects of iron chelation therapy in patients with SCD are scarce, but an ongoing extension phase of a 1-year deferasirox Phase II study is designed to provide up to 5-years' treatment data. *Aim.* To evaluate the cumulative 3.5-year safety and efficacy of deferasirox in patients with SCD and transfusional iron overload enroled in a 4-year extension to a 1-year comparative study. *Methods.* A 1-year comparative study has demonstrated that once-daily oral therapy with deferasirox was well tolerated and produced similar (dose-dependent) reductions in liver iron concentration (LIC) to deferoxamine (DFO) in SCD patients with iron overload. Eligible patients subsequently entered a 4-year extension phase and received deferasirox only; dose adjustments were based on monthly serum ferritin (SF) levels and safety assessments (adverse events [AEs] and laboratory parameters). Patients with abnormal renal function were excluded. *Results.* Data from 132 patients (mean age 19.3±10.7 years) entered the extension phase. The median duration of exposure to deferasirox was 37.4 months (3.1 years) at a mean dose of 18.4±6.2 mg/kg/day. Mean daily dose of deferasirox increased from 15.4±6.9 mg/kg/day at month 1 to 22.3±7.3 mg/kg/day at month 42. Mean iron intake over this period was 0.3±0.1 mg/kg/day. 72 patients (54.5%) continue to receive deferasirox. Reasons for discontinuation include: consent withdrawal (n=24, 18.2%), AEs (n=11, 8.3%), lost to follow-up (n=9, 6.8%), unsatisfactory therapeutic effect (n=4, 3.0%), and other reasons (n=11, 8.3%). One death was reported (post-liver transplantation, unrelated to study drug). The most frequent drug-related (investigator-assessed) AEs were nausea (n=20; 15.2%), diarrhoea (n=14; 10.6%), vomiting (n=8; 6.1%) and abdominal pain (n=6; 4.5%). The annual frequency of drug-related AEs generally decreased from year to year (Table). Nine patients (6.8%) had two consecutive increases in serum creatinine that were both >33% above baseline and above the upper limit of normal (ULN); however, there were no progressive increases. Five patients (3.8%) had an increase in alanine aminotransferase >10xULN on at least one visit; baseline levels were already >ULN in two patients. Median SF levels decreased from a baseline level of 3439 ng/mL (n=132) by 651 ng/mL (p=0.0533, Wilcoxon signed rank test; n=49). SF decreases were dose-dependent. *Summary and Conclusions.* Over 3.5 years of deferasirox treatment showed no evidence of progressive increases in serum creatinine in patients with SCD, who have a tendency to develop progressive renal disease. The incidence of drug-related AEs decreased after the first year. Deferasirox provided continued reduction in SF over the course of the study.

Table. Most common (>4% overall) drug-related AEs, by year of deferasirox treatment.

AE	Frequency, n (%)	Incidence			
		Year 1	Year 2	Year 3	Year 3.5
Nausea	20 (15.2)	17 (12.9)	2 (1.6)	–	1 (0.8)
Diarrhoea	14 (10.6)	13 (9.9)	–	1 (0.8)	–
Vomiting	8 (6.1)	7 (5.3)	1 (0.8)		–
Abdominal pain	6 (4.5)	5 (3.8)	–	1 (0.8)	–

0201

DEFERASIROX IN PAEDIATRIC PATIENTS WITH THALASSAEMIA MAJOR: 1-YEAR RESULTS FROM A LARGE PAEDIATRIC COHORT ENROLLED IN THE PROSPECTIVE, MULTICENTRE EPIC STUDY

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Background. Chronic anaemias including thalassaemia major (TM) are often diagnosed during infancy, requiring long-term red blood cell (RBC) transfusions. Iron overload can therefore occur as early as 2 years of age, hence the need to initiate effective and safe iron chelation therapy early in life to avoid significant clinical sequelae. The multicenter deferasirox EPIC trial, the largest conducted for an iron chelator, enrolled the largest cohort of paediatric patients ≥2 years in a single prospective study. *Aims.* To investigate the efficacy and safety of fixed starting doses of deferasirox according to transfusional iron intake, with subsequent dose titration in paediatric patients with TM. *Methods.* Patients enrolled in EPIC were ≥2 years with transfusion-dependent anaemias and serum ferritin (SF) ≥1000 ng/mL, or <1000 ng/mL with a history of multiple transfusions (>20 transfusions or >100 mL/kg of RBCs), and R2 MRI-confirmed liver iron concentration of >2 mg Fe/g dry weight. Deferasirox starting dose was 20 mg/kg/day for patients receiving 2-4 blood units/month. An initial dose of 10 or 30 mg/kg/day was considered for patients receiving less or more frequent blood transfusions, respectively. Protocol-specified dose adjustments of 5-10 mg/kg/day (range 0-40 mg/kg/day) were performed every 3 months based on SF trends and safety markers. The primary efficacy endpoint was change in SF from baseline at 1 year. Data from paediatric patients (aged ≥2-<16 years) with TM are presented. *Results.* 421 patients (234 male:187 female; mean age 8.8 [range 2-15] years) aged 2-<6 (n=118), 6-<12 (n=164) and 12-<16 (n=139) years were enroled. A mean 15.8 transfusions were received in the previous year and patients had been receiving transfusions for a mean 7.7 years. 85.5% had received prior chelation therapy for a mean 5.3 years: deferoxamine (66.5%), deferiprone (1.2%), deferoxamine/deferiprone combination (17.8%). 385 patients (91.4%) started on deferasirox 20 mg/kg/day or less. 343 patients (81.5%) received dose adjustments during the study, most commonly due to insufficient response (50.8%). The average actual deferasirox dose received was 23.8±5.2 mg/kg/day, with a final dose ≥30 mg/kg/day in 55% of patients. 405 patients (96.2%) completed 1 year; main reasons (>2 patients) for discontinuation were adverse events (AEs, n=6; 1.4%), consent withdrawal (n=3; 0.7%) and abnormal laboratory values (n=4; 1.0%). Drug-related serious AEs were reported in four patients (1.0%; Fanconi syndrome [n=1], gastric haemorrhage [n=1], rash [n=2]) and no deaths occurred. The most common (>2%) drug-related (investigator-assessed) AEs were rash (n=34; 10.4%) and abdominal pain (n=10; 2.3%). Nine patients (2.1%) had serum creatinine values >33% above baseline and the upper limit of normal (ULN) on two consecutive visits; there were no progressive increases. Four patients (1.0%) had increases in alanine aminotransferase >10xULN on two consecutive visits; levels were already elevated in three patients.

Table 1. Reduction in SF by dose cohort.

	<20 mg/kg/day (n=85)	≥20–<30 mg/kg/day (n=299)	≥30 mg/kg/day (n=35)	All patients (n=419)
Median SF at baseline	2317	3197	5093	3122
Median SF at end of study	2385	3282	3945	3181
Absolute change in SF	101	36.5	-1036	19
P-value	0.6318	0.7849	0.0295	0.5604
Mean iron intake (mg/kg/day)	0.48	0.56	0.44	0.53

Summary and Conclusions. Data suggest that in heavily transfused TM

paediatric patients, a higher starting deferasirox dose and/or prompt dose escalation provides more optimal control of iron burden. Deferasirox was generally well tolerated with a very low discontinuation rate and a safety profile consistent with previously reported data in paediatric patients.

0202

DELETION IN THE LDLR1 & 2 DOMAINS IN THE TMPRSS6 GENE IN A CHILD CAUSES IRIDA WITH EXTREMELY HIGH HEPCIDIN LEVEL

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Background. Matriptase-2 (Tmprss6), a type II transmembrane serine protease, plays an essential role in iron homeostasis as an inhibitor of hepcidine. Cases with mutations in both Tmprss6 genes have already been described and lead to severe microcytic anemia with iron deficiency state refractory to iron treatment (IRIDA). Aims and case report: We describe the first case of IRIDA in Switzerland and report our studies concerning the identification of the mutations in matriptase-2 gene. The propositus is a male child born at Bern in 2005. At 12 months laboratory analyses showed: Hb 77 g/L; MCV 51 fl; iron 2 µmol/L, transferrin saturation 3% and ferritin 49 µg/L. WBC and PLT count were normal. He received oral and i.v. iron with Hb 85 g/L at day 35 post treatment. Bone marrow showed iron in macrophages but not in erythroblasts; karyotype analysis and search for infection were negative. Subsequent infusion of fresh frozen plasma and iron in a month period raised Hb to 101 g/L. This effect was transitory although ferritin increased to 310 µg/L. CBC as well as iron status of both parents was normal. On June 2008 we infused 5×4 hours 25 mg/day of Venofer®. Within 3 weeks, Hb and RBCs increased from 70-103 g/L and 4.7-6.0×10^{12}/L, respectively. *Methods.* Hepcidine was dosed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry; TMPRSS6 exons were screened by PCR amplification of genomic DNA and sequenced using ABI 3130XL; HLA typing was performed by PCR-SSO. *Results.* Serum hepcidine was 30.7, <1.4, <1.4 and 2.2 ng/mL (propositus, and three healthy controls matched for the age). Sequencing of TMPRSS6 exons revealed a novel missense mutation in exon 8 (Ser304Leu), in heterozygosity in the propositus and the mother but absent in 200 control chromosomes as verified by RFLP analysis. Interestingly, the father was apparently homozygous for SNPs in exon 12 which were not present in his son. Paternity was confirmed by HLA typing. A deletion contributed by the father was therefore suspected and confirmed by PCR amplification. The deletion breakpoints were identified in exons 12 and 13 eliminating 1054 nucleotides and resulting in an in-frame deletion of 30 residues in LDLR1 and LDLR2 domains. *Conclusions.* In children of the same age as the propositus, serum hepcidin is low (<5 ng/mL with our method) most probably reflecting increased iron needs in that period of development. The hepcidin level (30.7 ng/mL) found in the propositus suggested matriptase-2 dysfunction, confirmed by DNA analysis. In fact a 30 amino acid deletion, most likely abolishing matriptase-2 function, and a missense mutation, Ser304Leu, affecting a residue conserved in all species studied so far were found in the propositus. As very little iron is absorbed or released from macrophages in such a situation, iron was given as continuous i.v. infusions to saturate serum transferrine. Obtained Hb value support this new therapeutic approach.

0203

OUTCOMES OF PATIENTS WITH SICKLE CELL DISEASE ADMITTED TO THE INTENSIVE THERAPY UNIT – A SINGLE INSTITUTION EXPERIENCE

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Background. King's College Hospital has a cohort of 500 adult patients with sickle cell disease. The commonest reason for hospitalisation is acute pain that can precipitate other more serious complications requiring intensive care. *Aims.* To examine the reasons for admission to the intensive therapy unit (ITU) in sickle patients, and to look at patients' outcome. *Methods.* The study includes adult patients (over 16 years old) over an eight year period, 1 January 2000 to 31 December 2007. We matched the King's database of general ITU admissions with the King's sickle database, to create a list of sickle-ITU admissions. Information on each patient was documented from the two databases, plus the hospital's electronic patient record system and each patient's medical notes. We collected information on the patient's past medical history, as well as specific information on that ITU admission (indication for admission, length of stay, organ failure, support required). *Results.* For the period studied, there were 46 admissions (19 male, 27 female) of 38 unique patients (14 male (12 HbSS and 2 HbSC), 24 female (20 HbSS and 4 HbSC)) to general ITU at King's. Out of the 46 admissions, 16 were to the high dependency unit, including all seven elective post-operative patients. The median age on admission to ITU was 31 years (range 17 to 59 years). The reasons for admission were categorised as "medical" (31.69%), "acute surgical" (4.9%), "trauma" (3.7%) or "planned post-operative" (7.15%). The "medical" reasons were further sub-classified as multi-organ failure (8), acute chest syndrome (14), renal (1), neurological (4) or other (4). The median stay in ITU was 3 days (range 1 to 68 days). Of 46 admissions, 37 survived to be discharged from ITU (i.e. 19.6% mortality), and 36 survived to hospital discharge. At one year post-ITU discharge, there were a further three deaths, bringing the total one year mortality to 28.2%, with none lost to follow up. Although a surprisingly high number of patients were genotypically HbSC (6 of 38), two of these admissions were for trauma, one was planned post-operatively, and only three were "medical" (two patients post cardiac arrest, one chest sepsis). Re-admission was common: during the 8 year period considered, for the 38 unique patients, six had re-admissions (one re-admitted four times and five twice), yielding a 16% risk of ITU re-admission. The organ support required by "medical" patients (typically with a reason for admission directly related to sickle cell disease) included: 48% receiving inotropes, 20% requiring ventilation, 16% requiring haemofiltration, 24% requiring transfusion and 76% requiring antibiotics. Many of the patients had pre-existing multi-organ damage, often sickle-related. *Conclusions*. Compared to a within-ITU mortality of 17.6% during the same period, in the same institution, sickle cell patients who are admitted to ITU have a similar mortality. Relatively more female patients (59% of total admissions) were admitted to ITU, contributing risk factors were gynaecological. Admission to ITU itself, is a significant risk factor for re-admission for sickle patients, possible causes are discussed.

0204

TREATMENT WITH DEFERASIROX EFFECTIVELY DECREASES IRON BURDEN IN PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Thalassemia intermedia encompasses a wide clinical spectrum of β-thalassemia phenotypes. Iron overload is frequently present in patients with thalassemia intermedia, and becomes evident mainly after the second and third decades of life. A variable rate of iron loading, reaching toxic levels in some patients, was seen in a series of intermittently transfused patients with β-thalassemia intermedia. Some patients were at risk of the toxic effects of iron overload, necessitating adequate chelation therapy. *Aim.* The aim of this study was to investigate the efficacy and safety of the once-daily oral iron chelator, deferasirox, in iron-overloaded patients with thalassemia intermedia. *Methods.* We evaluated 11 adult patients with thalassemia intermedia (5M/6F; mean age 41.2±6.5 years) who had serum ferritin levels >1000 ng/mL and who were sporadically transfused with <20 units of red blood cells before starting deferasirox treatment for up to 12 months. Nine and two patients were initially treated with deferasirox at 10 and 20 mg/kg/day, respectively, (mean initial dose 11.8±4.1 mg/kg/day). After 3 months, dose adjustments (increases) were allowed in increments of 5 mg/kg/day every 3 months as required to reduce markers of iron overload. Total iron burden was monitored by measuring serum ferritin levels before and monthly after starting deferasirox, while liver iron concentration and cardiac iron burden were measured by magnetic resonance imaging (MRI) T2 and T2* parameters at baseline and 12 months after deferasirox treatment. Left ventricular ejection fraction (LVEF) by MRI, and 24-hour proteinurea (Prot 24h) before and after treatment, were also measured. Hemoglobin (Hb) levels, serum creatinine, cystatin C, alanine (ALT) and aspartate aminotransferase (AST) were measured before and every month during deferasirox treatment. *Results.* The mean serum ferritin level was significantly reduced after 12 months of deferasirox treatment, while the mean baseline liver T2 and T2* significantly increased following 12 months of therapy (Table 1). Mean cardiac T2* and LVEF were normal at baseline and did not significantly change after 12 months of treatment. There were also no significant changes in mean serum creatinine, cystatin C, Hb or Prot 24h levels after 12 months of deferasirox treatment, while mean ALT and AST levels significantly decreased over 12 months. *Summary and Conclusions.* These data indicate that over 12 months deferasirox significantly reduced liver iron burden and serum ferritin levels in these iron-

overloaded patients with thalassemia intermedia. The decreases in ALT and AST are suggestive of an improvement in liver function. This study indicates that deferasirox provides effective iron chelation therapy in these patients without any significant adverse effects.

Table 1.

	Baseline	6 months	12 months
Serum ferritin, ng/mL	2030 ± 1340	1881 ± 1283 (*P*=0.23)	1165 ± 684 (*P*=0.02)
Liver T2, ms	20.1 ± 4.1	NM	23.7 ± 6.2 (*P*=0.01)
Liver T2*, ms	3.4 ± 3.0	NM	4.4 ± 3.0 (*P*=0.02)
Cardiac T2*, ms	38.9 ± 5.9	NM	39.8 ± 4.5 (*P*=0.64)
LVEF, %	66.3 ± 8.1	NM	66.9 ± 7.9 (*P*=0.76)
AST, U/L	64.8 ± 29.6	52.4 ± 21.7 (*P*=0.06)	42.5 ± 18.1 (*P*=0.04)
ALT, U/L	63.5 ± 29.5	53.7 ± 29.8 (*P*=0.12)	36.5 ± 17.6 (*P*=0.02)
Hb, g/dL	8.2 ± 1.9	8.3 ± 2.0 (*P*=0.75)	8.3 ± 1.9 (*P*=0.90)
Serum creatinine, mg/dL	0.67 ± 0.15	0.69 ± 0.15 (*P*=0.64)	0.75 ± 0.19 (*P*=0.07)
Cystatin C, mg/L	0.98 ± 0.23	1.13 ± 0.24 (*P*=0.12)	1.13 ± 0.27 (*P*=0.094)
Prot 24h, mg/24h	194.9 ± 104.7	NM	208.8 ± 160.8 (*P*=0.34)

NM, value not measured

0205

ACUTE SPLENIC SEQUESTRATION IN SICKLE CELL DISEASE

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Background. Acute splenic sequestration (ASS) is a life threatening event in sickle cell disease (SCD) during early childhood. Subsequent related mortality has been greatly reduced by neonatal screening enabling parents to be taught to recognize pallor and, if possible, to feel sudden enlargement of the spleen indicating splenic sequestration and consequently a need for immediate hospital attendance for blood transfusion. *Aims.* Our aim was to update spleen sequestration epidemiology in a French cohort followed after neonatal screening. *Methods.* We reviewed the medical files of SCD children born between 2000 and 2007, followed since birth in 5 pediatric centres. We included all SCD children who had presented acute splenic sequestration, defined as an acutely enlarging spleen with a fall in hemoglobin (Hb) level of at least 2 g/dL. *Results.* A total of 266 episodes of ASS occured in120 children, 69 boys and 51 girls. Genotypes were as follows: 9 S β° thalassemia, 2 SD-Punjab, 4 SC, 105 SS. In the SS subgroup, median age at first episode was 16 months [1-83] and 34.8% of these first episodes occurred before 12 months of age. Associated symptoms were found in 37 cases (isolated fever n=20, vasoocclusive crisis n=5, identified viral or bacterial infection n= 12) and did not influence the rate of recurrence. Mean Hb level during first ASS was 5.2 g/dL [3.3-7]. Fifty-nine % of SS patients had more than one episode. Mean ages at first episode of those who experienced only one episode (n=43) versus those who had more than one episode (n=62) were statistically different (28±22 months versus 18±10 months, p=0.002, Student test). Median interval between first and second episode was 3.5 months [1-31]; 31 patients had 3 or more episodes with a mean interval between the second and the third episode of 2 months [1-17]. There was no significant difference in the mean age at first episode in children who experienced 2, 3 or more than 3 episodes. There was only one fatal case due to acute anemia which occurred in a girl at first recurrence (Hb: 2.2 g/dL). After the second attack 14(22.5%) patients were splenectomized, 26 patients (41.9%) were started on a transfusion program (followed in 13 cases by splenectomy) and 21 remained in a standard care programme. Splenectomy was performed at a median age of 4.5 years. *Conclusions.* These results show that ASS remains a major concern in the management of SCD children and predictive factors need to be determined. We show a high rate of recurrence (59%) and that young age at the first episode favours this risk. This study confirms the efficacy of early diagnosis and parental education in the decrease of mortality. Prospective studies on spleen dysfunction are warranted.

0206

IRON CHELATION THERAPY WITH DEFERASIROX FOR TRANSFUSIONAL IRON-OVERLOADED PATIENTS WITH APLASTIC ANAEMIA AND OTHER RARE ANAEMIAS

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Background. Few studies have reported the efficacy of iron chelation therapy in transfusion-dependent patients with aplastic anaemia (AA) and other rare anaemias (RA). The deferasirox EPIC study included subsets of anaemia patients rarely evaluated in iron chelation therapy-related trials. *Aim.* To assess the efficacy of deferasirox over 1 year in reducing body iron in patients with AA and other RAs. *Methods.* Patients aged ≥2 years with transfusional iron overload indicated by serum ferritin (SF) levels of ≥1000 ng/mL, or patients with SF of <1000 ng/mL but with a history of multiple transfusions (>20 transfusions or 100 mL/kg of blood), and liver iron concentration >2 mg Fe/g dry weight confirmed by R2 MRI were enrolled. Initial deferasirox dose was 10-30 mg/kg/day depending on transfusion requirements. Dose adjustments in steps of 5-10 mg/kg/day (range 0-40 mg/kg/day) were based on SF trends and safety markers. The primary efficacy endpoint was change in SF from baseline at 12 months. Safety assessments included adverse event (AE) monitoring and assessment of laboratory parameters. *Results.* Data from 116 AA and 43 RA patients (red cell aplasia [n=20], hemolytic anaemia [n=11], pyruvate kinase deficiency anaemia [n=5], autoimmune hemolytic anaemia [n=3], acute porphyria [n=2], congenital anaemia [n=1], hereditary hemolytic anaemia [n=1], and thrombocytopenic purpura [n=1]) are reported. Transfusion requirements in the year prior to enrolment were 115.8 and 153.0 mL/kg of blood for AA and RA patients, respectively. 68.1% of AA and 30.2% of RA patients were also chelation-naive. In AA patients, after 12 months, median SF decreased significantly by 964.0 ng/mL (p=0.0003) at an average actual deferasirox dose of 17.6±4.8 mg/kg/day. In RA patients, median SF significantly decreased (-832.0 ng/mL, p=0.0275) at an average actual deferasirox dose of 18.6±5.6 mg/kg/day. Reductions in SF were observed in both groups at an actual mean dose of deferasirox ≥20-<30 and <20 mg/kg/day (Table 1). Overall, 76% (n=88) of AA and 70% (n=30) of RA patients completed the study. Five AA patients (4%) died (pneumonia [n=1], sepsis [n=3] and hepatic adenoma rupture [n=1]). Two deaths were recorded in RA patients (acute respiratory insufficiency and bladder tumour). No death was suspected by investigators to be drug-related. The most common (>10%) investigator assessed drug-related AEs in AA patients were: nausea (n=26, 22%), diarrhoea (n=18, 16%) and rash (n=13, 11%); in RA patients: diarrhoea (n=13, 30%), nausea (n=9, 21%), and abdominal pain (n=6, 14%). AEs were generally mild to moderate. 29 AA patients (25.0%; n=10 receiving concomitant cyclosporine) and eight RA patients (18.6%) had an increase in serum creatinine >33% above baseline and the upper limit of normal (ULN) on two consecutive visits; there were no progressive increases. Increase in alanine aminotransferase >10xULN on two consecutive visits occurred in one AA (0.9%) and one RA patient (2.3%); both had elevated levels at baseline. *Summary and Conclusions.* Deferasirox significantly reduced iron burden in transfusional iron-overloaded patients with AA and other RAs. Despite high iron burden at baseline, many patients had no prior chelation therapy, indicating a need for treatment. Deferasirox was generally well tolerated.

Table 1. Median change from baseline in SF (ng/mL) by average actual dose.

			Baseline		End of study		
Aplastic anemia	Average actual dose categories (mg/kg/day)	Mean iron intake ± SD, mg/kg/day	n	Median SF	n	Median change from baseline in SF	*P*-value versus baseline
	<20	0.21 ± 0.18	75	3263.0	75	−970.0	<0.0001
	≥20–<30	0.31 ± 0.20	41	3238.0	40	−883.8	0.2777
	All patients	0.25 ± 0.19	116	3254.0	115	−964.0	0.0003
Rare anemias	Average actual dose categories (mg/kg/day)	Mean iron intake ± SD, mg/kg/day	n	Median SF	n	Median change from baseline in SF	*P*-value versus baseline
	<20	0.43 ± 0.55	25*	2571.7	25	−846.0	0.0174
	≥20–<30	0.56 ± 0.86	17	4247.7	17	−770.5	0.4452
	All patients	0.49 ± 0.70	42*	3161.0	42	−832.0	0.0275

0207

A CLOSER LOOK AT CELLULAR IRON METABOLISM IN IRP2 DEFICIENT ERYTHROBLASTS

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Background. Developing red blood cells are the major consumers of body iron which is indispensable for the enormous production of heme for hemoglobin synthesis. The uptake of iron occurs via binding of iron-loaded transferrin to its cognate receptor (TfR). Thereafter the iron is shuttled to the mitochondria where it is incorporated into protoporphyrin IX to form heme. Excess iron is enclosed within the iron storage protein ferritin. Coordinated control between iron uptake and storage is mainly achieved by the post-transcriptional regulation of TfR1 and ferritin synthesis by the iron regulatory proteins IRP1 and IRP2. Recently, two groups independently created mice lacking either IRP1 or IRP2 and showed that only IRP2 deficient mice developed microcytic hypochromic anemia. Both groups observed a reduction in TfR1 protein expression levels in the developing red blood cells of IRP2 knockout animals and suggested that the decrease in receptor levels is responsible for the development of anemia. *Objective.* Aim of the study was a more detailed analysis of how the loss of IRP2 expression influences iron metabolism and hemoglobinization during terminal erythroid differentiation leading to microcytic hypochromic anemia. *Methods.* We isolated CFU-E-like erythroid cells from mouse fetal liver of wild type, IRP1 and IRP2 knock out animals. *in vitro* cultivation of these primary erythroid cells and their synchronous induction for differentiation allowed us to study their cellular iron metabolism at different time points. We analyzed the extent of hemoglobinization and cell size as well as the expression of ferritin and TfR1 during various stages of erythroid differentiation in IRP1, IRP2 and wild type cells. *Results.* In agreement with the published phenotype of microcytic hypochromic anemia, only erythroblasts lacking IRP2 exhibited a reduction in hemoglobinization and showed a significant increase in ferritin protein levels before and after induction of differentiation. In contrast, TfR1 protein expression levels on the cell surface were significantly decreased in IRP2 deficient cells until 24h of differentiation, but converged with those of wild type cells at 48h of differentiation at the time point at which hemoglobinization is fully in progress. Moreover, measurement of 59Fe uptake and its cellular distribution showed that there is significantly more 59Fe located in cytosolic ferritin of IRP2 knock out cells at all time points compared to their wild type counterpart. *Conclusion.* In summary, these results suggest that not only the reduced expression of TfR1, but also the up-regulation of ferritin, play important roles in the development of anemic phenotype in IRP2 knock out mice.

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0208

ASSESSMENT OF NON-TRANSFERRIN BOUND IRON IN THALASSEMIC SERA USING A NEW FLUORESCENCE BASED ASSAY

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Background. Transfusional iron overload associated with thalassemia leads to the appearance of non-transferrin bound iron (NTBI) in blood which is toxic and causes morbidity and mortality via tissue damage. A number of methods have already been described for quantification of NTBI based on chelation of iron. According to one method, NTBI is mobilized by a low affinity iron chelator, followed by its separation from serum proteins. The chelated fraction is then analyzed by HPLC1, or atomic absorption spectrometry. The second group of methods mobilizes and detects NTBI in the same reaction mixture without separation of the serum proteins from chelated iron using iron-sensitive fluorescence probes, such as fluorescence-labeled apotransferrin2. A limitation in these, is the interference from serum color and turbidity and thereby may not be discerning, particularly at low NTBI levels. In general large variations have been observed in measured values between different methods. *Aims.* Hence a highly sensitive and accurate assay of NTBI, with broad clinical application in both diagnosis and validation of treatment regimens for iron overload is important. Main focus of this study is on the application of bacterial siderophore as an analytical tool for assessing the free iron levels in biological fluids. *Methods.* The patient population consisted of 42 males and 21 females ranging between 2-25 years of age and suffering from β- thalassemia major. The protocol for the study included phlebotomy only and was approved by the Ethics Committee of All India Institute of Medical Sciences, New Delhi, India. Informed consent was obtained from all participating patients. Serum was separated within 1 hr of collection. Siderophore azotobactin with high affinity to Fe^{3+} has been used as the probe. The assay includes blocking of native apotransferrin iron binding sites, mobilization of NTBI, ultrafiltration of all serum proteins and finally addition of the probe molecule which also has a chromophore that fluoresces at 490 nm (Figure 1A). Binding of Fe^{3+} to azotobactin quenches the fluorescence in a concentration dependent manner (Figure 1B).

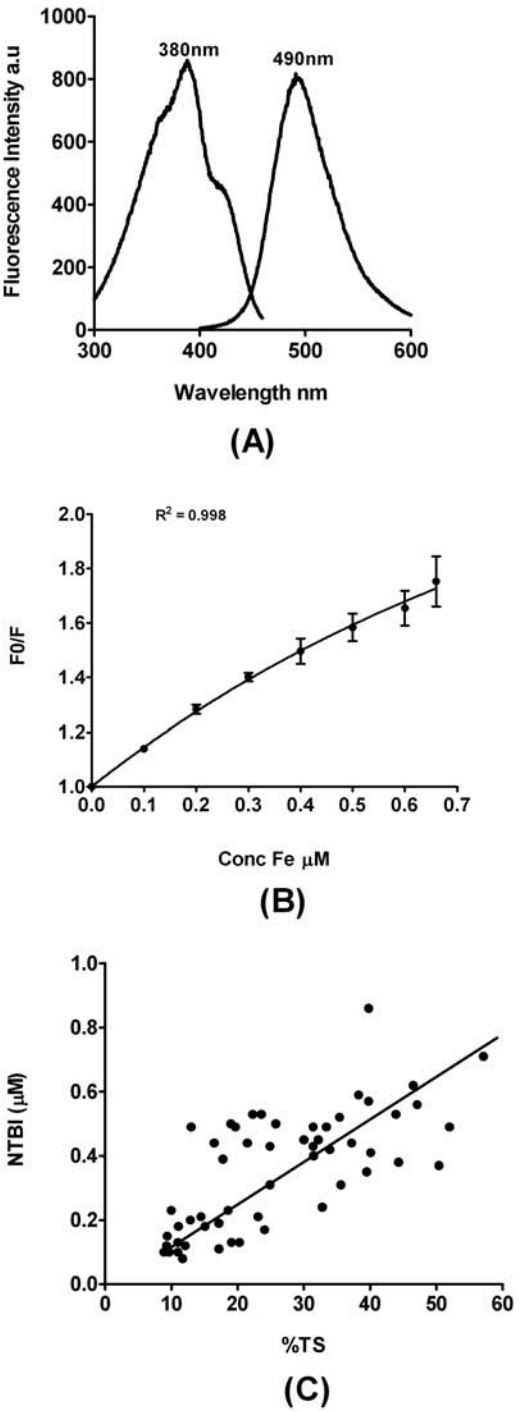

Figure 1. Measurement of NTBI and its correlations

Results. The present assay employs an intrinsically fluorescent bacterial siderophore, azotobactin, for sensing NTBI . The novelty lies in its being an iron chelator itself, along with the presence of the fluorescent chromophore as part of the molecule. Measured NTBI levels in 63 sera ranged from 0.07-3.24 µM (0.375±0.028 µM; mean ± SD). It correlates well with serum iron and percent transferrin saturation but not serum ferritin. Pearson's correlation coefficient was found to be 0.6074 (p<0.0001; Figure 1C) and 0.6102 (p<0.0001) for %TS and serum iron respectively. NTBI is reported to be present at concentrations varying between 1-10 µM3. The low values may be due to the patients being under regular chelation therapy even prior to sampling, indicating that the method is sensitive to very low levels of NTBI , allowing a detection limit much lower than the available methods. *Conclusions.* A simple, highly sensitive and elegant assay has been developed for accurate estimation of NTBI, not only in severe iron overload cases but also in cases where low to moderate levels of NTBI exist, when the patient is put on chelation therapy.

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0209

DEFERASIROX SIGNIFICANTLY REDUCES IRON BURDEN IN HEAVILY IRON-OVERLOADED PATIENTS WITH β-THALASSAEMIA: 2.7 YEAR RESULTS FROM THE ESCALATOR STUDY

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Background. The ESCALATOR study, evaluating deferasirox in heavily iron-overloaded β-thalassaemia major patients previously unsuccessfully chelated, showed that 76% of patients required dose increases at a median of 24 weeks within the 1-year core phase, thus suggesting that the effect of deferasirox on iron burden at 52 weeks may have been suboptimal due to insufficient dosing. An extension phase, which has now been completed, assessed at least 1 additional year of treatment at more optimal dose levels. *Aim.* To evaluate deferasirox efficacy and safety in patients entering the extension phase of the ESCALATOR study. *Methods.* All patients initially received deferasirox 20mg/kg/day (except three receiving 10 mg/kg/day). Deferasirox dose at the start of extension depended on the last dose received in the core trial. Dose adjustments, performed in steps of 5-10 mg/kg/day were based on serum ferritin (SF) levels and safety markers; dose increases above 30 mg/kg were permitted due to a protocol amendment in the extension phase. *Results.* Data presented here are based on 233 patients (162 paediatric [≥2-<16 years] and 71 adult [≥16 years]) who entered the extension study, excluding patients from site 602. 94% of patients completed the 1.7-year extension phase; reasons for withdrawal were: lost to follow-up (n=8, 4%), adverse events (AEs) [n=3, 1%], death (n=3 [cerebral haemorrhage, subarachnoid haemorrhage, respiratory failure], 1%), protocol violation (n=2, 1%), and consent withdrawal (n=1, <1%). Patients received deferasirox for a median of 140 weeks (2.7 years). Dose increases were performed in 137/233 patients (59%) during the extension, with increases to above 30 mg/kg/day in 112. Dose decreases and temporary treatment interruptions due to achievement of target SF level (″500 ng/mL x2 consecutive occasions) occurred in 5 and 15 patients, respectively. Mean liver iron concentration (LIC) and median SF at baseline, 1 year and end of study are shown in the Figure 1. At core baseline, 23 patients (9.9%) had LIC <7 mg Fe/g dw (4.5±1.7) and 210 had LIC ≥7 mg Fe/g dw (21.2±8.2). After 1 year's treatment the proportion of patients with LIC <7mg Fe/g dw had risen to 26.2%, increasing further to 44.4% after a median of 2.7 years. Safety analysis is based on 231 patients who received at least 1 dose of deferasirox during the extension phase (two patients did not receive the drug as their SF remained below 500 ng/mL throughout the study). High doses of deferasirox were well tolerated; drug-related AEs were lower in the extension than core phase (24% vs. 44%). Dose was decreased in six patients due to AEs or abnormal lab values. No progressive changes in markers of renal or liver function markers were seen. At end of study, left ventricular ejection fraction increased from baseline (65.2±6.8%) by 2.3±8.6% (p<0.0007). *Summary and Conclusions.* With optimal dosing, deferasirox therapy for a median of 2.7 years resulted in a significant reduction in LIC and SF in heavily iron-overloaded β-thalassaemia patients. More patients were able to achieve LIC <7 mg Fe/g dw with a longer course of deferasirox treatment. Despite dose increases to ≥30mg/kg/day in many patients, the overall safety was maintained with a low discontinuation rate in the extension phase.

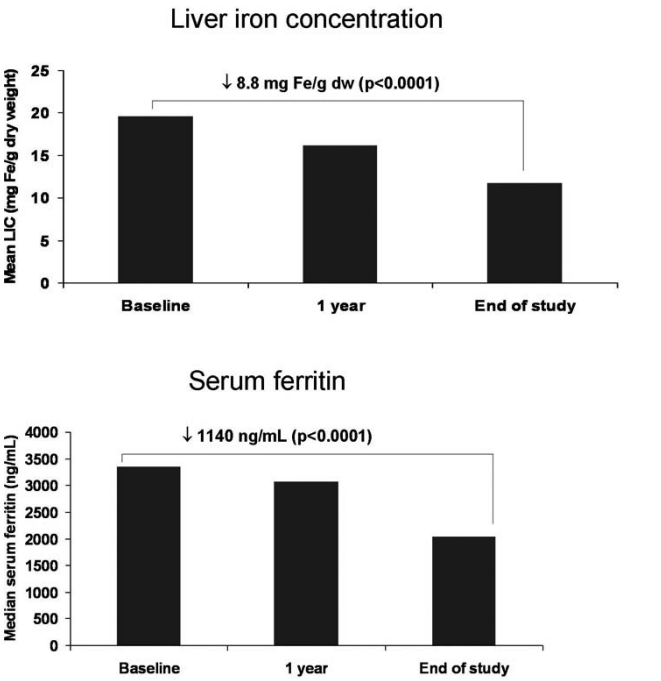

Figure 1. Mean LIC and median SF at baseline, 1 year and end of study

Red blood cells and Iron II

0210

DEFERASIROX FOR THE TREATMENT OF TRANSFUSIONAL IRON OVERLOAD IN SICKLE CELL ANEMIA: A 1-YR PROSPECTIVE STUDY

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Background. Majority of patients with sickle cell disease receive repeated blood transfusions by adulthood. Because the body has no physiological mechanism to actively excrete excess iron, chelation therapy is important for the management of iron overload and its complications, including iron deposition into the liver, heart and endocrine organs, eventual death. While studies are limited, progressive iron loading and subsequent tissue injury in SCD appears similar to other transfused populations. Deferasirox (Exjade, ICL670) is a once-daily, oral iron chelator that is approved as first-line treatment of chronic transfusional iron overload. Its safety, tolerability and efficacy in reducing body iron burden have been demonstrated in patients with β-thalassaemia major and in other chronic transfusion-dependent anaemias, including SCD. *Aims and Methods.* Objectives of this prospective, non-randomised, phase IV trial were to evaluate the iron overload status, before and after one year-treatment with deferasirox, using liver iron concentration [LIC, mg/d dry weight (dw)] by magnetic resonance imaging (MRI) hepatic, MRI cardiac (Cardiac T2*, ms), serum ferritin (SF, µg/L), and to evaluate the safety and tolerability of deferasirox. *Results.* A total of 31 paediatric (age >2y) and adult (age <65y) patients with SCD and iron overload, defined as the use of ≥20 units of RBC units and/or two SF levels ≥1000 µg/L during the 6 months preceding enrollment, received starting dose of 20 mg/kg/day of deferasirox. 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. Two patients discontinued treatment at 8 and 9 months, due to pregnancy and moving to other city, respectively. Mean ± SD and median (range) age 26.9±12.5y and 25.0y (9-49), respectively; 84% female, 90% afrodescendent, 61.3% on regular blood transfusion; mean ± SD and median (range) deferasirox dose (mg/kg/day) over 12 months were 19.7 ± 1.2 and 20 (15-20), respectively. Mean ± SD and median (range) SF levels (µg/L) did not significantly reduced at 12 months compared to baseline [from 2344.6±1077.09-2113.0 ± 1199.7 (p=0.052) and 2051.0 (1013.0-6074.0) to 2050.0 (407.0-6060.0), respectively]. The proportion of patients with SF levels <2000, 2000- <3000 and ≥3000 µg/L from baseline to 12 months by percentage of patients changed from 48.4% to 48.3%, 25.8% to 34.5% and 25.8% to 17.2%, respectively. Mean ± SD and median (range) MRI hepatic (LIC, mg/g dw) iron concentration significantly dropped at 12 months compared to baseline [from 14.0±7.4-10.5 ± 6.4 (p<0.001) and 14.0 (2.5-30.7) to 9.5 (2.0-19.5), respectively]. The proportion of patients with LIC levels (mg/g dw) ″7.0, >7.0-″14.0 and >14.0 from baseline to 12 months by percentage of patients changed from 13.6-36.4%, 40.9-27.3% and 45.5-36.4%, respectively. Mean ± SD and median (range) MRI cardiac (Cardiac T2*, ms) at baseline and 12 months varied from 40.5±6.0 to 39.0±5.8 (p=0.167) and 39.8 (27-51.2) to 39.0 (27.8-50.9), respectively. In all patients, Cardiac T2* was normal (> 20 ms) at baseline and 12 months of treatment. There was no significant difference between left ventricular ejection fraction values at baseline and after 12 months of treatment. The most common drug-related AEs were mild, transient diarrhea (7 patients), headache (7) and nausea (5). Maculopapular skin rash and serum creatinine increases upper limit of normal were observed in 2 (6.5%) patients. No patient experienced progressive increases in serum creatinine or renal failure. *Conclusions.* Our preliminary data confirms that deferasirox is effective in reducing body iron burden in transfused patients with SCD, well tolerated in pediatric and adult patients and with a clinically manageable safety profile. Although LIC significantly decreased over a 12-month-period of treatment, median SF did not. This aspect points to the fact that SF should not be the only laboratory parameter to be considered in the evaluation of iron chelation in SCD patients. 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.

0211

HPLC QUANTITATION OF ALPHA-GLOBIN HEMOGLOBINOPATHIES IS INFLUENCED BY CONCURRENT ALPHA-THALASSEMIA

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Cation-exchange high performance liquid chromatography (HPLC) has been shown to be a sensitive , specific and reproducible alternative screening method for diagnosis of hemoglobinopathies to electrophoresis. HPLC is emerging as the method of choice for sensitive direct identification and quantification of major and minor, normal and pathological hemoglobin fractions. Retention time, percentage hemoglobin and peak characteristics measures lead to hemoglobin identification. In instances of combined hemoglobinopathies, the abnormal hemoglobins are represented on HPLC as separated peaks. When α-thalassemia is concomitant with another hemoglobinopathy, there is only one peak but α-thalassemia can be suspected because of the increased height of the abnormal segment. In this manuscript we document the HPLC elution patterns of a concurrent α-thalassemia/hemoglobinopathy: α -3.7/α Setif (α 94 (G1)Asp→Tyr (α 2), with retention time of 6.28 minutes and percentage peaks of 23.6-37.6. Hemoglobin Setif alone is characterized by time retention of 6.18 minutes and percentage peak of 15.8. Molecular analysis (PCR and sequencing of the α-2 gene) was performed in order to confirm these compound heterozygosities. The increased height of the peak representing hemoglobin Setif is explained by the concomitant presence of α-thalassemia. HLC-G7 HPLC was design to identify β-talassemia but we found it is capable to diagnose α-talassemia in compound heterozygosities.

0212

GLUTATHIONE S TRANSFERASE GENE MUTATIONS AS THE CANDIDATE FACTOR RESPONSIBLE FOR THE OXIDATIVE STRESS BURDEN IN INDIAN HBE/B THALASSEMIA PATIENTS

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Background. Iron overload is the consequence in the HbE/ β thalassemia after repeated blood transfusions. Iron overload can generate the peroxidative status and the increase of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity. Severe oxidative damage is observed in erythrocytes due to the presence of excess α -globin chains. Thus, both the accumulation of excess α chains and iron overload causes oxidative stress in patients. The glutathione S-transferases (GST) are a family of enzymes that detoxify reactive electrophiles and products of oxidative stress. To our knowledge, there is no published study indicating role of GST deletions in HbE/β thalassemia patients. Indeed, there is supporting evidence that inheritance of GST deletions increases oxidative burden in anemia. Objective: Main objectives of the study were to compare the frequency of the null genotype for GSTM1, GSTT1, or both in HbE/βthalassemia patients to that in healthy controls, and to ascertain whether the oxidative burden increases due to coinheritance of specific genetic polymorphisms of GSTM1 and GSTT1. *Methods.* Subjects were 150 HbE/β thalassemia and 100 controls. The homozygous null polymorphisms GSTM1 and GSTT1 were determined using a modified multiplex PCR approach for simultaneous amplification of both genes and co-amplification of a housekeeping gene BCL2 as an internal control. Plasma MDA levels were determined by using the thiobarbituric acid reaction substance [TBARS] methods and Serum vitamin E levels were measured spec-throphotometrically. *Results.* The frequency of GSTM1/T1 null 11 (7.3%) and GSTT1 null 48 (32%) genotypes were significantly higher (p≤0.001, p=0.002 respectively) in patients when compared to controls (GSTM1/T1 0(0%), GSTT1 15(15%). Plasma Malandoaldehyde levels were significantly higher (p=0.01, p=0.01, p≤0.001) in patients positive for GSTM1 (2.7 nmol), GSTT1 (2.7 nmol) and GSTT1/M1 (5.6 nmol) null genotypes than patients negative (1.9nmol) for the same while Vitamin E levels were significantly lower for GSTM1 (4.5 mg/dL), GSTT1 (4.3 mg/dL) and GSTT1/M1 (0.89 mg/dL) (p=0.002, p≤0.001, p≤0.001) than those of negative for the same (5.9 mg/dL). *Conclusions.* GST deletions are more frequent in HbE/β thalassemia patients than normal individuals and its occurrence doubles the oxidative stress burden in these patients.

0213

ALTERATIONS IN CONTRACTILE RESPONSE OF DETRUSOR SMOOTH MUSCLE OF SICKLE CELL DISEASE TRANSGENIC MICE

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Introduction. Berkeley mouse model is well-accepted animal model of severe anemia that displays several clinical manifestations similar to those seen in humans. These animals have showed changes in the vascular and non-vascular smooth muscle reactivity. However, there is no study that has assessed the effect of detrusor smooth muscle (DSM) reactivity in sickle cell disease (SCD) mice. Bladder filing occurs by ability of the bladder to increase in volume at low intravesical pressure to prevent exposure to the upper urinary tract. Bladder emptying is accompanied by a reversal of function in which predominates DSM contraction. These DSM regulatory actions are promoted mainly by enhanced cholinergic-mediated contractions and decreased β-adrenoceptor-mediated relaxations. Activation of DSM muscarinic receptors promoted stimulation of phospholipase C with increased formation of inositol trisphosphate and diacylglycerol to release calcium from intracellular stores induced DSM contraction and causing emptying of bladder. Alterations in the contraction or relaxation mechanism of the DSM during the filling and emptying phases may contribute to bladder dysfunction. Thus, the aim of this study was to evaluate the contractile mechanism of isolated DSM in SCD transgenic mice. *Methods.* SCD transgenic mice (SS mice) and C57BL/6 mice (control) DSM was removed and placed in Krebs solution. The DSM strips were mounted in 10mL organ baths containing with Krebs at 37°C continuously aerated with a mixture of 95% oxygen and 5% carbon dioxide (pH 7.4). The tissues were stretched to a resting tension of 10 mN and allowed to equilibrate for 60min. Changes in isometric force were recorded using a PowerLab 400 Data Acquisition System (Chart, 5.2, AD Instruments, Colorado-USA). After the equilibration period, viability of the muscles was confirmed following addition of solution potassium chloride (KCl; 80 mM). Cumulative concentration-response curves were constructed for a muscarinic agonist, charbacol (CCh; 0.01-100 μM) and hyperpolarizing solution, KCl (1-300mM). Frequency-response curves for electrical field stimulation (EFS; 1-32Hz; 80V) were constructed in DSM strips in the presence and in the absence of atropine (1 μM) or suramin (100 μM). EFS were applied in strips placed between two platinum ring electrodes connected to a Grass S88 stimulator (Astro-Med Industrial Park, RI-USA). *Results.* Cumulative addition of the CCh produced concentration-dependent contractile responses in DSM segments, and potency (pEC50) did not change in SS mice (5.80±0.02), when compared with control mice (5.69±0.05), whereas the maximal response (Emax) was significantly reduced in SS mice (4.20±0.42mN), compared with control mice (9.96±1.77mN). Cumulative addition of the KCl produced concentration-dependent contractile responses in DSM segments and both pEC50 (0.98±0.08) and Emax (7.63±1.03mN) were significantly reduced in SS mice, compared with control mice (1.24±0.07 and 11.24±0.84mN, respectively). EFS-induced contractions in DSM of SS mice were significantly decreased in the higher frequency than compared to control mice. In addition, EFS-induced contractions in DSM were significantly decreased in the presence of atropine and suramin in both SS mice and control mice. *Conclusions.* In summary, this study is the first to demonstrate that SS mice exhibit a moderate contraction response in DSM, and suggesting a possible hypoactive bladder in SCD.

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0214

DO WE HAVE TO RECONSIDER FERRITIN OPTIMAL LEVELS IN MULTI-TRANSFUSED THALASSAEMIA MAJOR PATIENTS?

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Background. Serum Ferritin levels are considered as the gold standard for monitoring chelation treatment in iron overloaded Thalassaemia major patients (TMps). Over the time different values were proposed as the goal of optimal chelation. Nowadays, the introduction of T2* MRI have given new insight in iron deposition suggesting that even with low Ferritin levels a TMp may develop complications. *Aim.* to determine a safe margin of Ferritin levels in our TMps *Methods.* 1. Ferritin was measured by immunoturbidimetric assay and calculated as the mean of monthly determinations in order to eliminate false positive results, since ferritin is an acute phase reactant. 2. Cardiac and hepatic iron loading were investigated by Signa-MRI, 1.5-Tesla, T2*-sequences. 3. LIC:liver iron concentration was calculated by Ferriscan. 4. Cardiac function was assessed annually by echo-Doppler. 5. Endocrine function by dynamic tests (OGTT, GnRH) and hormonal screening annually.6. Statistical analyses were performed using SPSS ($p<0.05$ was considered significant). *Patients.* 50 transfusion-dependent TMps, mean age 30.8±2.03, switched from Desferrioxamine monotherapy to an intensive combined chelation with Desferrioxamine (40-60 mg/kg/d) and Deferiprone (75-100 mg/kg/d), on an individually tailored regimen. *Results.* after 3-7 years of combined chelation. 1) A trend analysis, PROC MIXED in SAS, revealed a negative trend of Ferritin over time ($p<0.0001$) with a rate of decline=-75 ng/mL/month and a cumulative decrease. In 90% of compliant TMps, mean Ferritin decreased dramatically from 3.421±882 ng/mL to 87±25ng/mL 2) MRI measurements led to significant reduction ($p<0.0001$) of iron load to virtually iron free organs (T2*Heart from 13.6msec to 35.5msec & T2*Liver from 1.5msec to 33.2msec). 3) In 12/50 TMp with pre-existing cardiac dysfunction on medication, symptoms reversed and heart medications were stopped. Ventricular dimensions and function in echo-Doppler shifted to normal. 4) Statistical correlations were observed, in 9/14 Diabetic TMps and 17/19 with Impaired Glucose Tolerance who normalized their glucose metabolism. The decrease in Glucose secretion ($p<0.0001$) and the increase in Insulin secretion ($p<0.05$) were correlated with a decrease in ferritin to 113±19 ng/mL (r= -0.592 $p<0.02$, r=0.568 $p<0.02$ respectively) and the normalization of MRI T2*L and LIC (r=0.629, $p<0.01$ & r= -0.619, $p<0.01$ respectively). 5) Also statistical correlations were noted between the increase of FSH & LH during GnRH test in Hypogonadal TMps and the decrease in ferritin 114±12ng/mL and the normalization of MRI T2*L (r=-0.936 $p<0.01$, r=0.723 $p<0.04$ respectively). Additionally in 7/14 males with pre-existing Hypogonadism who reversed and stopped testosterone injections, correlations were established between the increase of testosterone and the decrease in ferritin (108±32ng/mL) & LIC (r=-0.634 $p<0.04$, r=-0.679 $p<0.03$ respectively). 6) No serious adverse events which led to permanent interruption of combined chelation were observed in our TMps even with ferritin levels <500 ng/mL. *Conclusions.* The use of combined chelation, by achieving a negative iron balance and a reduction of total body iron, induces the reversal of iron-load complications in the majority of TMps. This was correlated with the dramatic decrease of ferritin to normal levels. Additionally the fact that no serious adverse events have occurred might lead us to reconsider Ferritin optimal levels in TMps.

0215

TREATMENT WITH DEFERASIROX EFFECTIVELY DECREASES IRON BURDEN IN PATIENTS WITH SICKLE CELL SYNDROMES

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Background. Iron overload was not thought to be an important issue in the past because of the short life-span of patients with sickle cell disease (SCD). However, the increase in longevity during the recent years has been associated with clinical evidence of iron overload in some SCD patients. The etiology of the latter is complex and comprises accumulation of transfusional iron, increased absorption associated with intensive erythopoiesis and iron deposition as a result of continuous hemolysis. Therefore, iron overload may play an important role in the severity of SCD and iron chelation has a definite indication in several SCD cases. *Aim.* The aim of this study was to investigate the efficacy and safety of the once-daily oral iron chelator, deferasirox, in iron-overloaded patients with sickle cell syndromes. *Methods.* We evaluated 18 adult patients with sickle cell syndromes (8M/10F; mean age 41.3±8.5 years) who had serum ferritin levels >1000 ng/mL and who were sporadically transfused with <20 units of red blood cells before starting deferasirox treatment for up to 12 months. Fifteen and three patients were initially treated with deferasirox at 10 and 20 mg/kg/day, respectively, based on the number of blood transfusions received before the initiation of treatment. After 3 months, dose adjustments (increases) were allowed in increments of 5 mg/kg/day every 3 months as required to reduce markers of iron overload. Total iron burden was monitored by measuring serum ferritin levels before and monthly after starting deferasirox, while liver iron concentration and cardiac iron burden were measured by magnetic resonance imaging (MRI) T2 and T2* parameters at baseline and 12 months after deferasirox treatment. Left ventricular ejection fraction (LVEF) by MRI, and 24-hour proteinurea (Prot 24h) before and after treat-

ment, were also measured. Hemoglobin (Hb) levels, serum creatinine, cystatin C, alanine (ALT) and aspartate aminotransferase (AST) were measured before and every month during deferasirox treatment. *Results.* The mean serum ferritin level was significantly reduced after 12 months of deferasirox treatment, while the mean baseline liver T2 and T2* significantly increased following 12 months of therapy (Table 1). Mean cardiac T2* and LVEF were normal at baseline and did not significantly change after 12 months of treatment. There were also no significant changes in mean serum creatinine, while cystatin C significantly increased after 12 months on treatment. However, cystatin C values between six and 12 months did not show any increased trend. Hb or Prot 24h levels did not change after 12 months of deferasirox treatment, while mean ALT and AST levels significantly decreased over 12 months. The side-effects of deferasirox were minimal and easily manageable, while the compliance was excellent. *Summary and Conclusions.* These data indicate that over 12 months deferasirox significantly reduced liver iron burden and serum ferritin levels in these iron-overloaded patients with sickle cell syndromes. The reductions in ALT and AST are suggestive of an improvement in liver function, if we take into consideration that liver is the target-organ of this disease. This study indicates that deferasirox provides effective iron chelation therapy in these patients and it was well tolerated without significant side effects.

Table 1.

	Baseline	6 months	12 months
Serum ferritin, ng/mL	1933 ± 997.94	1751 ± 1104.21 (*P*=0.19)	1106.44 ± 1016.31 (***P*<0.001**)
Liver T2, ms	21.13 ± 5.71	NM	27.48 ± 8.03 (***P*=0.001**)
Liver T2*, ms	4.14 ± 3.86	NM	6.05 ± 3.45 (***P*=0.013**)
Cardiac T2*, ms	37.49 ± 7.27	NM	37.23 ± 4.28 (*P*=0.98)
LVEF, %	65.35 ± 8.13	NM	66.09 ± 5.18 (*P*=0.67)
AST, U/L	52.67 ± 24.35	46.89 ± 25.34 (*P*=0.07)	44.22 ± 24.29 (***P*=0.01**)
ALT, U/L	46.56 ± 27.49	37.22 ± 18 (***P*=0.05**)	31.72 ± 17.60 (***P*=0.004**)
Hb, g/dL	8.29 ± 0.93	8.4 ± 1.31 (*P*=0.98)	8.51 ± 1.24 (*P*=0.36)
Serum creatinine, mg/dL	0.78 ± 0.16	0.87 ± 0.21 (***P*=0.009**)	0.811 ± 0.2 (*P*=0.24)
Cystatin C, mg/L	0.97 ± 0.32	1.13 ± 0.33 (***P*=0.001**)	1.12 ± 0.4 (***P*<0.001**)
Prot 24h, mg/24h	419.39 ± 632.9	486.67 ± 750.11 (*P*=0.075)	456.61 ± 586.375 (*P*=0.3)

NM, value not measured

0216

RED BLOOD CELL SEPARATION IN PK ACTIVITY ASSAY: A CRITICAL STEP FOR PK DEFICIENCY DIAGNOSIS

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Background. Pyruvate kinase (PK) deficiency is the most frequent enzyme abnormality of the glycolysis pathway causing hereditary chronic non spherocytic haemolytic anaemia (HNSHA). Since the haematological feature of PK deficiency are not always distinctive, the diagnosis of PK deficiency depends on the red blood cells (RBC) residual enzyme activity determination. In fact, PK activity assay represents an important tool to diagnose the PK deficiency, but some technical issues such as compensatory M2 isoform, high reticulocyte count and especially the incomplete white blood cells (WBC) removal, make this test not always reliable. *Aim.* In order to obtain accurate PK enzyme activity measurement, avoiding the PK activity interferences from other cell sources, and to improve the reference interval to better correlate the phenotype with the genotype, we optimized the RBC separation method, using the Ficoll procedure instead of saline separation, as suggested by the in-use kit. *Methods.* We assayed PK activity on 30 healthy subjects and on a PK deficient subject, utilizing a manual commercial Kit (PKD: Greiner Diagnostic GmbH). For RBC separation, we used both Ficoll and saline separation as suggested by ICSH recommendations and we compared the PK activity obtained with two methods. *Results*. The Ficoll procedure yelded a better reduction of the number of leucocytes (>95%) and platelets (>85%), as confirmed by cell count, with little loss of RBC (<4%). The obtained PK values after Ficoll separation are about 10% lower than those obtained after saline separation, so it was mandatory to redefine our PK reference intervals on 30 healthy subjects. Using the new reference values (70-180 IU/GR) instead of precedent values (60-220 IU/GR) we identify a subject with low PK activity. This subject, studied for PK-LR gene mutations, showed two mutations: p.R486W (c.1456C>T) and p.M403I (c.1209G>A). The first is a common mutation in Southern Europe while the second one has been reported only once in compound heterozygosis with the other. Moreover, we studied the two daughters of this patient. One of these showed normal PK values, carrying the c.1456C>T mutation, the other showed low PK values, carrying the c.1209G>A mutation, suggesting an hypothetical dominant transmission of this mutation, as reported by Fermo *et al. Conclusions.* The clinical manifestations of PK deficiency are widely variable ranging from mild to severe haemolytic anemias. In the mild deficient forms it is important that the PK deficient diagnosis was performed as soon as possible. The suggested Ficoll RBC separation is easy, timesaver, reproducible technical work and since it eliminates false negative results due to leukocytes and platelets, allows a better identification of the PK mild deficient subjects. In addition, since the genotype-phenotype correlation is a very important field of investigation to understand the effect of the mutations on the functional role of each part of the enzyme, it is very important to utilize a sensitive PK activity assay method.

Reference

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0217

RELEVANCE OF ALPHA HEMOGLOBIN STABILIZING PROTEIN POLYMORPHISMS IN SICKLE CELL DISEASE PATIENTS FROM OMAN

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Background. Interaction of the genetic modifying factors play a significant role in makeup of the phenotype and has important implications. α-Hemoglobin stabilizing protein (AHSP), a chaperone of α-globin, is a potential modifier of the genotype by virtue of its ability to detoxify excess free α-globin. *Aims.* To study the impact of AHSP polymorphisms on the hematological parameters in Sickle cell disease patients from Oman. *Methods.* Sickle cell disease (SCD) patients following in the outpatient clinic at the Sultan Qaboos University Hospital were prospectively enrolled after an informed consent. Genomic DNA was isolated using the semi-automated 6100 nucleic acid extractor. Sickle mutation was defined by direct sequencing of the β globin gene (ABI 3100 genetic analyzer). Sickle haplotypes were defined using previously described techniques by RFLP using specific restriction enzymes(HincII5'to ε, XmnI 5' to Gγ, Hind III within Gγ and Aγ, Hinc II within and 3'to β, Hinf I and RsaI5' to β, and Taq1 within Gγ and Aγ).

Table 1. Distribution of Hp and Red cell indices in SS-SCD with respect to the AHSP Haplotypes.

		RBC count [RR4.5-5.8]	Hb [RR11.5-15.5]	MCV [RR78-96]	MCH [RR26-33]	MCHC [RR31-35]	RDW [RR11.5-16.5]
AHSP - High	Mean ± SD	3.9 ± 0.77	9.6 ± 1.53	69.6±5.08	23.6±1.56	34.7±0.97	18.9±3.12
N=35	Range	2.68-6.04	7.4 – 14.2	60-79.7	20.7-27.6	32.1+36.1	14.2-31.2
AHSP- Low N=7	Mean ± SD	2.74±0.37	7.75±0.75	74.1±6.1	25.6±3.4	35.2±0.73	22.03±3.8
	Range	2.33-3.25	7-8.6	65-79.8	22-29.7	34.3-36.4	18.3-29.3
AHSP - Intermediate N=32	Mean ± SD	3.87±0.9	9.6±1.52	70.98±5.3	24.1±2.46	34.2±1.3	19.9±3.56
	Range	2.49-5.98	7.1-13.2	60.8-80.5	19.9-29.4	31.6-36.9	13.6-28.4
p value	H v/s L	0.0007	0.0049	0.060	0.022	0.027	0.04
	H v/s I	0.733	0.879	0.296	0.337	0.810	0.265
	L v/s I	0.0051	0.0062	0.208	0.194	0.115	0.195

Direct sequencing of AHSP gene (coding exons & exon-intron junctions) was performed and three AHSP haplotypes were constructed as

High, intermediate, and low AHSP expression using six SNPs (rs4499252, rs5816533, rs8050390, rs4296275, rs17677 & rs10843). *Results.* 93 patients (50 males) aged between 5-42 years, mean age 20.9±8.6(SD) were consecutively enrolled. 19(20.5%) patients were genotyped as Sickle-Thal, whereas 74 (79.5%) were homozygous SS cases. βs haplotyping amongst the SS group revealed that 29,11, and 9 cases were homozygous Benin, Bantu and Arab-Indian haplotypes respectively, with the remaining 25 being mixed heterozygous. AHSP haplotyping with regards to AHSP protein expression amongst the SS group revealed that 35(47%), 32 (43%) and 7(10%) showed High, Intermediate and Low expressor haplotype. There was a significant difference in the hemoglobin and red cell indices amongst the High and Low AHSP expression haplotypes (Table 1). *Conclusions.* Apart from the number of α globin genes and an inherent capacity to produce Hb F, the proteolytic capacity of the erythroid precursors in catabolizing the excess α globin chains has been suggested as another factor in modifying the genotype. AHSP polymorphisms are common and represent a potential mechanism through which genetically determined variations in AHSP expression could influence the underlying genotype.

0218

NON-TRANSFERRIN-BOUND IRON: A NOVEL INDICATOR OF IRON OVERLOAD IN PATIENTS WITH SICKLE CELL DISEASE

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Background. The use of serum ferritin and liver iron concentration (LIC) to detect iron overload in patients with sickle cell disease (SCD) has been established. However, ferritin levels alone are not fully acceptable because they may display considerable variations due to inflammation, infection, or other chronic disorders. Moreover, the discomfort and risk that a liver biopsy causes to patients can potentially limit its diagnostic value. Assessment by R2 magnetic resonance imaging has been proven reliable but its use remains limited due to cost and the need for specialized equipment. Several studies have demonstrated that non-transferrin-bound iron (NTBI) is a good indicator of iron overload in thalassemia patients; however, data in patients with SCD is limited. *Aims.* The current study evaluates whether NTBI is a useful indicator of iron overload in non-chelated patients with SCD by assessing the relationship between NTBI, serum ferritin, LIC, total number of lifetime transfusions, patient- and disease-related characteristics. *Methods.* This was a cross-sectional study of randomly selected patients with SCD treated at two comprehensive SCD centers: Nini hospital and Rafik Hariri University Hospital (RHUH), Lebanon. The sampling frame consisted of 200 non-chelated SCD patients >2 years of age and a simple random sample was obtained. Patient charts were reviewed and a medical history compiled, which included details of drug use, transfusional history, status of the spleen, and comorbid illnesses or infections. Blood samples were obtained for assessment of NTBI, steady state serum ferritin, and liver enzyme levels. Direct determination of LIC was performed using R2 magnetic resonance imaging. Written informed consent was provided by all patients. *Results.* Data from 52 SCD patients were included in the analysis (Table 1).

Table 1. Patient, disease and iron overload characteristics

Parameter	Value
Mean age ± SD, years (range)	18.54 ± 9.0 (4-49)
Male/Female	5/8
Splenectomized, (%)	16 (30.8)
Persistent splenomegaly[a], (%)	21 (42)
Mean transfusions[b] ± SD, n (range)	50.9 ± 61.4 (0-300)
Mean hemoglobin ± SD, g/dL (range)	8.6 ± 1.4 (5.6-11.9)
Mean SF ± SD, ng/mL (range)	987.5 ± 1230.3(16-6190)
Mean LIC ± SD, mg Fe/g dw (range)	5.9 ± 9.4 (0.3-50.0)

SF = serum ferritin at steady state; LIC = liver iron concentration; dw = dry weight. [a]For more than 6 years. [b]Total number of lifetime transfusions.

None of the patients had evidence of hepatitis C or B infection, or elevation in liver enzymes. A significant positive correlation was observed between NTBI and LIC (Pearson correlation 0.466; p=0.001). There was also a significant correlation between NTBI and serum ferritin (Pearson correlation 0.514; p<0.0001). Moreover, a significant positive correlation was noted between age and NTBI (p=0.028) but not with serum ferritin or LIC. The total number of transfusions was positively correlated to NTBI, serum ferritin and LIC (p=0.003, <0.0001, <0.0001; respectively). Although female gender, splenectomy, and persistent splenomegaly were associated with higher levels of NTBI, serum ferritin and LIC; the relationships did not reach statistical significance. *Summary and Conclusions.* Significant correlation was found between NTBI and both serum ferritin and LIC, confirming the value of this method for assessing iron overload in SCD patients. Neither splenectomy nor persistent splenomegaly seem to significantly affect NTBI levels, thus questioning the role of the spleen in scavenging iron free radicals, including NTBI, in SCD patients. Increasing age and total number of lifetime transfusions are associated with higher NTBI levels, thus, suggesting its accumulation over time. The value of NTBI in monitoring iron chelation therapy is to be evaluated.

0219

OUTCOME OF PREGNANCY IN SICKLE CELL SYNDROMES. A SINGLE CENTER STUDY

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Background. Pregnancy has been associated with exacerbation of sickle cell disease and may place patients and their infants at an additional risk. However since 1980 many studies have reported a decline of maternal and fetal complications. Aims. The aim of this retrospective observational study is to examine maternal and fetal outcomes of pregnancy in women with sickle cell syndromes. *Methods.* During the period 1991 to 2009, 161 women with sickle cell syndromes are attended in our center. We present the gynecologic and obstetric history from 78 women with sickle cell syndromes (S/β 59, S/S 17,S/C 1, S/δβ 1), median age at time of study 49.5 years (range 25-72). Data were recruited from our hospital records and were all verified with interview of the patients. Results. In the group of our patients the median age of menopfania is 14 years (range 11-17), primitive amenorrhea referred two patients, irregular menstrual cycles 7 and menorrhalgia 6, under hormone therapy are 4 women. There were 120 pregnancies in 57 of the 78 patients, the others 21 never become pregnant. Median age of pregnancy is 27 years (range 17-41). The outcome of these pregnancies is: 32 (26.6%) elective and 27 (22.5%) spontaneous abortions (first trimester: 22, second trimester: 2, third trimester: 3), 61 (50.8%) labors and delivery of 63 alive infants (two twin pregnancies). Birth weight average is 2.791 (1.300 -4.800). Ten of the newborns needed intensive care, from one to thirty days and one received exchange transfusions because of jaundice. From these 63 infants one died at the age of ten months, because of a genetic cardiac problem and another at sixteen months of age because of neurological problems because of a preterm labor continuing a severe painful crisis of the mother. All the others are alive and healthy. During these pregnancies, increase in the frequency and severity of pain episodes documented in 6 women, while decrease in 33. From our group of patients, 33 were supported with transfusions during pregnancy, 3 of them received exchange transfusions. Three women were transfused only during labor. Severe maternal complications included: a gastric bleeding during 4th month, one pre-eclampsia during 8th month, an acute chest syndrome in one woman after delivery and a severe uterus bleeding after labor. Five patients receiving hydroxyurea, manage to become pregnant after discontinuation of the medicine. They were supported with transfusions, or exchange transfusions, carried out their pregnancies without problems and gave birth to five healthy infants. The mode of delivery was: vaginal in 27/61 (44.2%) and cesarean section in 34/61 (55.7%). Cesarean section was performed for medical and obstetric reasons in 6 cases, all the others were programmed. Gestational age at delivery was higher than 37 weeks in 51 (83.6%), while preterm labor was reported to occur in 10 (16,4%) deliveries, (8 between 32-36 weeks, 1 at 30 w and 1 at 27w). *Conclusions.* Our study suggests that women with sickle cell syndromes, with appropriate management, can carry their pregnancy to term, without serious maternal and fetal complications.

0220

INCREASED PLATELET AND NEUTROPHIL ADHESION IN SICKLE CELL DISEASE CORRELATES DIFFERENTLY TO VARIABLE LEVELS OF HEMOGLOBIN A

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Sickle cell disease (SCD) is a group of disorders in which the inheritance of the gene codifying the abnormal hemoglobin S (HbS) occurs in homozygosity (SCA), or in heterozygosity with other abnormal β globin genes, such as HbC, HbD or β thalassemia, among others. SC disease and HbS-β thalassemia account for most of the compound heterozygote forms of SCD. These conditions may have a clinical phenotype similar to sickle cell anemia, as in HbS-β0 thalassemia, while SC disease and HbS-β+ thalassemia, depending on the β globin mutation, present with a milder form of the disease. Polymerization of HbS and vaso-occlusive events in SCD generate a chronic inflammatory state, but differences in the inflammatory markers among genotypes have not yet been determined. The aim of this study was to evaluate the expression of adhesion molecules, adhesion properties of platelets and neutrophils and neutrophil chemotaxis in SCD patients. Neutrophils and platelets were isolated from peripheral blood samples of healthy controls and patients with SCA, SC disease and three groups of HbS-β thalassemia (HbS/β0-CD39 C->T; HbS/β+-IVS1-nt5 G->C - severe phenotype - and HbS/β+-IVS1-nt6 T->C - mild phenotype), transfusion-independent patients and not on hydroxyurea treatment. Basal adhesions were compared using static adhesion assays and data are represented by % of cells adhered. Surface protein expression in neutrophils was evaluated by flow cytometry. Chemotaxis of control and patients neutrophils (4×10^6 cells/mL in RPMI medium) was assessed using static adhesion assays and a 96-well chemotaxis chamber assay (ChemoTX, Neuroprobe). Flow cytometry showed no differences in neutrophil positivity for CD11a, CD11b and CD49d adhesion molecules among SCD groups compared to control individuals. SC and SCA neutrophils had higher chemotactic activity than controls ($3.8\pm0.6\times10^5$mL; $4.1\pm0.5\times10^5$mL vs $1.98\pm0.2\times10^5$mL, p=0.0034; p=0.0027, respectively). No significant differences were observed in chemotaxis of the three groups of HbS-β thalassemia compared to control group. Mean hemoglobin A levels in HbS-β thalassemia groups were 5.2±1.3% and 26.3±1.4% for HbS/β+-IVS1-nt5 and HbS/β+-IVS1-nt6 groups, respectively. Our data suggest that platelet adhesion is increased in all forms of sickle cell disease, while lower neutrophil adhesion seems to correlate with higher levels of hemoglobin A production as occurs in HbS/β+-IVS1-nt6. This may explain why *in vitro* analysis of SC and SCA groups were similar despite being clinically different. Further studies may contribute to our understanding of the differences in the clinical presentation of these patients.

Table.

	N	Neutrophils (%)	P value	Platelets (%)	P value
Control	n≤21	7.9 ± 0.53		11.7 ± 1.2	
SS	n≤14	14.5 ± 1.1	<0.0001	29.4 ± 3.8	0.0005
SC	n≤29	14.4 ± 1.1	<0.0001	26.4 ± 3.0	0.0001
HbS/β⁰-CD39	n≤8	13.6 ± 2.1	0.03	31.3 ± 3.6	<0.0001
HbS/IVS1-nt5	n≤15	11.5 ± 1.4	0.02	30.2 ± 2.6	<0.0001
HbS/IVS1-nt6	n≤17	10.4 ± 1.2	NS	22.4 ± 2.5	0.0017

0221

IMPLEMENTATION, EFFICACY AND SAFETY OF A BLOOD CONSERVATION PROTOCOL IN PATIENTS UNDERGOING SURGERY FOR HIP FRACTURE REPAIR. AN ANALYSIS OF 399 CONSECUTIVE PROCEDURES

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Background. To restore hemoglobin levels and avoid deleterious effects of anemia, 30-70% of patients undergoing perthrochanteric (PHF) or subcapital hip fracture (SHF) repair receive allogeneic blood transfusion (ABT). *Aims.* We prospectively investigated the effect of a blood conservation protocol on ABT requirements in 384 consecutive patients. *Methods.* The conservation protocol consisted of the application of a restrictive transfusion trigger (Hb<8 g/dL) and the perioperative administration of IV iron sucrose (3×200 mg/48 h). Additionally, patients with Hb <13 g/dL may received recombinant human erythropoietin (EPO 40,000 IU sc) on admission to the orthopedic ward. Perioperative clinical data were collected. *Results.* Overall, 136 (35.4%) received at least one ABT unit (2.1 ± 0.9 U/patient). However, ABT rates were significantly lower in SHF than in PHF (29.5% vs. 40.3%, respectively; p=0.028), but not pre-transfusion Hb or transfused volume. In both groups, patients receiving EPO (n=96) presented with lower preoperative Hb than those who did not (n=288), but there were not significant differences in ABT rates or postoperative Hb between them. There was a higher incidence of postoperative complications and 30d mortality among transfused patients (n=136) than among non-transfused patients (n=248). Only 7 mild IV iron adverse effects were witnessed, but 50 patients received only 400 mg IV iron. *Conclusions.* Our blood conservation protocol seems to be safe and effective in reducing ABT in hip fracture patients. However, a persisting awareness among the medical staff and nurses is crucial to avoid protocol violations and to limit further limiting the exposure to ABT and ABT related risks.

0222

OXIDANT-ANTIOXIDANT SYSTEM AND LYMPHOCYTE DNA DAMAGE IN BETA THALASSEMIA CHILDREN

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Background. Thalassemia is an autosomal recessively inherited hemoglobinopathy which basicly characterised by ineffective eritropoesis, hemolysis and anemia. Metabolic disorders, iron overload, chronic hypoxia and cell damage are added to these findings. Oxidative stres is recently being investigated in β-Thalassemia major (TM) patients as well as in cardiovascular diseases, cancer, renal diseases, infection and neurological diseases. Oxidative stress develops as a result of imbalance between formation and neutralisation of pro-oxidants. Pro-oxidant / antioxidant equilibrium disorders may cause oxidative stress and DNA damage in cellular structures. *Aims.*The aim of this study was to detect and correlate iron overload with the oxidant / antioxidant status and DNA damage in transfusion dependent b-TM patients. *Methods.*The patient group was constituted from 83 patients with transfusion dependent b-TM whose mean age was 7,34±4,21 years. 40 sex and age-matched children with non-anemia, served as control group. The damage of mononuclear DNA were assessed with comet assay method and total oxidant status (TOS) and total antioxidant capacity (TAC) measurement by using Erel's methods. *Results.* In the b-TM patients, mean DNA damage level was 10,65±6,58 AU, mean TOS level was 15,98±9,44 (μmol H2O2 Eqv./L), mean TAK level was 1,62±0,27 (mmol Trolox Eqv./L), mean Oxidative Stres Index (OSI) level was 11,11±8,42 AU and mean lipid hydroperoxide (LOOH) level was 5,44±1,80 (μmol H2O2 Eqv./L) detected. In control group, mean DNA damage level was 1,45±2,02 AU, mean TOS level was 7,18±3,74 (μmol H2O2 Eqv./L), mean TAK level was 1.76±0.38 (mmol Trolox Eqv./L), mean OSI level was 4,66±3,46 AU and mean LOOH level was 2,44±0,86 (μmol H2O2 Eqv./L) detected. When compared to the controls, DNA damage was detected to be increased in b-TM patients (p<0.001). When oxidant-antioxidant system were evaluated, while TOS, OSI and LOOH levels were significantly increased, TAC levels were decreased in b-TM patients compared to controls (p<0.001; p<0.001; p<0.001 and p=0.018 respectively). There was a statistically significant positive correlation between serum ferritin levels and DNA damage, TOS, OSI and LOOH; however a negative correlation was observed between serum ferritin and TAC levels in the b-

TM patients. There was a statistically significant positive correlation between the serum iron and ferritin levels and AST, ALT and transfusion years in the β-TM patients. TOS and OSI levels were positively correlated with ALT, however a negative correlation was observed between TAC and ALT levels in the b-TM patients. Serum AST and ALT levels were detected to have a stastistically significant positive correlation with transfusion years in the b-TM patients. In the b-TM patients the DNA damage was detected to have a stastistically significant positive correlation with TOS, OSI, LOOH, AST and ALT respectively; however a negative correlation was observed between DNA damage and TAC levels. *Conclusions.* There were increased iron dependent oxidative stress and DNA damage present in patients with b-TM. The combination of effective iron-chelatory agents with natural or synthetic antioxidants can be very helpful in the clinical practice and in decrease of the oxidant stress and DNA damage of patients with β-TM.

0223

DISORDERS OF GLUCOSE METABOLISM ARE A MAJOR ENDOCRINE COMPLICATION IN OMANI THALASSAEMICS

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Background. The thalassaemia centre at Sultan Qaboos University hospital (SQUH) opened in 1991 and is now the largest thalassaemia referral centre in Oman. Compared to european centres, iron chelation for the majority of our patients started relatively late in 1985 with regular chelation only since 1991. Although endocrine dysfunction secondary to iron overload is a major problem in thalassaemia major, there is very little data on the prevalence of endocrinopathies in Arab thalassaemics. *Aims.* To assess the overall prevalence of endocrine complications in Omani patients with thalassaemia major and compare two cohorts of patients; those born before 1990 and those born after, to assess if early access to iron chelation has reduced endocrinopathies in the latter group. *Methods.* Endocrine screening is performed yearly after the age of 11 years in all patients at the Day Care Thalassaemia unit in SQUH. MRI cardiac and liver T2* measurements are also part of routine management of all patients over the age of 10 years. We reviewed data of 104 thalassaemia major patients between the ages of 12 and 35 years, (55 females and 49 males). 66 patients were born before, (Group 1) and 38 after (Group2), 1990. *Results.* Delayed puberty, hypogonadotrophic hypogonadism, arrested puberty or secondary amenorrhoea were seen in 62 patients (59.6%) overall with 46 and 16 in groups 1 & 2 respectively (Chi Square 2.04; p=0.15). Amongst the 40 patients who developed pancreatic endocrine dysfunction, 17 (16.3%) patients were frankly diabetic and 23(22%) had impaired glucose tolerance [Total 40; 35 and 5 in groups 1 and 2 respectively(Chi Square 6.86; p=0.0088)]. 9 patients had hypoparathyroidism (8.6%) and 3 patients (2.8%) were hypothyroid. A further 5 patients had subclinical hypothyroidism and one had congenital hypothyroidism, unrelated to iron overload. Cardiac T2* was available for 102 patients. There were 24 patients with cardiac T2* <10ms, 30 in the range of 10-20ms and 48 were >21ms. We found a low correlation, (Pearson's r -0.3), between the degree of cardiac siderosis and presence of an endocrinopathy. *Conclusions.* The incidence of gonadal failure is similar to that noted in other thalassaemia centers around the world. However the prevalence of diabetes and deranged glucose tolerance (38%) is markedly higher. Optimizing chelation seems to have reduced this complication significantly [p=0.008] in the younger cohort who had the opportunity of better chelation facilities at an early age. In contrast to data from the Italian working group, few of our patients have developed hypothyroidism, but hypoparathyroidism is an increasing problem. Expectedly the younger cohort of patients had a significantly lower prevalence of endocrine complications. This was particularly true of gonadal and glucose dysfunction. However, longer follow up is needed to see if the improvement is sustained. Early intervention with diet, exercise and chelation is essential in Omani thalassaemics to prevent the endocrine complications, and in particular diabetes, as it has a high prevalence in the general population(diabetes 16.1%, impaired fasting glucose 7.1%).

0224

OXYDATIVE STRESS AND ERYTHROPOIETIN REDUCTION DURING AND AFTER A FOURTEEN DAYS SCUBA DIVE

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Background. Six scuba divers (three men and three females), lived for 14 days under the sea, controlled by a physician's team. The conditions applied in this experiment were different compared to those of others previously conducted. *Aims*. Since oxygen is the main regulator of Epo synthesis, we investigated the effects of a so long staying underwater, breathing Air in hyperbarism (HyperBaric Air-HBA), on s-Epo production. In addition, we evaluated some markers related to the oxidative stress to elucidate if the last could influence Epo production. *Methods.* During the entire length of the experiment, the six scuba divers stayed at a depth of 8-10 metres (22-28 feet), breathing air (oxygen 21%) at a pressure ranging between 1,8 and 2 ATA. Serum Erythropoietin (s-Epo) (Immulyte-Medical System), complete and differential blood count (CBC) (ADVIA 120 Bayer Diagnostics) as far as percentage and absolute reticulocyte count (Beckman Coulter LH 750) and the most important routine hemato-chemical parameters were assayed. Moreover, serum nitric oxide (NO) (Nitric Oxide Colorimetric Assay, Roche), glutathione, cysteine and homocysteine (HPLC with fluorimetric detection according Araki *et al.* 1987) were evaluated. All these exams were performed before immersion (TIME 0), 7 days (TIME 1) and 14 days (TIME 2) after the beginning of the dive, two hours (TIME 3) and 24 hours (TIME 4) after the resurfacing. *Results.* As shown in the Table Hgb, did not change whereas s-Epo and other data not shown such the ratio between s-Epo predicted and observed (O/P ratio), and reticulocyte (absolute and percentage) declined progressively from TIME 0 to TIME 3. At TIME 4, s-Epo increased again. All these changes were statistically significant (Fisher test for non parametric data). Among oxidative stress parameters, NO, glutathione and cysteine vary similarly to s-Epo, decreasing during the diving and then turning up at resurfacing. Homocysteine follows an opposite trend. However the statistical significance of these changes is reached only for NO (p=0.001), Cysteine (p=0.001) and Homocysteine (p<0.05). Only a trend of statistical significance is present for Glutathione (p=0.08). *Conclusions.* These observations suggest that during the dive, a progressive rise of oxidative stress occurs. In fact, the reduction of the antioxidants, probably as an effect of their consumption, and the homocysteine elevation support this conclusion. Moreover, the results obtained can give an explanation of the s-Epo reduction. Since Hgb does not vary during the dive, the prolonged hyperoxia could be a factor lowering the s-Epo production through the oxidative stress. In fact, our data show significant direct correlation between s-Epo and glutathione (r 0.41; p=0,04) and indirect correlation between s-Epo and homocysteine (r -0,61; p=0,002) (Spearman test).

Table.

T	Hgb g/dl	Ht	s-Epo mU/ml	O/P ratio	Glutatione µmol/L	Cysteine µmol/L	NO µmol/L	Homocysteine µmol/L
0	14,03±1,25	41,32±2,81	11,58±3,09	0,89±0,10	4,38±2,68	150,35± 8,48	46,9±28,1	21,35±12,32
1	13,72±1,39	40,03±3,37	6,28±3,20	0,60±0,19	3,42±0,42	144,26 ±8,37	29,7±12,9	23,4±15,62
2	13,33±1,80	38,83±4,35	4,23±1,59	0,46±0,10	2,93±0,53	135,72 ±18,24	20,8±6,2	31,25±20,90
3	13,40±1,43	39,77±3,38	4,50±1,73	0,49±0,17	2,94±1,00	163,71 ±22,71	92,5±58,3	19,22±9,41
4	13,67±1,35	39,82±3.14	14,02±5.05	0,90±012	3,30±0,67	166,38 ±5,87	20,1±5,0	21,29±20
P	n.s.	<0.0001	<0.0001	<0.0001	0,08	0,001	0,001	<0.05

0225

ENDOSOMAL TRANSPORT OF TRANSFERRIN TO MITOCHONDRIA IS ESSENTIAL FOR EFFICIENT UTILIZATION OF IRON FOR HEME SYNTHESIS

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An exquisite relationship between iron and heme in hemoglobin (Hb)-synthesizing cells makes blood red. Erythroid cells are the most avid consumers of iron (Fe) in the organism and synthesize heme at a break-neck speed. Developing red blood cells (RBC) can take up Fe only from the plasma glycoprotein transferrin (Tf). Delivery of iron to these cells occurs following the binding of Tf to its cognate receptors on the cell membrane. The Tf-receptor complexes are then internalized via endocytosis, and iron is released from Tf by a process involving endosomal acidification. Iron, following its reduction to Fe^{2+} by Steap3, is then transported across the endosomal membrane by the divalent metal transporter, DMT1. However, the post-endosomal path of Fe in the developing RBC remains elusive or is, at best, controversial. It has been commonly accepted that a low molecular weight intermediate chaperones Fe in transit from endosomes to mitochondria and other sites of utilization; however, this much sought iron binding intermediate has never been identified. In erythroid cells, more than 90% of iron must enter mitochondria since ferrochelatase, the final enzyme in the heme biosynthetic pathway that inserts Fe^{2+} into protoporphyrin IX, resides in the inner part of the inner mitochondrial membrane. In fact, in erythroid cells, strong evidence does exist for specific targeting of Fe toward mitochondria. This targeting is demonstrated in Hb-synthesizing cells in which Fe acquired from Tf continues to flow into mitochondria even when the synthesis of protoporphyrin IX is suppressed. Based on this, we have formulated a hypothesis that in erythroid cells a transient mitochondrion-endosome interaction is involved in iron translocation to its final destination. Recently, we have collected strong experimental evidence supporting this hypothesis: we have shown that Fe, delivered to mitochondria via the Tf pathway, is unavailable to cytoplasmic chelators. Moreover, we have demonstrated that Tf-containing endosomes move and contact mitochondria in erythroid cells, that vesicular movement is required for iron delivery to mitochondria and that "free" cytoplasmic Fe is not efficiently used for heme biosynthesis. Additonally, performing flow cytometry on cell lysates from reticulocytes incubated with two different fluorescent markers for endosomes and mitochondria, we have identified three distinct populations: endosomes, mitochondria, and a population of particles labelled with both fluorescent markers. The size of the double-labelled population increases with the incubation time and plateaus in ~30 min. Reticulocyte re-incubation with unlabelled Fe^{2}-Tf leads to a time-dependent decrease, and ultimate disappearance, of the double-labelled population, indicating a reversible nature of mitochondria-endosome interactions As mentioned above, the substrate for the endosomal transporter DMT1 is Fe^{2+}, the redox form of iron which is also the substrate for ferrochelatase. These facts make the above hypothesis quite attractive, since the "chaperone"-like function of endosomes may be one of the mechanisms that keeps the concentrations of reactive Fe^{2+} at extremely low levels in oxygen-rich cytosol of erythroblasts, preventing ferrous ion's participation in a dangerous Fenton reaction.

Platelets and thrombocytopenia I

0226

REDUCED CORTICOSTEROID IN ADULTS WITH ITP RECEIVING ROMIPLOSTIM

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Background. Romiplostim, a novel thrombopoietin mimetic agent, was recently approved for the treatment of chronic immune thrombocytopenia (ITP) in adults. ITP is an autoimmune disease caused by increased platelet destruction and suboptimal platelet production. Patients are commonly given oral corticosteroids as first-line treatment. Although initial response rates with corticosteroids are high, their long term use can be associated with serious side effects and complications, including excessive weight gain and obesity, mood changes, diabetes, and osteoporosis. Furthermore, the therapeutic effects of corticosteroids often cease when treatment is stopped. *Aims.*To assess the proportion of patients with chronic ITP able to reduce or discontinue concurrent corticosteroid treatment while being treated with romiplostim in two randomised, double-blind, placebo-controlled phase 3 trials. *Methods.* Altogether 125 patients (63 splenectomised, 62 non-splenectomised) with chronic ITP and a mean of three platelet counts 30×10^9/L or less were enrolled and randomised in a 2:1 ratio to receive either romiplostim (n=42, splenectomised, n=41 non-splenectomised) or placebo (n=21 in both studies). Informed consent was obtained from all patients prior to performing any study specific procedures. Details of the treatment regimen used in the studies have been published previously (Kuter. D, J *et al.* 2008). Overall platelet response was defined as four or more weekly platelet counts above 50×10^9/L in the absence of rescue medication. Patients could enter the studies while receiving concurrent corticosteroids and reductions in these were permitted when platelet counts were above 100×10^9/L. In a post-hoc analysis, the proportion of patients able to reduce their corticosteroid dose by at least 25%, or discontinue corticosteroids permanently by the end of week 25 was investigated. Analyses were performed on the full analysis set. *Results.* With regards to efficacy, in both studies overall platelet response was higher for the romiplostim-treated patients compared with placebo (Table). The percentage of patients in the romiplostim group receiving corticosteroids at study entry was similar to that in the placebo group. By the end of week 25, all except two of the romiplostim-treated patients had reduced their corticosteroid dose by at least 25% or discontinued corticosteroids permanently, compared with approximately half of the placebo-treated patients. Furthermore, with the exception of two patients, the reductions in corticosteroids observed in the romiplostim-treated patients were sustained until the end of week 25 and subsequent dose increases were not required. *Conclusions.* Romiplostim, a new non-immunosuppressive therapy for adult ITP, allows the majority of patients to reduce or discontinue concurrent corticosteroid treatment while raising and sustaining platelet counts.

Table.

	Overall platelet response* n (%)	Corticosteroid use at study entry n (%)	Corticosteroid use by wk 25	
			Reduced by ≥ 25%† n (%)	Permanently discontinued† n (%)
Splenectomised				
Romiplostim (N=42)	33 (79%)	12 (29%)	5 (42%)	7 (58%)
Placebo (N=21)	0 (0%)	5 (24%)	1 (20%)	1 (20%)
Non-splenectomised				
Romiplostim (N=41)	36 (88%)	11 (27%)	5 (45%)	4 (36%)
Placebo (N=21)	3 (14%)	8 (38%)	2 (25%)	3 (38%)
Pooled data				
Romiplostim (N=83)	69 (83%)	23 (28%)	10 (43%)	11 (48%)
Placebo (N=42)	3 (7%)	13 (31%)	3 (23%)	4 (31%)

*p<0.0001 for all comparisons (romiplostim versus placebo); †categories mutually exclusive, percentages calculated from number receiving corticosteroids at study entry.

Reference

Kuter, D. J et al.Lancet 2008;371: 395-403.

0227

MULTICENTER STUDY OF LOW-DOSE ANTI-CD20 VELTUZUMAB FOR TREATMENT OF RELAPSED IMMUNE THROMBOCYTOPENIA

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Background. Veltuzumab, a 2nd-generation humanized anti-CD20 monoclonal antibody with structure-function differences from rituximab, is clinically active in non-Hodgkin's lymphoma, even at lower doses than used with rituximab. Since immunotherapy with rituximab has demonstrated activity in immune trombocytopenia (ITP), we hypothesized that low-dose veltuzumab would also be effective in this disorder. *Aims.* A multicenter, phase I/II study was undertaken to evaluate low-dose veltuzumab in adults who had ITP for at least 6 months, who failed >1 standard therapy, and presented with platelets <30 K/μL, but no major bleeding. *Methods.* Initially, veltuzumab was given by intravenous (IV) infusion, but after the development of a high-concentration formulation, is being administered by subcutaneous (SC) injection. All patients received 2 doses of veltuzumab 2 weeks apart (without steroids) and were evaluated for up to 12 weeks, with responding patients continuing in long-term follow-up. Efficacy was assessed by platelet level increases, with responses confirmed at least 1 week apart classified as complete (CR, >150K/μL), partial (PR, 50-150K/μL), or minor (MR, 30-50K/μL). Adverse events and safety laboratories were evaluated by NCI CTC v3 toxicity grades. Other evaluations included circulating B-cell levels (CD19), veltuzumab serum levels, and human anti-veltuzumab antibody (HAHA) titers. *Results.* The Phase I dose-escalation portion of this study has been completed with 17 patients (6M/11F, 21-67 years old, 5 post-splenectomy) receiving IV doses at 80 (N=3), 120 (N=3), or 200 mg (N=1), or SC doses at 80 (N=3), 160 (N=4), or 320 mg (N=3) veltuzumab. They had ITP for up to 18 years (median 1.9 yr) and previously were treated with steroids (N=17), IVIG/anti-D (N=11), azathioprine/danazol (N=6), rituximab (N=3), chemotherapy (N=2), and TPO-R agonists (N=1). One patient had a Grade 3 infusion reaction to veltuzumab after receiving ~100 mg at first IV dose, but otherwise veltuzumab was well tolerated with other infusion/injection reactions limited and transient Grade 1-2 events, and with no other safety issues. B cells were depleted rapidly with both IV and SC dosing and are continuing to be followed for recovery. Even at these low doses, IV veltuzumab achieved expected serum levels (mean Cmax, 20.3 and 46.0 μg/mL at 80 and 120 mg, respectively) without evidence of rapid clearance or sequestration (mean post-treatment half-life ~1 week), while SC veltuzumab had slower release over several days with lower serum levels, but comparable availability/exposure. Two patients developed low-level HAHA titers of uncertain clinical significance. Of 13 patients with post-treatment results currently available, 8 (62%) responded, including 4 (31%) CRs, 2 (15%) PRs, and 2 (15%) MRs. Responses, including CRs, occurred with both SC and IV administrations, and across all dose levels, even 80 mg. The PR and MRs were short lived (3-12 weeks), but all CRs are still continuing, now up to one year later. *Conclusions*. Low-dose veltuzumab (2 doses, 2 weeks apart), given IV or SC without steroids, demonstrates promising activity in relapsed ITP, including durable complete responses. Based on these findings, Phase II is proceeding, with patients being recruited to provide additional/confirmatory data for further clinical development.

0228

SAFETY AND EFFICACY OF LAPAROSCOPIC SPLENECTOMY IN PATIENTS WITH REFRACTORY IMMUNE THROMBOCYTOPENIC PURPURA. A LONG-TERM SURVEY

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Background. Despite the popularity of splenectomy has decreased dramatically in the past few years, the surgical approach remains the therapy of choice for patients with refractory Immune Thrombocytopenic Purpura (ITP) in terms of high and durable rate of response (Vesely *et al.*, Ann Intern Med 2004;140:112). The recent introduction of anti-CD20 antibodies and thrombopoietins of second generation such as AMG 531 and Eltrombopag may have a relevant role (Kuter *et al.*, Lancet 2008; 371: 362) but their long-term safety and efficacy have not been still established. In parallel with new drugs, there has been an evolution in the surgery of splenectomy as well (Dolan *et al.*, Am J Hematol 2008; 83: 93). Actually, the laparoscopic surgery is considered the standard approach ant the ITP represents the most common indication in 50-80% of all the laparoscopic splenectomies. *Methods.* The aim of this work is to evaluate the long-term complete and partial response (CR + PR), as well as the short and long-term complications, of 40 patients (30 females and 10 males; median age: 38 years - range 6-71) with unresponsive ITP after one or more medical approaches and underwent laparotomic splenectomy at our Institution from 1999 through 2006. The 40 patients accounted for 22.2% of 181 patients diagnosed in those years. An abdominal CT scan to evaluate the presence of accessory spleens was performed in all cases. All patients received meningococcal, pneumococcal and haemophilus influenzae vaccine one week before splenectomy. For 4 or 5 days before splenectomy the patients were treated with high doses of intravenous Immunoglobulins. Anti-thrombotic prophylaxis was performed with low molecular weight heparin (LMWH) for 10 days and afterwards with cardioaspirin (ASA) if the platelet count exceeded 500×10^9/L. *Results.* No cases required conversion to laparotomic splenectomy. An accessory spleen was found in 2 patients (5%). Immediate haematological response rate was of 100%. At date, after a median follow-up of 78 months (range 28-112 months), 36 patients (90%) remain in CR or PR with a platelet count more than 50×10^9/L and 2 patients are taking ASA. Four patients (10%) relapsed, out of which 2 patients have a platelet count less than 10×10^9/L. Short and long-term mortality rate was 0%. Immediate postoperative complications rate was 5%: we observed 2 cases of hemoperitoneum related to a trocar's tube and to an active bleeding respectively, both resolved with laparoscopic approach. The mean postoperative hospital stay was 4,5 days (range 4-8). Neither cases of bacterial sepsis in the postoperative or during the follow-up time, nor cases of splenic-portal vein thrombosis (SPVT) and no cases of neoplasms occurred. *Conclusions.* Our experience suggests that laparoscopic splenectomy is an excellent approach to patients with refractory ITP in terms of safety, efficacy and costs. With respect to laparotomic splenectomy, the use of laparoscopy is likely to make the splenectomy even safer and therefore suitable for a larger number of patients. Undoubtedly there is a great expectation for the new drugs (Rodeghiero *et al.*, Am J Hematol 2008;83:91) and we agree that only controlled comparative clinical trials (Vianelli *et al.*, Haematologica 2005; 90: 72) will be able or not to say a final word and to challenge the role of splenectomy.

0229

USE OF FONDAPARINUX IN PATIENTS WITH SUSPECTED HEPARIN-INDUCED THROMBOCYTOPENIA

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Background. Heparin-Induced thrombocytopenia (HIT) is a rare side effect of heparin therapy. Although the use of fondaparinux in this situation is not approved, it could be a good alternative in these patients. *Aims.* We studied a total of 21 patients that were treated with fondaparinux because of suspected heparin HIT. The primary endpoint was platelet recovery after fondaparinux initiation. Secondary endpoints were the appearance of thrombosis or bleeding. *Methods.* Retrospective observational study in one center. Twenty-one patients with suspected HIT, in which fondaparinux treatment was started, were included. Platelet counts were obtained daily until platelet recovery and fondaparinux dose was adjusted according to anti-factor Xa levels. We also registered hemorrhagic and thrombotic complications. *Results.* Eleven (52%) patients were men and 10 (48%) women. The median age at the moment of admission was 67 (range 41-83) years. Most patients were admitted in the cardiology (5, 21%) or cardiac surgery (6, 28%) wards. All patients presented a decrease in platelet count after heparin initiation. Nine (44%) patients received low molecular weight heparin (LMWH), 8 (39%) unfractionated heparin and 4 (17%) patients recieved both types were administered. All these patients presented a drop in platelet count after initiating heparin. HIT was suspected in all them. The grade of HIT suspicion was high in 9 (45%), intermediate in 7 (30%) and low in 5 (25%) patients. Heparin-dependent IgG antibodies were tested in all

patients. In 11 patients the test was positive. Heparin was replaced by fondaparinux at a dose between 1.5 and 7.5 mg daily. The dose depended on the indication of anticoagulant therapy and patient creatinine clearance and weight. After three days tratment an incresed of at least 30% occured in 17 (80%) patients. Treatment with fondaparinux was maintained a median of 39 days (range 1-180). Seven patiens (30%) were undergoing hemodyalisis. In six cases the procedure was hemodiafiltration and in one conventional hemodyalisis. In these patients fondaparinux dose was adjusted according to anti-factor Xa levels. A median of 5 (range 1-12) controls were made. Only one thrombotic complication was registered during fondaparinux treatment, a deep vein thrombosis of the leg seven days after discontinuation of heparin. No hemorrhagic complications were registered. *Conclusions*. This study suggests that fondaparinux may be an alternative to heparin therapy in patients in who HIT is suspected. Fondaparinux seems to be safe even in patients with renal failuire undergoing hemodialysis. The dose in these patients should be adjusted according to anti-Xa levels. Anyway, we should be aware of thrombotic complications during fondaparinux therapy in patient affected of HIT.

0230

THE HOSPITALIZATION NEED AND COST OF ADULT PRIMARY IMMUNE THROMBOCYTOPENIA TREATMENT: A MONOCENTRIC EVALUATION

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Background. Primary immune thrombocytopenia (ITP) results in an increase of healthcare resource utilization and hospitalization rate, for diagnosis, medical therapy, surgery and treatment of bleeding complications. However, there are not accurate data about ITP related cost. Few studies on the burden of illness were carried out, with some unavoidable limitations, such as the patient selection bias, due to retrospective analysis. Aims. To overcome these difficulties, a prospective study of a consecutive cohort of ITP patients was carried out, focused on the hospital based costs, calculated as the reimbursements to the hospital by the Italian Health Service (HIS), using the Disease Related Group tariffs, for essential thrombocytopenia (ICD-9-CM, code 287.3). In addition, the cost of highly expensive drugs (intravenous immune-globulins, IVIg, and rituximab) prescribed for rescue therapy in ITP was considered. *Methods.* All patients with a consecutive diagnosis of primary ITP (ASH 1996 criteria), from January 1997 to May 2007, age above 18 years and platelet count <50×10^9/L were enrolled. Data on patients demographics characteristics, medical history and hospital admission were collected. *Results.* From the 170 patients diagnosed during the observation time at our Institution (male 61, 35%, median age 41, range 18-95 years), 18 were excluded from the evaluation because never treated or admitted. 152 patients (median platelet count at the start of therapy: 10×10^9/L, range 1-48) received at least one line of treatment; 80 patients required at least one hospital admission, for a total of 126 admissions (1.5/patient): 45 at the time of diagnosis, 37 for relapse or ITP-related complications, 44 at Surgery Department for splenectomy (ICD-9-CM code: 41.2). Patients with significant bleeding symptoms (WHO score 2 or 3) were more frequently admitted at diagnosis than non or mild-bleeders, WHO 0 or 1 (35/72, 48%, vs. 10/80, 12.5%, Fischer exact test <0.001). The total admission days was 775 (median 6 days/admission; 10 days/patient). The cycles of IVIg infusion were 198 (one cycle = 1 g/Kgw/day x 2 or 400 mg/Kw/day x 5); of rituximab infusion were 10 cycles (one cycle = 375 mg/m^2 weekly x 4). The cumulative reimbursement (at 2005 tariffs) by HIS was 227.000 euro and 418.000 euro for medical and surgical discharged, respectively. The cost of IVIg and rituximab at the hospital price purchased was 994.000 euro; the total cost was thus 1.639.000 euro (10.780/patient). At last follow-up visit, (after a median of 6 years) 101 patients were in remission and out of treatment (complete response, platelet count >100×10^9/L or response, platelet count >30×10^9/L). 40 (26%; 12 after splenectomy) were in non-response and required continuous medical therapy; limiting the analysis to this subgroup, the median cost for patients was 20.125 euro. 11 patients were deceased (one for hemorrhagic complications). Conclusions. Taking into account the limitations of this analysis (DRG reimbursement tariffs do not reliable the real cost of assistance; moreover, resource medical utilization for outpatients care were not considered) the median costs for the hospital assistance for ITP appear to be in the lower range of the most common chronic disease; however, major expenses were due to splenectomy interventions and recurrent admissions for high-cost drugs as rescue therapies. Our estimates offer a rough basis for the comparison of the treatment cost incorporating new drugs.

0231

DURABLE AND OVERALL PLATELET RESPONSES IN PATIENTS WITH CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA DURING LONG-TERM TREATMENT WITH ORAL ELTROMBOPAG BY SPLENECTOMY STATUS: THE RAISE STUDY

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Background. Eltrombopag is the first, oral, small molecule, non-peptide, thrombopoietin receptor agonist being studied for the treatment of thrombocytopenia related to a variety of conditions. *Aim.* To assess the efficacy of eltrombopag as measured by a durable platelet response in previously treated adults with chronic immune thrombocytopenia (ITP) and platelet counts <30 Gi/L, by splenectomy status. *Methods.* RAISE was the largest 6-month, randomized (2:1 [eltrombopag:placebo]) double-blind, placebo-controlled phase 3 study in patients with chronic ITP. The primary efficacy analysis has been previously presented (Cheng *et al.* Blood 2008;112:400). Additional post-hoc analyses have been performed to ascertain the effect of eltrombopag on durable and overall response by splenectomy status. Durable response was defined as having a platelet count elevation ≥50 and ″400 Gi/L for at least 6 of the last 8 weeks in the 6-month treatment period. Overall response was defined as having either a durable response or a transient response with platelet count elevations ≥50 and ″400 Gi/L for at least 4 consecutive weeks at any time during the 6-month treatment period. All patients who received a rescue medication (conservatively defined as any increased dose of baseline ITP medication, a new ITP medication, platelet transfusion, and/or splenectomy) during the 6-month treatment period were not considered to have a durable platelet response, irrespective of platelet counts. Platelet count elevations occurring during periods of rescue and up to the time platelet counts fell below 50 Gi/L were not included in the assessment of transient response. *Results.* Durable response was achieved by 60% of patients in the eltrombopag group versus 10% in the placebo group. Overall response was achieved in 81% of patients in the eltrombopag group versus 18% of patients in the placebo group, and similar overall responses were observed in both splenectomized (70%) and non-splenectomized (88%) cohorts treated with eltrombopag. Fifty-one percent (51%) of splenectomized and 66% of non-splenectomized patients treated with eltrombopag also achieved a durable platelet response. *Conclusions.* In this 6-month study, both splenectomized and non-splenectomized patients achieved more durable and overall platelet responses when treated with eltrombopag compared with placebo.

Table 1. Summary of Durable and Overall Response During the 6-Month On-Therapy Period – ITT Population

		Treatment Group	
		Placebo N=39	Eltrombopag N=95
All Patients	Durable Response, n (%)	4 (10)	57 (60)
	Overall Response, n (%)	7 (18)	77 (81)
Baseline Splenectomy Status			
Splenectomy	Splenectomized, n	12	37
	Durable Response, n (%)	1 (8)	19 (51)
	Overall Response, n (%)	2 (17)	26 (70)
No Splenectomy	Non-splenectomized, n	27	58
	Durable Response, n (%)	3 (11)	38 (66)
	Overall Response, n (%)	5 (18)	51 (88)

0232

STUDY OF THE *IN VITRO* EFFECT OF THE NONPEPTIDE THROMBOPOIETIN -RECEPTOR AGONIST SB-497115 (ELTROMBOPAG) ON MEGAKARYOPOIESIS OF PATIENTS WITH LOW/INTERMEDIATE-I RISK MYELODYSPLASTIC SYNDROME

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Background. Thrombocytopenia remains a major problem in myelodysplastic syndromes (MDS) because an effective thrombopoiesis-stimulating agent has not been available so far for these patients. The aim of the present study is to evaluate the *in vitro* effect of the nonpeptide thrombopoietin-receptor agonist SB-497115 (Eltrombopag) on bone marrow (BM) megakaryopoiesis of patients with MDS. This agent has been

successfully given as an oral agent for the treatment of patients with Idiopathic Thrombocytopenic Purpura. *Methods.* BM aspiration was performed in 5 MDS patients with low/intermediate-1 risk according to the International Prognostic Scoring System (IPSS) and 3 haematologically normal controls and the BM mononuclear cells (BMMCs) were isolated after gradient centrifugation. We evaluated the growth of the megakaryocytic colony forming units (CFU-Meg) in the BMMC fraction using the commercially available Mega-Cult medium in the presence or absence of different concentrations of SB-497115 (0.1 μg/mL-30 μg/mL). CFU-Megs were scored after fixation and staining of culture slides with anti-CD41 monoclonal antibody using the alkaline phosphatase anti-alkaline phosphatase technique (APAAP). We also studied the effect of the above concentrations of SB-497115 on the survival characteristics of BMMCs, following overnight incubation, by means of flow-cytometry and 7-amino-actinomycin D (7AAD). *Results.* The number of CFU-Meg in MDS patients increased significantly following 14-day incubation of BMMCs with 0.1 μg/mL SB-497115 (97.80±23.93 CFU-Meg per 5×10^4 BMMCs) compared to baseline (73.60±34.20 CFU-Meg per 5×10^4 BMMCs; p=0.047, paired t-test). An increase was also obtained in the number of CFU-Meg obtained from healthy controls following incubation of BMMCs with 0.1μg/mL SB-497115 (239±37.32 CFU-Meg per 5×10^4 BMMCs) compared to baseline (176.33±53 CFU-Meg per 5×10^4 BMMCs; p=0.116, paired t-test). In the presence of higher concentrations (1.0 μg/mL-30μg/mL) of SB-497115, a dose-dependent decrease was obtained in the frequency of CFU-Meg in both patients and healthy controls in comparison to untreated cultures although not at a statistically significant level (p=0.4751 and p=0.290, respectively; one-way ANOVA). In accordance with the CFU-Meg data were the flow-cytometry results. In the presence of 0.1 μg/mL SB-497115 the proportion of apoptotic (7AADdim+bright) BMMCs remained unchanged in both patients and healthy controls (12.98±11.84% and 2.13±2.13%, respectively) compared to baseline (12.64±11.93% and 2.27±2.37%, respectively) suggesting a non-toxic effect of this concentration. However, in the presence of higher doses (1.0 μg/mL-30 μg/mL) of SB-497115, the proportion of apoptotic BMMCs increased at a dose-dependent manner in both patient and control groups although not at a statistically significant level (p=0.4018 and p=0.1377, respectively). A pooled analysis of patient and control data showed a statistically significant increase in the proportion of apoptotic BMMCs in the presence of SB-497115 at a dose ≥1 μg/mL (1.0μg/mL-30μg/mL), compared to baseline (p=0.0402, one-way ANOVA). *Summary and Conclusions.* These preliminary data suggest that SB-497115 at a dose of 0.1μg/mL displays a beneficial effect on megakaryopoiesis of MDS patients with low/intermediate-I IPSS without any adverse effect on BMMC survival. These encouraging *in vitro* data indicate a possible clinical beneficial effect of Eltrombopag in this group of patients. Studies investigating the *in vitro* effect of lower SB-497115 concentrations are in progress.

0233

IMPROVEMENT IN FATIGUE AND HEALTH-RELATED QUALITY OF LIFE (HRQOL) WITH LONG-TERM ELTROMBOPAG THERAPY IN CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA: RESULTS OF PHASE 3, DOUBLE-BLIND STUDY(RAISE)

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Background. Eltrombopag is the first, oral, small molecule, non-peptide, thrombopoietin receptor agonist for the treatment of chronic idiopathic thrombocytopenic purpura (ITP). *Aim.* To assess the effects of long-term eltrombopag treatment on HRQoL in patients with chronic ITP. *Methods.* RAISE was a 6-month, randomized, double-blind, placebo-controlled, phase 3 study that evaluated the efficacy and safety of eltrombopag in previously treated adults with chronic ITP and platelet counts <30.000/μL. Patients were stratified by splenectomy status, use of baseline ITP medication, and platelets ″15.000/μL. Efficacy results (eg, platelet count, bleeding, reduction in ITP medications), and safety results were previously reported (Cheng *et al.* Blood 2008;112:400). HRQoL was self-reported at baseline, week 6, week 14, and upon completion or withdrawal from study using the SF-36v2, fatigue sub-scale of FACIT-Fatigue, and a 6-item subset of the FACT-thrombocytopenia (FACT-Th) that focuses on concern with bleeding and bruising, and limits to physical and social activities. Maintenance of eltrombopag therapy over time on mean outcomes was assessed using longitudinal generalized estimating equation models and exchangeable correlation structures. Minimally important differences in responses were defined as improvement from baseline of at least 1/2 the standard deviation of the baseline score. Further adjustment was made for stratification variables. *Results.* One hundred ninety-seven (197) patients (eltrombopag, 135; placebo, 62) were enrolled in RAISE; baseline characteristics were balanced: in both arms ~50% of patients had platelet counts ″15.000/μL, ~50% were receiving baseline concomitant ITP therapies, ~35% were splenectomized, and >15% had received at least 3 prior ITP medications. During 6 months of eltrombopag treatment, patients reported meaningful improvements in vitality, emotional and physical role domains, and in overall mental health. Patients consistently reported clinically and statistically significant benefits in physical and social activities and in concerns for bleeding and bruising symptoms. The odds of observing a minimal important difference during treatment with eltrombopag was significantly greater for the eltrombopag group compared with the placebo group, further supporting the reported results (data not shown). No decreases (relative to baseline) in mean HRQoL scores in any category were reported during eltrombopag treatment. *Conclusions.* This evidence suggests that long-term treatment with eltrombopag improves vitality/fatigue, physical role, and emotional role for patients with chronic ITP. With eltrombopag treatment, patients perceived a reduction in fatigue symptoms and an improved ability to participate in activities of daily living.

Table.

Patient-Reported Outcome: Instrument and Domain	Average Effect of Eltrombopag vs Placebo on Score Change From Baseline[a] (ITT population) Estimate of Score Change	95% CI	*P* value
SF-36v2			
Physical Function	2.8	-1.1, 6.7	0.154
Physical Role	5.4	0.5, 10.3	0.030[b]
Bodily Pain	5.1	-0.5, 10.6	0.074[c]
General Health	2.4	-1.6, 6.5	0.243
Vitality	3.9	0.1, 7.7	0.045[b]
Social Function	4.1	-0.6, 8.9	0.089[c]
Emotional Role	5.4	0.8, 10.1	0.023[b]
Mental Health	2.5	-0.9, 6.0	0.154
Physical Component Summary[d]	1.3	-0.2, 2.9	0.092[c]
Mental Component Summary[e]	2.1	0.2, 4.0	0.030[b]
FACIT-Fatigue	1.6	-0.2, 3.5	0.082[c]
FACT-Th (6 selected items)	1.5	0.5, 2.5	0.004[b]

CI, confidence interval; ITT, intent to treat

a. Estimated from a longitudinal regression model for the effect of eltrombopag vs placebo of on-therapy score changes from baseline.
b. Indicates significant at 5% level of significance.
c. Indicates significant at 10% level of significance.
d. Physical Component Summary includes all domains but Physical Function, Physical Role, Bodily Pain and General Health contribute proportionately the most to the aggregate scores.
e. Mental Component Summary includes all domains but Vitality, Social Function, Emotional Role and Mental Health contribute proportionately the most to the aggregate scores.

0234

USEFULNESS OF PRETEST CLINICAL SCORES ('4T'S) AND THEIR CORRELATION WITH THE RAPID ID-HEPARIN / PF4 ANTIBODY TESTING FOR THE DIAGNOSIS OF HEPARIN INDUCED THROMBOCYTOPENIA

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Background. Heparin-induced thrombocytopenia (HIT) is a serious thrombocytopenic and thrombotic condition. A prompt diagnosis and immediate replacement of heparin with an alternative anticoagulant is required to prevent life threatening thrombotic complications of HIT, however, differing operational characteristics of the laboratory assays and the common occurrence of thrombocytopenia makes diagnosis of HIT difficult. *Aim.* We evaluated the usefulness of a pretest clinical scoring system "the 4T's" (BCSH Guidelines 2006) in combination with a rapid Particle Gel Agglutination Assay (PAGIA) in diagnosing the probability of HIT in 111 patients at a single centre. *Materials and Methods.* The "4Ts" includes (i) the severity (ii) timing (iii) association with thrombosis and (iv) likely cause of the thrombocytopenia and scores patients into low (0-3), intermediate (4-5) and high (6-8) risk groups of having HIT. ID-PAGIA (Diamed, Dalkeith, Scotland) shows agglutination of polymer particles coated with Heparin/PF4 (HPF4) complexes in the presence of anti - heparin/PF4 complex antibodies. 4T score was calcu-

lated either retrospectively (58 unselected patients) or prospectively after a request form including the score and management advice had been devised to aid clinicians (53 patients). *Results.* None of the low "4Ts" were reported test positive for HIT (36/36). The risk of a positive HIT test increased with the increasing 4Ts scores (29% of intermediate (15/51) and 61% of high risk (14/23) respectively). PAGIA showed a false negative rate of 20% when judged against the clinical decision having HIT. Amongst the patients having clinical and serological diagnosis of HIT (29/111), 86% had a platelet fall of >50%, 24% had thrombosis and 63% had suspicion on another potential cause for thrombocytopenia. *Conclusions.* The pretest clinical scoring can be difficult due to involvement of other potential aetiologies for the thrombocytopenia. However, the "4Ts" appear very useful in ruling out HIT. Patients scored as low risk do not seem to need lab testing (32% avoidable requests in this audit). The risk of HIT is significant with intermediate and particularly with the high scores.

Table 1.

Characteristics of HIT positive patients 25% (n = 29)	
Indications for Heparin	
Cardiothoracic surgery (n= 56);	**40%** *(n = 12)*
Haemofiltration (n = 23)	**28%** *(n = 8)*
Treat PE/DVT/ACS (n= 11)	**11%** *(n = 3)*
Prophylaxis DVT + others (n=20)	**21%** *(n = 6)*
Day of HIT testing (1st day of heparin = day 0)	
< 5 days (with prior Heparin exposure)	**14%** *(n = 4)*
5 – 14 days	**76%** *(n = 22)*
> 14 days	**10%** *(n = 3)*
Platelet fall > 50% of baseline	**86%** *(n = 25)*
Platelets on 1ST day of heparin Rx	67 – 456 *(213)*
Platelets on day of HIT testing	8 – 104 *(49)*
Thrombotic phenomenon	**24%** *(n = 7)*
Alternative cause for low platelets evident	**63%** *(n = 18)*
4T's scores	
0 – 3; Low risk (n = 36)	**0%** *(n = 0)*
4 – 5; Intermediate (n = 52)	**49%** *(n = 15)*
6 – 8; High risk (n = 23)	**51%** *(n = 14)*
Definite UFH *(+/- LMWH)*	**76 %** *(n = 22)*

0235

PROSPECTIVE STUDY OF ABBREVIATED COURSE RITUXIMAB FOR AUTOIMMUNE HAEMATOLOGICAL DISORDERS REFRACTORY TO STANDARD IMMUNOSUPPRESSIVE THERAPY

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Background. Profound B cell depletion by rituximab (R) has led to its off-label use for antibody-mediated autoimmune haematological disorders (AHD) adopting standard anti-lymphoma dosing (375 mg/m^2 weekly x 4). There are limited data, however, on the efficacy of abbreviated schedules which may be more cost-effective. *Aim.* To investigate prospectively the efficacy of abbreviated dose R in a variety of AHD refractory to conventional immunosuppression (IS). *Methods.* Informed consent was obtained in all eligible adult patients (pts) who received one dose of R (375 mg/m^2) as part of salvage therapy. A repeat dose was indicated if no response (NR) was seen 4-8 weeks after first infusion, or relapse after achieving complete (CR) or partial response (PR). Planned follow-up was 36 months and disease-specific autoantibodies, lymphocyte subsets and serum R levels were obtained. Concomitant IS was tapered at physician's discretion after two weeks according to response using standard criteria. *Results.* 30 eligible patients with median age of 55 years (range 21-84) were enrolled from March 2007 to February 2009 with 13 pts receiving two doses. Haematological conditions included refractory or recurrent immune thrombocytopenia (ITP), thrombotic thrombocytopenic purpura (TTP), refractory autoimmune haemolysis (AIHA), and miscellaneous conditions. Table 1 describes the results in ITP and TTP. ITP: 11 pts were on prednisolone (median dose 25 mg/d) for a mean duration of 2 weeks and 4 pts received additional IVIG 1 week prior to R; 2 pts had prior splenectomy. The median platelet count immediate prior to R was 21 (range 4 to 54) $\times 10^9$/L. 2 pts treated at relapse by single dose of R alone have attained CR for 15+ and 31+ wks respectively; in 5 other complete responders, prednisolone was ceased after a median of 6 weeks post R. TTP: 4 pts were given single dose R as maintenance after achieving CR at completion of plasma exchanges (PE) during first episode. R was combined with PE as salvage therapy in 5 others with refractory or relapsed TTP. AIHA: 2 pts had unsustained PR (defined as transfusion-independence and positive haemolytic markers) requiring splenectomy and 1 pt remains in CR for 25+ wks. Responses in other disorders: cold agglutinin disease [n=1] - PR for 76+ wks; refractory Evans syndrome [n=1] - failed; acquired factor VIII inhibitor in first relapse [n=1] - CR for 12+ wks; combined autoimmune neutropenia and haemolysis [n=2] with one unsustained and one ongoing CR. In the whole study population, no grade 2-4 infusion toxicity was observed. 2 pts died from refractory TTP and stroke. Grade 3 infection was observed in 2 pts. In pts receiving a single infusion, peripheral blood B cells were detectable at or above the lower limit of normal (i.e. $\geq 0.05\times10^9$/L) earliest at week 24; the median B cell number in these patients at 12, 24, 48, and 72 weeks was 0.01, 0.03, 0.26 and 0.31×10^9/L; serum R levels peaked at week 2 at a median of 30 mcg/mL and became undetectable beyond week 8. *Conclusions.* The preliminary data suggest abbreviated R is safe and sustained response is achievable in refractory/relapsing AHD.

Table 1.

	N=	Response (number)	Median time to attain CR	Duration of CR (weeks)
ITP	13	CR# after 1 dose: 7 CR after 2 doses: 1 PR^:2 NR:3	3 weeks (range: 1-10)	7+, 10*, 11+, 15+, 31+, 34*, 40+, 43+
TTP: first episode Post PE	5	CR: 4 NR: 1 (death)	N/A	58+, 59+, 101+, 104+
TTP: relapsed	4	CR: 4	N/A	7+, 18+, 66+, 73+

CR#: platelet $\geq 100\times10^9$/L; PR^: platelet $50\text{-}100\times10^9$/L; * denotes relapse

0236

CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA THERAPIES: THE PATIENTS' PERSPECTIVE ON BOTHERSOME EFFECTS

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Background. Multiple therapies are available for treatment of chronic idiopathic thrombocytopenic purpura (ITP); however, these treatments are associated with side effects that are poorly tolerated. Little information is currently documented on the impact of these side effects from the patients' perspective. *Aim.* The objective of this study was to quantify patient-perceived burden associated with frequently used chronic ITP therapies. *Methods.* Patients who were ≥18 years, had a self-reported history of chronic ITP (>6 months), had been exposed to corticosteroids, IVIg, anti-D, or rituximab, and belonged to a patient ITP support association in the United States were surveyed. Patients completed an online questionnaire that was developed from a literature review using clinician and patient input. Data on demographics, patient disease histories, ITP therapy-related side effects and associated levels of bother were collected in this IRB-approved study in September 2008. The level of bother for each of 26 potential side effects associated with treatment was measured using a 5-point Likert scale in which 1 = not bothered at all and 5 = extremely bothered. *Results.* Five hundred eighty-nine (589) patients completed the survey. Most were female (78%), Caucasian (89%), and diagnosed with ITP for ≥5 years (59%). Corticosteroids were the most frequently reported therapy (n=542, 92%), followed by IVIg (n=322, 56%), rituximab (n=213, 36%), and anti-D (n=209, 36%). Seventy-three percent (73%) and 98% of respondents reported ≥1 side effect associat-

ed with rituximab and corticosteroid treatments, respectively. Corticosteroid-treated patients reported significantly more side effects, compared with other therapies (mean[SD]): corticosteroids (10.7[5.7]); anti-D (2.4[2.2]); IVIg (2.3[2.2]); rituximab (2.1[2.2]) (p<0.0001 for all). The mean level of bother reported for corticosteroids side effects was substantially higher compared with other therapies (mean[SD]): corticosteroids (3.67[0.78]); anti-D (2.98[1.39]); IVIg (2.97[1.39]); rituximab (2.77[1.41]) (p<0.0001 for all). Between 22% (rituximab) and 39% (corticosteroids) of respondents reported the need to stop or reduce their dose of the medication due to side effects of treatment. *Conclusions.* ITP patients recognize, remember, and report multiple bothersome side effects that may lead to dose reduction or treatment discontinuation. Corticosteroid treatments are associated with the greatest number of bothersome and potentially serious side effects. Clinicians and patients may want to consider patient-perceived burden of ITP therapies when making treatment choices.

0237

THROMBOPOIETIN RECEPTOR EXPRESSION IN INHERITED AND ACQUIRED PLATELET DISORDERS

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Background. The interaction between thrombopoietin (TPO) and thrombopoietin receptor (cMpl) appears to be critical in the initial phases of thrombopoiesis (platelet production) promoting $CD34^+$ differentiation, megakaryocytes (MK) proliferation and maturation via cytoplasmatic down-stream signalling. The second consequence of TPO/cMpl interaction is the regulation of TPO level. On platelet surface, there are about 100 high affinity cMpl binding sites that bind, internalize and degrade TPO by a time course process constituting the main regulation of TPO levels. This model explains the high amount of TPO observed in thrombocytopenia secondary to MK hypoplasia. However, TPO levels are unexpected low in immune thrombocytopenia as well in inherited macrothrombocytopenia, and unexpected variable in patients with primary thrombocythemia (ET) where cMpl is underexposed. These observations may be potentially explained by different expression of the receptor on platelets and/or MK in platelet disorders. *Aim of the study.* We aimed to investigate platelet cMpl expression in several familial and acquired platelet disorders in order to clarify their pathogenesis and to potentially better address the treatment of immune thrombocytopenia with the cMpl receptor agonists. *Material, methods and patients*. We studied 34 patients suffering from inherited and acquired platelet disorders: immune thrombocytopenic purpura (ITP),10 patients; megakaryocyte hypoplasia thrombocytopenia (MK-Hyp) 9 patients; inherited thrombocytopenia (FAM) 8 patients: MYH9 related disorder, 1 patient; Bernard-Soulier 1 patient; heterozygous Bernard-Soulier type N41H, 4 patients; heterozygous Bernard-Soulier Bolzano type 2 patients; essential thrombocythemia (ET), 7 patients. We studied 13 healthy subjects as control group. cMpl expression has been evaluated by Western-Blot on 2×10^7 platelet lysates using 1 μg/mL rabbit polyclonal anti-Human cMpl antibody. Anti CD41-clone SZ22 monoclonal antibody against platelet GPIb complex was used as control on the same membranes, after stripping anti-cMpl antibody. For each sample, expression of both cMpl and anti CD41 was quantitated by densitometry analysis of Western Blot. The results are given as mean cMpl/CD41 expression ratio for each group of patients, and as the percent of value of healthy controls set as 100%. *Results.* The results are shown in Table 1.

Table 1. cMpl expression in platelet disorders.

	N	Platelet count $x10^9$/L (mean ±SD)	cMpl/CD41 ratio ($2x10^7$ Plt) (mean ±SD)	cMpl, % of controls (mean)
Controls	13	254 ± 46	0.136 ± 0.103	----
ITP	10	30 ± 20	0.434 ± 0.291	320%
FAM	8	87 ± 25	0.276 ± 0.188	203%
MK-Hyp	9	54 ± 18	0.314 ± 0.243	231%
ET	7	865 ± 372	0.109 ± 0.094	80%

In all groups of patients we observed a heterogeneous pattern of cMpl expression. However, all thrombocytopenic patients showed a greater platelet cMpl expression than controls and ET patients where cMpl is lower. Among thrombocytopenic patients, the increase in cMpl was more pronounced in ITP patients than in those with inherited and MK-Hyp thrombocytopenia. *Conclusions.* Our data seem to confirm that cMpl expression might be inversely regulated by platelet count. Surprisingly, cMpl expression shows a similar pattern in ITP and MK-Hyp patients, despite opposite levels of TPO that have been described in these two types of thrombocytopenia. This observation may be potentially explained by a different cMpl expression on platelet surface in spite of the same total amount of cMpl.

0238

EFFICACY AND SAFETY OF INTRAVENOUS ANTI-D TREATMENT OF PATIENTS WITH REFRACTORY AUTOIMMUNE THROMBOCYTOPENIC PURPURA

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Background. Autoimmune thrombocytopenic purpura (AITP) is an autoantibody-mediated hematological disorder in which platelets are prematurely destroyed by the reticuloendothelial system. The initial treatment, according to the American Society of Hematology practice guidelines for ITP in 1996, is a steroid course but a significant percentage (20%-30%) of adult patients remain with PLT levels<20000/μL and need immediate therapeutic intervention to avoid severe bleeding. Intravenous Immunoglobulin treatment and intravenous (IV) Anti-D treatment, both with similar action mechanisms, have been used for this cause. *Aims.* In this study we investigate whether repeated infusions of intravenous anti-D globulin could allow adults with Autoimmune Thrombocytopenic Purpura (AITP) who had failed an initial steroid course to postpone and ultimately avoid splenectomy. *Methods.* Eleven Rhesus positive ITP patients, [9 under relapse and with resistant to previous treatments (steroids or IVIg) disease and 2 on diagnosis] received IV anti-D globulin in repeated doses of 25 μg/Kg every 21 days. Prior to treatment all the patients had PLT levels <30000/μL and had denied splenectomy. Attainment of PLT levels >50000/μL after 7 days was defined as response to treatment. The patients' median age was 36 years (extreme values: 18-82). *Results.* Eight patients (80%) completely responded to treatment with a PLT median value of 89000/μL (extreme values: 50000-150000/μL). 1/8 relapsed after interruption of treatment for 64 days and repeated it successfully with doses of 40 μg/Kg. 7/8 patients have completed 12 cycles of treatment. 1/8 patients completed 10 cycles and maintained normal PLT levels. 3/8 patients are to the present day healthy without any maintenance therapy. 1/11 responded partially, having received only one cycle of treatment, and remains with PLT levels around 50000/μL 12 months later. 2/11 patients did not respond at all. Headache was recorded on 3 patients and 1/7 suffered hemolysis (-b=9 gr/dL) after receiving the second treatment cycle in less than 21 days. *Conclusions.* Despite the small number of patients, it seems that repeated low-dose IV anti-D globulin treatment: 1) is a safe and effective treatment for patients with chronic ITP and 2) removes the need of splenectomy without, anyhow, offering a permanent solution. For safer conclusions, by all means, the necessity of a study of a larger number of patients for a longer period of time should be under imperative consideration.

0239

IMPROVEMENT IN FATIGUE AND HEALTH-RELATED QUALITY OF LIFE WITH LONG-TERM ELTROMBOPAG TREATMENT IN ADULTS WITH CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA: THE EXTEND STUDY

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Background. ITP is characterized by autoantibody-induced platelet destruction and reduced platelet production, leading to low platelet

counts(<150Gi/L). Eltrombopag treatment is aimed at elevating and maintaining platelets to ≥50Gi/L to minimize risk of bleeding, to reduce or discontinue concomitant medications including corticosteroids, and to improve health-related quality of life (HRQoL). *Aim.* To assess effects of eltrombopag on fatigue and other HRQoL measures among chronic immune thrombocytopenia (ITP) patients in the EXTEND study. *Methods.* EXTEND is an ongoing, open-label extension study in chronic ITP patients previously enrolled in an eltrombopag trial. Interim efficacy and safety analyses have been reported (Bussel *et al.* Blood 2008;112:3432); however, HRQoL data have not. In this study, both eltrombopag and concomitant ITP medications could be dose-adjusted to the minimal treatment necessary to maintain platelet counts in a safe range. HRQoL, self-reported at baseline and upon progression toward establishing an effective dose, was measured with: 1) SF-36v2 (comprises 8 domains, mental component summary [MCS], and physical component summary [PCS]); 2) FACIT-Fatigue subscale; and 3) 6 items from FACT-Thrombocytopenia (FACT-Th) focusing on bruising and bleeding concerns, and limits in daily physical and social activities.

Table.

HRQoL Measure	Baseline Score (95% CI)	Dosing Initiation (Stage 1)	Minimizing ITP Medications (Stage 2)	Optimizing Dosing (Stage 3)	Maintenance Dosing (Stage 4)
		Estimated Change From Baseline (95% CI)			
SF-36v2 Physical Component Summary (PCS)	46.1 (45.0–47.2)	NS	NS	2.2 (0.6–3.7) *P*=0.007	2.1 (0.4–3.8) *P*=0.015
Physical Functioning Domain	73.0 (69.8–76.1)	NS	NS	5.2 (1.1–9.2) *P*=0.013	NS
Physical Role Domain	68.8 (65.1–72.4)	4.6 (0.8–8.3) *P*=0.016	NS	6.1 (0.9–11.2) *P*=0.020	NS
Bodily Pain Domain	74.1 (70.7–77.5)	NS	NS	NS	NS
General Health Domain	52.1 (49.2–55.0)	NS	NS	6.0 (1.6–10.3) *P*=0.007	6.4 (1.4–11.4) *P*=0.012
SF-36v2 Mental Component Summary (MCS)	46.0 (44.4–47.5)	2.1 (0.5–3.7) *P*=0.009	3.9 (1.5–6.3) *P*=0.001	NS	NS
Vitality Domain	54.3 (51.1–57.4)	NS	6.3 (0.7–12.0) *P*=0.026	NS	NS
Social Functioning Domain	75.3 (72.1–78.6)	4.9 (1.6–8.2) *P*=0.003	NS	5.9 (1.1–10.7) *P*=0.016	NS
Emotional Role Domain	76.2 (72.9–79.5)	3.6 (0.1–7.2) *P*=0.042	4.4 (0.2–8.6) *P*=0.039	NS	NS
Mental Health Domain	68.7 (66.1–71.4)	3.2 (0.5–5.9) *P*=0.021	6.8 (2.4–11.1) *P*=0.002	NS	NS
FACIT-Fatigue	36.1 (34.5–37.7)	NS	NS	NS	NS
FACT-Th	14.3 (13.4–15.2)	2.0 (1.2–2.7) *P*<0.001	2.1 (0.8–3.4) *P*=0.002	2.9 (1.9–3.8) *P*<0.001	2.7 (1.2–4.3) *P*=0.001

CI, confidence interval; NS, not statistically significant.
Covariates included: time since baseline visit, stage, prior splenectomy, baseline platelet count ≤15 Gi/L, age, ethnicity, BMI, region, sex, country, prior exposure to eltrombopag, and treatment termination status.

Treatment effect was assessed using longitudinal models with generalized estimating equations methodology and an exchangeable correlation structure. *Results.* At time of this analysis, 144/207 patients in EXTEND had both baseline and on-therapy HRQoL assessments. At baseline, patients had a median age of 50 years, 67% were female, 33%(n=69) were receiving concomitant ITP medication, 40%(n=82) had been splenectomized; 57% had ≥3 prior therapies. Baseline SF-36 domain and summary scores were consistent with those reported in other eltrombopag studies (Bussel *et al.* New Engl J Med 2007) reflecting impaired HRQoL in chronic ITP patients compared with general US population (Ware *et al.*, 2000). Similarly, baseline FACIT-Fatigue scores were worse than observed for general US population (Cella, 2002). Improvements from baseline in HRQoL were observed throughout EXTEND. Statistically significant, clinically meaningful improvements from baseline were reported in SF-36v2 MCS during initiation of treatment with eltrombopag (p=0.009) and during reduction of concomitant ITP medications (p=0.001). Improvements from baseline in SF-36v2 PCS were reported during eltrombopag dose adjustment (p=0.007) and maintenance dosing (p=0.015). Improvements in vitality, social functioning, mental health, and emotional role were reported during initiation of eltrombopag dosing and minimization of concomitant ITP medication, whereas improvements in physical functioning, physical role, and general health domains of the SF-36v2 tended to be observed once a stable platelet count elevation was achieved and maintenance dosing of eltrombopag was initiated. Improvements from baseline in the FACT-Th were observed throughout therapy(p″0.002) evidenced as reductions in bleeding/bruising concerns and improved capacity for physical and social activities. *Conclusions.* In the EXTEND study, health status and fatigue among those with chronic ITP is worse than reported for the general US population. EXTEND study results also suggest improvement in HRQoL, across measures and domains. With eltrombopag treatment, patients initially report improvement in MCS and subsequent improvement in PCS; the difference in response time may reflect both increased confidence in activities from improvements in MCS and reduction in concomitant medications.

0240

MICOPHENOLAT MOPHETIL IN THE TREATMENT OF REFRACTORY ADULT CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA

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Aim. The authors evaluate the treatment with mycophenolate mophetil (MM) of refractory chronic immune thrombocytopenic purpura (ITP) and outcome over a period of 8 years in a single institution. *Patients and Methods.* data from 16 patients (pts) suffering from ITP followed in our Institution between 2001 and January 2009. refractory to more than two therapeutic modalities were collected. The diagnosis required a platelet count <50×10^9/L and exclusion of other haematological and non haematological disorders. There were 11 females and 5 males with median age of 51 years. In 11 pts (67%) the haemorrhagic syndrome (purpura and petechial bleeding) was present, 1 pts (6%) had gastrointestinal bleeding and 5 pts (24%) were asymptomatic. 9 pts (56%) had severe thrombocytopenia (<10^9/L) at diagnosis. Splenectomy was performed in 10 patients and all had relapsed after splenectomy. Ten pts showed untoward effects to danazol and prolonged corticosteroids administration. In 8 pts several treatment modalities were applied including azathioprin, ciklosporin, i.v. immunoglobulins and long infusion of vincristine without effect. Median platelet count before MM was 7×10^9/L .MM was administered per orally in doses 2000 mg daily. *Results.* MM therapy was tolerated exceptionally well without any clinically significant side effect. At a median of follow up of 10,3 months (range 2-32), 14 patients responded to treatment, 9 (56.2%) with complete remission and 5 (31.2) with partial response, 2 (12.5%)pts did not respond. Four patients maintain platelet count within normal range after withdrawal of MM, whereas 6 have platelet count between 50-100×10^9/L without reccurence of severe ITP. *Conclusion.* Micophenolat mophetil appears as a favorable immunomodulatory agent for the treatment of chronic autoimmune thrombocytopenia purpura or in the case of old patients with contraindication to splenectomy and/or unresposivness to other treatment modalities.

0241

RITUXIMAB IN THE TREATMENT OF PATIENTS WITH REFRACTORY IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Aims. To evaluate the efficacy and safety of Rituximab in patients with refractory idiopathic thrombocytopenic purpura as well as to analize potential factors in relation with the results. *Material and Methods.* A retrospective study has been performed at our medical centres involving the use of Rituximab in 17 patients who had been diagnosed of idiopathic thrombocytopenic purpura. All of them, who had been unresponsive to steroids and/or immunoglobulin, received Rituximab (375 mg/m^2 weekly for 4 doses) as rescue therapy between 1996 and 2007. It was used as second or third line therapy in 13 of 17 patients, and 3 of them had been previously splenectomized. The median time from the diagnosis was 13 months (range,0-314). There were 13 women and 4 men, with a median age of 48 years (range,5 to 82). The median platelet count at baseline was 5,5×10^9/L (range,1 to 54) and 15 of 17 had mucocutaneous hemorrhagic syndrome at the beginning. We found no etiology for 11 patients and we only detected platelet antibodies in 7 of them. *Results.* Complete Remission (>100-10^9/L) was achieved in 12 patients (71%) and Partial Remission (>50-10^9/L) in another 2 (11%), only 3 of them showed No Response. The median follow-up was 7 months (range 2-32) and the median time from the first Rituximab dose to any response was 4 weeks (range, 1-8). Only 4 patients (28%) experimented relapse beyond the twelveth month of follow-up (range, 7-32). There were no severe adverse events during drug administration, and increased number of infections

were not recorded either. We found some response related factors with different scientific evidence: age (remission in patients older than 18 years was 100% while it was only reached in 25% patients younger than 18 years: p=0.001), number of treatment regimens received before Rituximab (remission when Rituximab was used as second or third line therapy was 91% while it was 67% when Rituximab was used at least as fourth line: p=0.21) and detection of platelet antibodies (remission when platelet antibodies were not found was 90% while it was 67% when they were positive: p=0.24). *Conclusions.* The use of Rituximab in refractory idiopathic thrombocytopenic purpura showed a global response rate of 82%. Relapse 30 months after the use of Rituximab was found in 28% patients, and none of them have reached any kind of response with any other later treatment. The factors associated with no Rituximab response were paediatric age, higher number of previous treatment regimens and presence of platelet antibodies. No splenectomized patient was able to reach any kind of response.

0242

CORTICOSTEROIDS FOR THE TREATMENT OF CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA: PATIENT-PERCEIVED BURDEN

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Background. Pharmacologic management of chronic idiopathic thrombocytopenic purpura (ITP) relies heavily on corticosteroids as a first-line treatment. Until now, data on patient-perceived burden associated with corticosteroids have been anecdotal, assumed, or based on the product information. *Aims.* This study evaluated types and numbers of side effects associated with corticosteroids and their level of bother as reported by exposed patients with chronic ITP. *Methods.* Patients ≥18 years with self-reported history of chronic ITP (>6 months) and exposure to corticosteroids were recruited from a US ITP patient support association and surveyed online using a questionnaire developed using a literature review, and clinician and patient input. Demographics, patient and treatment history, and corticosteroid-related treatment side effects data were collected in this IRB-approved study in September 2008. The levels of bother for 26 potential side effects associated with treatment, generated with clinician and patient input, were measured using a 5-point Likert scale (1 = not bothered at all, 5 = extremely bothered). Bother responses were aggregated to express overall burden of reported side effects. Regression modeling predicted level of corticosteroid burden, controlling for factors in patient history, including demographics and clinical characteristics. *Results.* A total of 542 patients completed the survey. Most were female (77%) and Caucasian (90%), and the mean age was 47.5(±14.4). Fifty-nine percent (59%) had been diagnosed with ITP for ≥5 years. Thirty-one percent (31%) were taking corticosteroids at the time of the survey. Fifty-five percent (55%) reported having experienced ≥11 adverse effects or adverse experiences of treatment with corticosteroids, although the constellation of side effects for each patient was unique. Weight gain (83%), changes in personality/mood (77%), problems sleeping (75%) and moon face (67%) were experienced most frequently. More than 50% of patients were highly bothered (4 = bothered quite a bit or 5 = extremely bothered) by these 4 most frequent side effects. The mean level of bother was highest for weight gain (4.2) and moon face (4.2) and averaged 3.67 across all side effects. In a regression model, greater levels of corticosteroid bother were associated with female gender (p<0.001), number of perceived side effects (p<0.0001), and the need to reduce the dose due to side effects (p=0.0011). *Conclusions.* Corticosteroids are the most frequent first-line therapy for the treatment of chronic ITP; however, corticosteroids are associated with multiple side effects, which are substantially bothersome and burdensome to patients. These patient-reported data may make clinical discussions between patient and physician more informative regarding the selection and consequences of treatment with corticosteroids for chronic ITP.

0243

COMPARATIVE EVALUATION OF PLATELET COUNTING ON CELLAVISION DIFFMASTERTM OCTAVIA VERSUS OPTICAL FLUORESCENT PLATELET COUNTING ON SYSMEX XT-2000I AUTOMATED HEMATOLOGY ANALYZER

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Background. CellaVision DiffMasterTM Octavia (DiffMaster) is an automated image analysis system for peripheral blood smears. One of the advantages of the system is the ability to review cases (slides) from a remote location (via internet). The system provides functionality for platelet morphologic features and for estimation of platelet count. The Sysmex XT-2000i incorporates two platelet methodologies, impedance and fluorescent optical (PLT-O). PLT-O is counted by flow cytometry in the reticulocyte channel using a semiconductor laser and nucleic acid fluorescent dye. The PLT-O measurement is an accurate platelet count in specimens where small red blood cells or large platelets are present, because overlapping of populations is avoided. *Aim.* The aim of this study is to evaluate the platelet count on DiffMaster in samples containing microcytic erythrocytes (mean corpuscular volume, MCV ″79fl). *Methods.* All samples included in this study [n=80, MCV 66.5±8.0fl, hemoglobin 11.4±1.5 gr/dL (mean±SD), platelet 58-573×10^3/μL (range)] had been analyzed on Sysmex XT-2000i in the reticulocyte channel. Blood smears were prepared in duplicate with a semiautomated method and stained with May-Grünwald - Giemsa stain. Two laboratory physicians (A, B) counted the platelets (on four images consisting of 36 patched areas of the slide). A calculated estimate of the platelet concentration is made based on the counts per image and a platelet estimate factor. We calculated the mean platelet count of the two observers (score A + score B/2) for each sample. Two comparisons were made: one between the counts of the two physicians and one between DiffMaster (score A + score B/2) and Sysmex XT-2000i PLT-O values. Statistical analysis: the data were analyzed with Pearson correlation and regression analysis. Statistical significance was set at p<0.05. *Results.* Neither fragmented erythrocytes, nor platelet clumping were reported. For each comparison the correlation coefficients (r), linear regression equations, slopes and P values are summarized in Table 1. The agreement between the two examiners A and B was very good (r=0.94, p=0.001). *Conclusions.* The evaluation has shown that the CellaVision DiffMasterTM Octavia and Sysmex XT-2000i PLT-O have excellent correlation on platelet count (r=0.92, p=0.001). Platelet count on CellaVision DiffMasterTM Octavia tended to be the same as the corresponding values reported by XT-2000i PLT-O as suggested by a slope of 0.9876. As digital images can be reviewed remotely, small laboratories could rely on laboratory physicians in remote locations by using the CellaVision DiffMasterTM Octavia system.

Table 1.

Platelet count	r	linear regression equation	slope	P
score A versus score B	0.94	y = 0.8609x + 26.153	0.8609	0.001
DiffMaster vs PLT-O	0.92	y = 0.9876x + 2.7413	0.9876	0.001

0244

MEDICAL CONTRAINDICATIONS FOR SPLENECTOMY AMONG ADULTS WITH IMMUNE THROMBOCYTOPENIC PURPURA: DATA FROM THE GENERAL PRACTICE RESEARCH DATABASE

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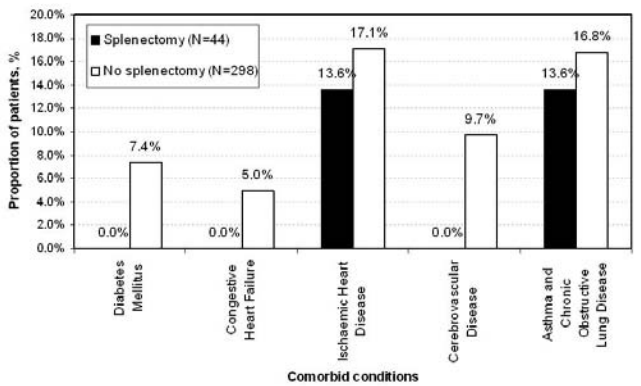
Background. Splenectomy is a potential treatment for chronic immune thrombocytopenia purpura (ITP), generally recommended for patients with severe disease who have a risk of serious bleeding and fail to respond to first-line treatment. It is thought that a subgroup of ITP patients may not be able to undergo splenectomy due to medical con-

traindications. These contraindications have not been clearly described. *Aims.* To describe characteristics of unsplenectomised ITP patients and compare with characteristics of splenectomised ITP patients. *Methods.* We first performed a review of the literature to identify potential medical contraindications for splenectomy. We then assessed the prevalence of these contraindications among a group of splenectomised incident adult ITP patients. We compared this to the prevalence of contraindications among unsplenectomised adult ITP patients. ITP patients were identified from the UK General Practice Research Database (GPRD). Newly diagnosed patients aged between 18-100 years who had at least one medical code for ITP and received at least one prescription for prednisolone were included in the study. *Results.* Potential medical contraindications identified from the literature included diabetes mellitus, congestive heart failure, ischaemic heart disease, cerebrovascular disease, asthma and chronic obstructive pulmonary disease and older age (over 70 or 80 years). The prevalences of these conditions were examined among 342 incident adult ITP patients receiving prednisolone treatment, of whom 44 (13%) were splenectomised. None of the ITP patients who had coexistent diabetes mellitus, congestive heart failure and cerebrovascular disease were observed to have undergone splenectomy (Figure). Forty of the 44 splenectomies (91%) were performed on adults aged less than 75 years. The median age of splenectomised adult ITP patients (52 years, interquartile range (IQR): 36-65) was substantially younger compared to non-splenectomised individuals (median 68 years, IQR: 47-76; p=0.0001 (t-test)). The proportion of splenectomised ITP patients did not differ by gender or by calendar year of ITP diagnosis. *Summary and Conclusions.* Data from routine clinical practice support findings from the literature that certain subgroups of ITP patients may not be indicated to undergo splenectomy. However, it should be noted that some of the ITP patients included in our study may have responded to first-line treatment and/ or had less severe disease. Therefore, some of the patients in our non-splenectomised group may not have been indicated for splenectomy for reasons other than their coexisting medical conditions.

Figure. Comorbidities in adult ITP by splenectomy status.



0245

COMPARATIVE EVALUATION OF 4 AUTOMATED HEMATOLOGIC ANALYSERS (ADVIA120, PENTRA120DX, LH750, XE2100) WITH IMMUNOLOGICAL REFERENCE METHOD IN THROMBOCYTOPENIC HAEMATOLOGICAL PATIENTS

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Background. The appropriate prophylactic platelet transfusion threshold (TT) in haematologic patients was lowered in the last two decades from 20-10×10^9/L without significantly increasing the haemorrhage risk. Recently, a further reduction of TT to 5×10^9/L has been suggested, though the potential inaccuracy of standard routine haematology analysers for very low platelet count (PC) is a major concern. It has been showed that with PC below 20×10^9/L the failure to discriminate platelets from other cell fragments and debris of a similar size or the presence of giant platelets increases the PC variability. *Aims.* Our aim was to evaluate, in adult haematological thrombocytopenic patients, the PC accuracy of 4 different haematology analysers utilizing different technologies: i) optical ADVIA120 (Siemens); ii) impedancemetry-based PENTRA120DX (Horiba Medical) and LH750 (Coulter); iii) both technologies XE2100 (Siemens). The obtained results were compared to the immunological reference method validated by the International Society of Laboratory Haematology. *Methods.* 90 blood samples with PC less than 150×10^9/L were collected from 55 thrombocytopenic haematological patients, 66 samples (73%) showed PC less than 50×10^9/L. The samples were analyzed within 4 hours from phlebotomy, while the immunological PC was performed within 24 hours. Spearman correlation was used to analyze correlation between methods. Mean value was compared by t test. Comparison between methods were made through Bland Altman graph and Roc curve was used to evaluate sensibility, specificity and possible better cut off. *Results.* Data (Table 1) show a good correlation between immunological reference values and counts provided by automatic analysers: 0.946 for ADVIA120; 0.957 and 0.942 for XE2100 impedancemetry and optical method respectively; 0.938 for LH750; 0.954 for PENTRA120DX.

Table 1.

	Advia 120	XE 2100i	XE 2100o	LH 750	Pentra 120dx
N	87	90	72	89	90
Correlation (r)	0.9469	0.9572	0.9423	0.9389	0.9544
Mean differences	4.79	3.59	0.18	12.10	6.57
Regression coefficient (b)	0.84	0.86	1.04	0.67	0.80
Cut off 20x10^9/l					
Sensibility	93.33	90.32	76.92	80.65	74.19
Specificity	100.00	98.31	100.00	100.00	100.00
Correctelly classified	97.70	95.56	91.67	93.26	91.11
Recalculated best cut off	20	23	21	24	24
Sensibility	93.33	96.77	81.48	98.28	96.61
Specificity	100.00	98.31	92.87	90.32	87.10
Correctelly classified	97.70	96.81	91.89	95.51	93.33
Cut off 10x10^9/l					
Sensibility	61.11	77.78	73.33	55.56	66.67
Specificity	97.10	95.83	96.49	97.18	95.83
Correctelly Classified	89.66	92.22	91.67	88.76	90.00

All analysers over-estimated the PC compared with the immunological method by a mean of 4.79×10^9/L for ADVIA120, 12.10×10^9/L for LH750, 6.57×10^9/L for PENTRA120DX, 3,59 and 0.18×10^9/L for XE2100 impedancemetry and optical method respectively. Anyway there is a good sensitivity (range 74.19-93.33) and excellent specificity (range 98.31-100) at a cut-off set to 20×10^9/L platelets. The 10×10^9/L cut-off is critical for all the analyser due to the worsened sensitivity (range 55.56-77.78) and specificity (range 94.2-96.49). The best accuracy (higher number of patients correctly classified) is obtained with the following cut-off: 20×10^9/L for ADVIA120; 23 and 21×10^9/L for XE 2100 impedance and optical method respectively; 24×10^9/L for LH750 and Pentra120DX. *Summary and conclusions.* The analysers show a good correlation with the immunological reference method in thrombocytopenic hematological patients. Their best accuracy is obtained when PC is equal or higher 20, 23, 21 and 24×10^9/L depending on the utilized analyser (Table 1), However, the analyzer performances are poor when PC is very low (″10×10^9/L). Moreover, all analysers over-estimated the PC when compared to the immunological reference method. Therefore, despite dramatic advancements in automated blood count technology have been made, to reduce the current accepted platelet prophylactic TT from 10.0-5.0×10^9/L continues to be a challenge for the future.

Myelodysplastic syndromes I

0246

LENALIDOMIDE ABROGATES THE CLONAL ADVANTAGE OF DEL(5Q) MDS STEM CELLS VIA ALTERATION OF NICHE INTERACTIONS

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Background. The myelodysplastic syndromes (MDS) constitute a heterogenous group of clonal disorders of hematopoietic stem cells (HSC) leading to ineffective hematopoiesis in one or more lineages in the bone marrow. A range of cytogenetic abnormalities can be identified in around 50% of cases, with the most frequent cytogenetic entity being the 5q-syndrome, characterized by loss of the 5q31-33 region. Recently, the thalidomide analogue lenalidomide has been demonstrated to induce cytogenetic response and restored erythropoiesis in this group of patients. While inhibition of angiogenesis, cell adhesion, and proliferation, as well as modulation of pro-apoptotic cytokines, have all been put forward as putative modes of action of lenalidomide, the exact mechanism has remained unclear. Interestingly, the more primitive hematopoietic compartment seems to possess a clonal advantage where del(5q) HSC are able to outcompete non-5q HSC. Whether the molecular mechanism for clonal advantage is intrinsic to the stem cell clone or a result of a compromised niche is unclear. However, we have previously demonstrated that lenalidomide is able to abrogate this clonal advantage. *Aims.* Our previous results implicated the matri-cellular protein SPARC to be upregulated in CD34$^+$ del(5q) cells in response to lenalidomide. Thus, we sought to test the hypothesis that a putative decrease in SPARC expression of a clonal hematopoietic progenitor should give rise to a population of cellular descendants that exhibit an increased adhesion to the microenvironment and more active rates of proliferation, thus explaining the clonal advantage of del(5q) MDS stem cells. *Methods.* We therefore studied the basal adhesion of del(5q) CD34$^+$ progenitor cells to the murine stromal cell line MS-5 using a gravitational-force upon-inversion assay. Using multi-parameter flow cytometry, we studied proliferation and apoptosis in del(5q) and normal HSC and how these parameters are affected by lenalidomide and recombinant SPARC-protein. *Results.* Our data show that del(5q) CD34$^+$ progenitors exhibited increased adhesion to the murine stromal cell line MS-5 compared to normal CD34$^+$ cells. In short-term *in vitro* cultures, lenalidomide and recombinant SPARC were able to abrogate this increased adhesion. Multiparameter flow cytometry revealed a decreased rate of spontaneous apoptosis in del(5q) HSC compared to progenitors. Lenalidomide increased apoptosis in both compartments and this effect was specific to del(5q) cells. However, neither lenalidomide nor SPARC appeared to have any effect on the proliferation of CD34$^+$ progenitors from normal or del(5q) bone marrow. *Summary and Conclusions.* These studies suggest that decreased expression of SPARC leads to increased adhesion of del(5q) HSC/progenitor cells to their microenvironment and may explain why del(5q) HSC are able to outcompete the remaining healthy HSC. Our studies suggest that lenalidomide is able to abrogate this clonal advantage partly via its increase in SPARC expression with a consecutive decrease in adhesion and increase in apoptosis

0247

EVALUATION OF COMORBIDITY AS PROGNOSTIC VARIABLE IN 419 PATIENTS WITH MYELODYSPLASTIC SYNDROMES: A STUDY OF THE AUSTRIAN MDS WORKING GROUP

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Background. Myelodysplastic syndromes (MDS) are a group of clonal hematologic neoplasms characterized by ineffective erythropoiesis, peripheral cytopenia(s), and an increased risk to transform to secondary acute myeloid leukemia (AML). The prognosis in MDS is variable and depends on the disease variant, other disease-related features, and patient-related parameters. The evaluation of comorbidity is of increasing importance in patients with hematologic disorders. *Patients and Methods.* In the present study, the influence of comorbidity on survival and AML evolution was analysed retrospectively in 419 patients with *de novo* MDS (observation period: 1985-2007). The median age was 71 years (range 18-91 years). Two different scoring systems for comorbidity, the hematopoietic stem cell transplantation comorbidity index (HCT-CI) and the Charlson comorbidity index (CCI) were applied. *Results.* The HCT-CI was found to be a significant prognostic factor for survival (OS, $p<0.05$) as well as event-free survival (EFS, $p<0.05$) in our patients, whereas the CCI was of prognostic significance for OS ($p<0.05$) but not for EFS. For AML-free survival (AFS), neither the HCT-CI nor the CCI were of predictive value. In a next step, a multivariate analysis including age, LDH, serum ferritin, karyotype, number of cytopenias, FAB-groups, and comorbidity was performed. Analysing the entire group of patients neither the HCT-CI nor the CCI were independent prognostic parameters for OS or EFS after addition of ferritin to the multivariate cox model. However, chronic comorbidity was found to be an independent prognostic factor for both OS and EFS when good risk MDS patients (IPSS low or int-1) were analyzed separately. In particular, the HCT-CI was a prognostic variable for OS ($p<0.05$) and EFS ($p<0.05$) in this group of patients, independent of age, LDH, ferritin, FAB-subgroup, number of cytopenias, and karyotype. In contrast, the CCI was not of prognostic significance neither for OS nor EFS. With regard to int-2 or high risk MDS patients, both, HCT-CI and CCI were not of prognostic significance. *Conclusions.* Together, our data show that comorbidity as defined by the HCT-CI is an independent risk factors for OS and EFS in patients with low or int-1 MDS. The HCT-CI is a superior prognostic score system in MDS.

0248

A NOVEL RECURRENT GENETIC ABNORMALITY IN MYELODYSPLASTIC SYNDROMES: REARRANGEMENTS AND AMPLIFICATIONS OF IER3

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Background. IER3 (Immediate Early Response 3; also known as IEX-1) has an important role in regulating death receptor-induced apoptosis, interaction with NF-kB pathways, and cellular response to genotoxic stresses such as ionizing irradiation. While murine models have suggested a role for IER3 in neoplasia (e.g., forced overexpression results in development of a lupus-like syndrome and lymphoproliferative disorders), and gene expression microarrays in various solid tumors and 2 independent CD34$^+$ microarray experiments in myelodysplastic syndromes (MDS) have identified IER3 as a striking expression outlier (Hofmann W-K *et al.* Blood 2002; Prall WC *et al.* Int J Hem 2009), definitive evidence for involvement of IER3 in a human disease has been lacking. During breakpoint cloning of an isolated tranlocation in a patient with MDS, we identified an IER3 rearrangement, and then detected IER3 abnormalities in other patients with MDS. *Methods and Results.* The propositus exhibited t(6;9)(p21.3;q34) as an isolated chromosomal abnormality in 18 of 20 metaphases; FISH assays for DEK/CAN fusion and ABL rearrangements were unrevealing. To clone the breakpoints efficiently without resorting to a large library of BAC clones, we generated somatic hybrid murine/human cell lines from a buffy coat; microdissected derivative chromosomes with laser capture microscopy; and hybridized to 6p/9q custom comparative genomic hybridization (CGH) arrays. The CGH results considerably narrowed the 6p and 9q breakpoints (within 800 bp for 6p), which were then amplified by long-range PCR and sequenced to define the rearrangement. The IER3 gene at 6p21.3 was found to be separated from all but 40 bp of its upstream regulatory elements in this rearrangement and translocated to a transcript-poor region of 9q, dramatically downregulating expression in the patient compared to healthy controls, as confirmed by RT-PCR and Western blotting. We then designed split-signal and locus-specific FISH probe sets for IER3. We examined archival bone marrow cell pellets from 204 additional patients with various clonal hematological disorders and chromosomal rearrangements involving 6p21 or 6p22 (i.e., translocations, inversions, deletions, or additions), and found IER3 abnormalities in 8 additional patients: 3 split signals and 6 amplifications (one patient had both abnormalities); all 8 patients had MDS. FISH studies in 157 additional MDS patients (90 higher-risk, 67 lower-risk) with normal karyotype were unrevealing. RT-PCR in 46 MDS patients without 6p rearrangements demonstrated down-regulation of IER3 by >4-fold in 16 patients (35%, mostly higher-risk) and down-regulation by >4 fold in 12 patients (26%, mostly lower-risk) when compared to the mean for 23 healthy controls. Direct sequencing of the IER3 coding region and the

promoter region in 74 patients without 6p rearrangements revealed no point mutations. Hematopoietic progenitor growth from IER3-/- mice was compared to wild-type controls, and we noted no differences in BFU-E, CFU-E, and CFU-GM growth under unstressed conditions. *Conclusions.* IER3 rearrangements and amplification represent a novel clonally-restricted recurrent genetic abnormality in MDS. This is the first time IER3 has been linked to human disease, and the combination of somatic hybridization + *array painting* may facilitate cloning of other breakpoints in hematological neoplasms. Dysregulation of IER3 expression is common in MDS (61% had either >4-fold increase or decrease in expression), even in patients without 6p rearrangements. In early MDS, lack of IER3 is expected to contribute to excessive apoptosis and hematopoietic failure; in later MDS, amplification or overexpression of IER3 would favor cell survival and progression to AML. Better understanding of the role of IER3 in MDS is likely to yield new insights into MDS pathobiology.

0249

NEW INSIGHTS INTO THE BIOLOGY OF REFRACTORY ANEMIA WITH RING SIDEROBLASTS (RARS) AND IDENTIFICATION OF ERYTHROID G-CSF TARGETS BY GENE EXPRESSION PROFILING

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RARS is characterized by severe ineffective erythropoesis and mitochondrial iron overload. Granulocyte-CSF inhibits erythroid apoptosis and mitochondrial leakage of cytochrome c *in vitro* as well as *in vivo*. The molecular mechanisms underlying the ineffective erythropoiesis in RARS and the effects of G-CSF were studied by gene expression profiling (GEP) of erythroblasts from normal (NBM) and RARS bone marrow after first confirming their clonal origin by HUMARA analysis. $CD34^+$ selected marrow cells were cultured for 7-14 days in Iscove´s medium with 15% BIT9500, and an aliquot of cells were treated with G-CSF at day 7. GEP was determined using Affymetrix, U133 Plus2.0 chips (Pellagatti *et al.*, 2006). The expression of several genes involved in cellular iron metabolism was significantly downregulated in the RARS samples: ABCB7, mutated in X-linked sideroblastic anemia with ataxia and involved in maturation of cytosolic Fe/S enzymes and Sfxn, mutated in mice with siderocytic anemia. Other downregulated genes included, FANCC, a key mutated gene in Fanconi anemia, and FOXO3A, a transcription factor regulating oxidative stress in erythropoiesis. None of these genes were, however, influenced by G-CSF, in spite of its anti-apoptotic effect. By contrast, the expression of another key down-regulated gene, MFN2, involved in mitochondrial membrane stability, was reverted back to the normal range by G-CSF, in line with previous findings of G-CSF inhibition of cytochrome c release (Tehranchi, 2003). Moreover, we studied methylation status by MCA-Methylation assay and confirmed that DNA methylation was not a significant cause of low mRNA expression. Importantly, both ABCB7 and MFN2 expression increased 2-3-fold during normal erythroid differentiation, while decreasing by 2-3-fold in the RARS cultures, as assessed by realtime-PCR. By comparing GEP between $CD34^+$ cells and erythroblasts, it was clear that a number of genes, including ALAS2, that were upregulated in $CD34^+$ was normalized or less dysregulated in the erythroblast fraction, indicating that the $CD34^+$ expression pattern may be strongly influences by the composition of that cell compartment. The expression level of HSPA1B and HSPA9B, two members of the HSP70 family, which were slightly up-regulated in RARS increased significantly after G-CSF treatment. HSPA1B regulates erythropoiesis by preventing caspase-3-mediated cleavage of GATA-1 during differentiation and HSPA9B acts as an anti-aopototic protein via inactivation of P53. Increased expressions of HSPA1B and HSPA9B are thus consistent with the observed *in vivo* response of RARS patients to G-CSF. In conclusion, our data supports the strength of assessing GEP in differentiating, cohorted cells. We have identified a number of key downregulated genes involved in mitochondrial function and iron transport, whose expression decreased during differentiation, while other genes involved in erythropoiesis and cell death / survival remained more or less normal in intermediate erythroblasts, in spite increased cytochrome c release and other apoptotic features in the cultures. G-CSF seems to act through upregulation of hsp 70 genes, but also has a very interesting direct effect of key mitochondrial function genes, such as MFN2.

0250

ABT 737 BCL-2 INHIBITOR TARGETS LEUKEMIC STEM CELLS IN MOUSE MODELS OF MYELOID (PRE)LEUKAEMIA

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Background. Combinations of oncogenes that transform cells have been described; however, few studies have analyzed the progression of genetic events leading to the expansion of the leukemic clone. Animal models enable us to understand disease progression and provide us with reagents to test various therapeutic strategies. *Aims.*We have previously developed an animal model of myelodysplasia/acute myelogenous leukemia (MDS/SAML) progression using mutant NRAS, and overexpression of human hBCL-2. Here we plan to further characterize these models and compare them to the human disease. Using a mimetic inhibitor ABT 737, we aim to evaluate the effects of targeting BCL-2 in our preclinical models. *Methods.* Transgenic mice bearing the NRASD12 oncogene was crossed with mice bearing hBCL-2 driven by either the MMTVLTR or MRP8 promoters and were followed for disease my measuring their blood counts. The diseased mice were treated with 15x75mg/kg of the small molecule ABT 737 (Abbott) every other day. Cells were harvested and characterized. *Results.* Expanded leukemic stem cells (LSC) were identified as Lin-/Sca1+/KIT+ populations by flow cytometry, with increased myeloid colony growth and were transplantable. Increased hBCL-2 expression in the RAS-GTP complex are observed in both MDS/AML diseases using the RAS pulldown assay. Gene expression profiling studies indicate that the signatures of the AML-like disease resemble human AML with an underlying MDS. The MDS-like disease had increased apoptosis assayed by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) on liver sections, whilst the AML-like mice had liver apoptosis patterns similar to wild type. The single mutant NRAS line also had increased apoptosis. *in vivo* imaging by single-photon emission computed tomography (SPECT) using Tc-99m-labelled AnnexinV confirmed these findings. In the MDS mice the majority of the RAS and BCL-2 doubly stained cells localized to the plasma membrane, where active pro-apoptotic RAS is normally located, whereas in the AML disease RAS and BCL-2 co-localize in the mitochondria, where BCL-2 is normally found. This is concordant with the signalling profile measured by conventional western blotting and the nanoimmunoassay (NIA) (Firefly TM, Cell Bioscience) with phosphorylated ERK isoform expression patterns associated with disease progression. These patterns were also observed in patients with increased RAS/BCL-2 co-localizations correlating with clinical features. Increased genomic instability in the Sca1+ compartment provides a mechanism for disease progression. We have used these models to determine the efficacy of a BH3 mimetic inhibitor ABT 737. We have shown that treatment with this reagent targets primitive progenitors using flow cytometry and colony assays, induces apoptosis, measured by TUNEL and SPECT and clears the liver infiltrations in both MDS and AML mouse models with the disruption of the RAS/BCL-2 complex, measured by western blotting and confocal microscopy and increases in phosphorylated ERK isoforms by NIA. *Conclusions.* Emerging technologies of genomics, proteomics and imaging have been employed in the MDS/AML models to characterize disease progression and follow response to treatment in order to gain molecular insight in the evaluation of the efficacy. ABT 737 appears to target LSCs and induce apoptosis, regulating appropriate pathways.

0251

FISH PROBES DERIVED FROM ARRAY CGH (ACGH) REVEAL NOVEL CHROMOSOMAL LESIONS IN MDS PATIENTS WITH A NORMAL KARYOTYPE

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In MDS the cytogenetic pattern is one of the most important parameters to predict overall survival (OS) and the risk of MDS/AML evolution. However, in about 40-50% of patients, especially low-rish MDS, conventional cytogenetics (CC) fails to reveal clonal chromosomal defects. FISH with probes specific for chromosomal regions most commonly involved in MDS can improve CC results (Bernasconi *et al.*, 2003). Currently, aCGH revealed that MDS patients, independently of the cytogenetic pattern, harbour novel chromosomal lesions targeting unsuspected regions. Thus, our study was aimed at evaluating whether probes derived from a recent aCGH (Starczynowski *et al.*, 2008) were truly able to unmask cryptic lesions in chromosomally normal MDS patients, whether these defects consisted in either chromosomal gains/losses or balanced rearrangements and whether they influenced OS and disease evolution. The twenty-one patients entered the study were examined between January 2005 and May 2008. They were six females and fifteen males, their median age was 64 years (range 24-75). According to WHO classification, 3 patients were classified as RA, 8 as RAEB-1 and 10 as RAEB-2. Considering IPSS score, 4 patients were considered low-risk, 8 intermediate-1 risk and 9 intermediate-2 risk. Median follow-up was seven months (range 1-46). At the time of the study no patients have died, whereas 4 have evolved to RAEB-2 and 2 to AML. FISH probes were chosen based on the frequency of their involvement in MDS (Starczynowski *et al.*, 2008) and their Mb position determined using UCSC genome browser on Human Mar. 2003 assembly. They were obtained from BACPAC Resources Center at C.H.O.R.I. (Oakland, USA), labelled and applied as previously described. We used the following probes: RP11-912d8 (19q13.2); RP11-196p12 (17q11.2); RP11-269c4 (14q12); RP11-351o1 (10q21.3); RP11-144g6 (10q11.2); RP11-122a11 (7q34); RP11-951k18 (5q13.1); RP11-100m20 (4p14); RP11-544h14 (2q33). For i-FISH, cut-off values, obtained from the analysis of 300 nuclei from ten normal samples, were fixed at 10%. An abnormal FISH pattern was revealed in 10 patients (47.6%): 5 presented a 19q13.2 deletion, 2 an amplification of band 4p14, 2 a 14q12 deletion and one a potential rearrangement of band 10q11.2. One of these patients harboured two defects, namely a 19q13.2 deletion and an amplification of band 4p14. An abnormal FISH pattern was observed in 2/3 RA patients, in 3/8 RAEB-1 and in 5/10 RAEB-2 and in 2/4 IPSS low-risk, in 4/8 intermediate-1 risk and in 4/9 intermediate-2 risk MDS patients. Disease evolution occurred in the only 2 RA patients with an abnormal FISH pattern and in 2 of the 4 RAEB-1/RAEB-2 patients with an abnormal FISH pattern. Additional studies are warranted to assess the prognostic significance of cryptic chromosomal lesions. In conclusion our data suggest that FISH with probes derived from aCGH studies i) reveals novel unsuspected chromosomal lesions in about 47% of chromosomally normal MDS patients; ii) these chromosomal lesions mostly consist in gains/losses, whereas balanced rearrangements seem to be very rare; iii) an abnormal FISH pattern seems to correlate with disease progression even if this observation should be confirmed on additional patients.

0252

ALLOGENIC STEM CELL TRANSPLANT IN MDS: RESULTS OF THE SPANISH REGISTRY

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Background. The only curative treatment known so far for myelodysplastic syndromes (MDS) is the allogenic stem cell transplantation (AlloSCT). Although pre-transplant characteristics which allow to predict the outcome after alloSCT remains to be clearly determined. *Aims.* Our objective is to describe the characteristics and outcome of the 190 MDS patients included in the Spanish Registry who have received an alloSCT. *Methods.* 190 patients who have received an alloSCT in 11 Spanish transplant centers between 1987 and 2008, have been included. The main characteristics of the patients are as follows: median age was 49 years (range 16-75); male / female ratio was 64.5 / 35.5; MDS diagnosis was established according to the WHO classification (1999): refractory anemia with/without ringed sideroblasts (RA+/-RS) 6.9%, refractory cytopenia with multilineage dysplasia (RCMD) 7,6%, refractory anemia with excess of blasts (RAEB)-1 15,2%, RAEB-2 23,4%, 5q- syndrome 1,4%, MDS unclassifiable 2,1%, myelodysplastic/myeloproliferative disease (MDS/MPD) 13,1%, secondary acute myeloid leukaemia (AML) 27%, and other MDS 2,8%. Patients with MDS/MPD such as cronic myelomonocytic leukaemia (CMML) (n=19) were also included in the analysis. *Results.* 42% of patients were treated with chemotherapy previous to the transplantation, and their disease status at transplant was: complete remission (CR) 25,7%, partial response (PR) 7,3% and relapse (Re) 8,3%. The rest of the patients received the alloSCT without previous chemotherapy (No-ch) 53,2% and 3,7% underwent alloSCT in progression (Pr) without previous chemotherapy. The mean of the percentage of blasts in the bone marrow, at transplantation, was 4.5 (0-10.7). Myeloablative conditioning (MC) treatment was used in 72,3% of patients while 27.7% received reduced intensity conditioning (RIC). Overall survival (OS) and event free survival (EFS) of the whole group were 51% and 48%, respectively (Figure 1) at ten years of follow up. WHO classification was predictive for both OS and EFS.

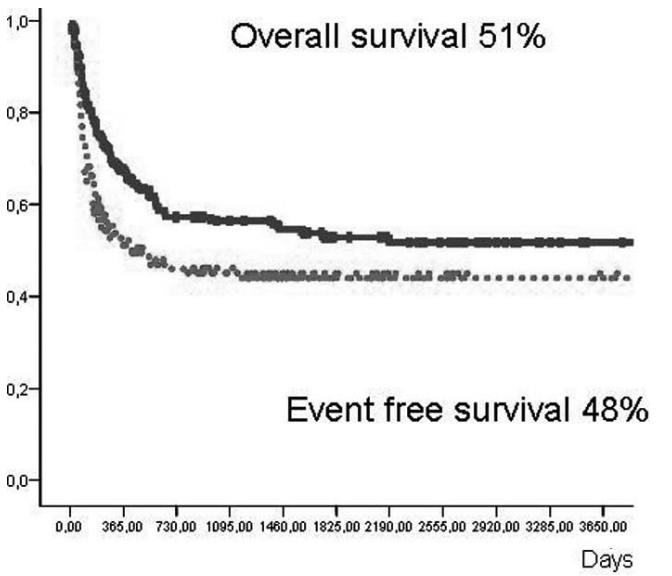

Figure 1.

None of the following variables significantly influenced OS or EFS: blast percentage and ferritin levels at the time of alloSCT, age, conditioning regimen (MC vs RIC) (50-48% for OS and 46-42% for EFS for MC vs RIC, respectively) although patients receiving RIC were significantly older (42 vs. 55 years). When only high risk patients (RAEB-1, RAEB-2, AML and MDS/MPD) were considered, disease status at transplant significantly influenced the outcome with better results being observed for patients in CR or No-ch. *Summary.* In summary, a significant percentage of patients with MDS, including high-risk patients, remain alive more than ten years after transplantation showing that this approach remains the only curative one for these patients. The WHO classification and the state of disease at transplantation in the high risk group are the most powerful predictors for survival.

0253

MICRORNA SIGNATURE OF CD34+ CELLS IN MYELODYSPLASTIC SYNDROME PATIENTS WITH DEL(5Q)

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Background. An interstitial deletion of the long arm of chromosome 5, del(5q), is the most commonly reported deletion in *de novo* myelodysplastic syndrome (MDS). The del(5q) occurs as the sole karyotypic abnormality in 5q- syndrome, which is the most distinct of the MDS. The 5q- syndrome has a good prognosis and a low probability of transformation to acute myeloid leukemia. *Aims.* Pathophysiological basis of the 5q- syndrome is likely associated with haploinsufficiency for genes mapping to the deleted region at 5q; however, other mechanisms may contribute to the failure of normal bone marrow. Because microRNAs (miRNAs) have been shown to regulate hematopoiesis and their aberrant expression contributes to the pathogenesis of clonal hematopoietic disorders, we searched for differently expressed miRNAs in CD34+ progenitors of MDS patients. *Methods.* Bone marrow CD34+ cells from 5q- patients (n=8) and healthy donors (n=4) were sorted by magnetic columns. Gene expression profiling of miRNAs was performed by quantitative real time PCR based array platform with 365 miRNA probes. *Results.* Out of the miRNA panel, transcripts of ~202 miRNAs were expressed at the detectable level. Hierarchical clustering analysis defined miRNA signatures in 5q- patients distinct from those of controls. Comparative analysis revealed differential expression of 38 miRNAs in 5q- patients at $p<0.05$; up-regulation of 23 miRNAs and down-regulation of 15 mRNAs. For example, increased expression was observed in let-7b, miR-199a, miR-338, and miR-410. Especially, miR-34a showed abundant level of transcript in 5q- patients. This miRNA is a direct proapoptotic transcriptional target of p53 and may play a role in increased apoptosis of bone marrow progenitors seen in low-risk MDS. Down-regulation was found in miR-135b, miR-203, miR-369-5p, miR-429, and miR-589 etc. miR-203 is often hypermethylated in hematopoietic malignancies and targets ABL1 gene whose expression induces tumor cell proliferation. Three miRNA genes (miR-143, miR-145, miR-378) are mapped within the deleted region 5q31-q32. In the patients, miR-143 and miR-145 was expressed at the control level and only miR-378 showed 2fold decrease of expression. miR-146a is mapped close to the deletion and was down-regulated in the patients. Its target gene is TRAF6 whose induced expression in mouse bone marrow leads to the neutropenia, anemia, and thrombocytosis. In addition, mRNA profiles of CD34+ cells in the tested subjects were assayed using whole-genome microarrays with 24 500 probes and 245 significantly deregulated genes ($p<0.01$) in 5q- patients were found. An integrative analysis of microarray data with in silico target predictions (TargetScan and Miranda software) defined potential downstream targets of the deregulated miRNAs. We observed significant down-regulation of some putative targets of miR-299-5p (H3F3B, TP53INP2), miR-34a (KLF4, LEF1, NR4A2) and miR-223 (EBF3, PDE4B, PLEKHM1, RHOB). *Conclusions.* Our study demonstrates that the specific miRNA signature is associated with 5q- phenotype and that the aberrantly expressed miRNAs may be involved in the pathogenesis of MDS by a modulation of their target genes. MicroRNA profiles of other MDS patients (RAEB-2, RCMD) with del(5q) are being analyzed. Supported by IGA: NR/9236 MZ CR.

0254

THE CAPACITY OF THE HYPOMETHYLATING AGENTS AZACYTIDINE AND DECITABINE TO INDUCE APOPTOSIS AND CELL CYCLE ARREST DEPENDS ON THE ACTIVATION OF THE DNA-DAMAGE RESPONSE PATHWAY

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Background. The hypomethylating agents azacytidine (AZA) and decitabine (DEC) have shown clinical efficacy in patients (pts) with MDS. There is *in vitro* evidence that both agents, in addition to their hypomethylating effect, also function by inducing apoptosis, cell cycle arrest and/or the activation of a DNA damage response (DDR). However, the exact contributions of those mechanisms of action and their functional interdependence remain to be defined. Aims and *Methods.* A panel of MDS (P39, MDS-1)- and AML (HL-60, KG-1)-derived cell lines were incubated with increasing dosages of AZA (1-2 μM) and DEC (1-2 μM) and the drugs capacity to induce apoptosis (DiOC6(3)/PI), cell cycle arrest (PI) and/or a DDR (immunoflourescence staining of P-ATM, P-Chk-1, P-Chk-2, γ-H2AX) were assessed in absence and presence of the ATM-inhibitor KU-55933 and the Chk-1 inhibitor UCN-01. *Results.* We show that both drugs induced dose-dependent apoptosis in myeloid cell lines: whereas AZA increased apoptosis in KG-1 and HL-60 by about 10% (48h, 2 μM) the respective incubation with DEC augmented apoptosis by about 20% (HL-60) to 30% (KG-1). P39 cells were resistant to AZA and increased apoptosis by 15% after 48h of 2 μM DEC, and MDS-1 cells were resistant to both drugs. In addition, both drugs induced a G2/M-arrest in P39 (+15% after 48h with 2 μM of AZA or DEC) and HL-60 (+20% after 48h with 2 μM of AZA or DEC) cells, but not in KG-1 and MDS-1 cells. Noteworthy, both drugs induced a DDR in the apoptosis-sensitive KG-1 cells (but not P39 cells) as evidenced by the appearance of nuclear P-ATM and Á-H2AX foci. Surprisingly, this activation of P-ATM did not induce the nuclear translocation of P-Chk-1-Ser317 or P-Chk-2-Ser68. To more clearly define the importance of the DDR in AZA- and DEC-induced apoptosis and G2/M-arrest, experiments were recapitulated in the presence of the ATM-inhibitor KU-55933 and the Chk-1 inhibitor UCN-01. Inhibition of ATM abrogated the apoptosis-inducing activity of AZA and DEC in KG-1 cells (without influencing cell cycle progression), whereas inhibition of Chk-1 remained without effect. In contrast, in P39 and HL-60 cells, inhibition of ATM neither affected cell cycle progression, nor sensitivity towards the drugs. Nevertheless, inhibition of Chk-1 by UCN-01 completely abrogated the G2/M-arresting effect of AZA (and diminished that of DEC) in P39 and HL-60 cells. *Conclusions.* We provide novel evidence for the cell-type dependent capacity of the hypomethylating agents 5-azacytidine and decitabine to induce apoptosis, cell-cycle arrest and DDR in cell lines representing different subtypes of MDS and AML. Moreover, we show the crucial role of ATM and Chk-1 activation - as part of the DDR - in mediating apoptosis-inducing and cell cycle-arresting effects of AZA and DEC, respectively, providing evidence that hypomethylating agents confer their beneficial effects by employing different pathways of the DDR.

0255

TREATMENT OUTCOME ACCORDING TO PRETREATMENT RISK GROUP IN MDS PATIENTS WHO WERE TREATED WITH AZACITIDINE

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Background. The use of hypomethylating agents improved significantly the clinical outcome of myelodysplastic syndrome (MDS) patients. The stratification of patient group can help design risk adopted therapy or predicting the prognosis in the MDS patients receiving azacitidine. *Aims.* We analyzed the outcomes of the patients with MDS treated with azacitidine. *Methods.* From Aug. 2006 to Jun. 2008, a total of 129 patients with MDS who had anemia and/or thrombocytopenia were treated with azacitidine at a dose of 75 mg/m^2 per day SQ for 7 days, which was repeated every 28 days. The median age of the patients was 64 years (range, 20-82). Fifty-six patients had refractory anemia with excess blasts. Intermediate-2 and high risk in IPSS were in 43 patients (33.4%), and high and very high risk in WPSS were in 61 patients (47.3%). Of the 129 patients, 126 patients treated more than 2 cycles were analyzed. *Results.* Sixty patients (47.6%) had responses at median 3 cycles (range, 1-5). Seventeen patients showed response after 3rd cycles and 16 after 4th cycles. After 5th cycles, only 3 patients showed responses. Complete response (CR) including marrow CR was observed in 21 patients (16.7%), partial response (PR) in 6 patients (4.8%), and hematologic improvement (HI) in 33 patients (26.2%). The 3-year overall survival (OS) rate was 48.4±10.0% in young age group (<60) and 28.7±9.3% in old age group (≥60; $p=0.333$). The 3-year OS rate according to the IPSS risk group was 41.5±7.8% in low and intermediate-1 group and 29.3±12.7% in intermediate-2 and high group ($p=0.082$), and according to the WPSS risk group 71.6±10.4% in very low and low group, 31.6±8.9% in intermediate group, and 9.7±8.9% in high and very high group ($p<0.001$). Progression to acute leukemia was more common in advanced risk group; IPSS (22.0% vs. 8.2%, $p=0.030$) and WPSS (23.7% vs. 7.1%, $p=0.002$). But complete response to azacitidine did not prevent the progression to acute leukemia; 19.0% in CR and marrow CR vs. 11.4% in others ($p=0.338$). *Conclusions.* Pretreatment risk group was an important prognostic factor to predict the outcomes of the patients with azacitidine treatment. The response to 5-AZA did not prevent disease progression. Advanced risk group should consider more effective therapy.

0256

RUNX1 MUTATIONS IN *DE NOVO* MDS AND CMML AT BOTH DIAGNOSIS AND AML TRANSFORMATION: A COMPARATIVE ANALYSIS ON 80 MATCHED PAIRED MARROW SAMPLES

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Background. Transcription factor RUNX1 is essential for normal hematopoiesis. Somatic mutations of RUNX1 have been described in *de novo* myelodysplastic syndrome (MDS). We also observed a high frequency (37%) of RUNX1 mutations in chronic myelomonocytic leukemia (CMML). The role of RUNX1 mutations in the progression of MDS and CMML to acute myeloid leukemia (AML) is not defined. Aims. We aimed to analyze RUNX1 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 with clinicohematologic features and outcome. *Materials and Methods.* Eighty matched paired bone marrow samples including 53 high risk MDS (17 RAEB1, 22 RAEB2 and 14 RCMD)/AML and 27 CMML (15 CMML1 and 12 CMML2)/AML were analyzed for RUNX1 mutations. Mutational analysis was performed by direct sequencing of all RT-PCR or DNA-PCR products amplified with different primer pairs covering the entire coding sequencies of RUNX1b gene. Results. Thirteen (7 RAEB1, 4 RAEB2 and 2 RCMD) of 53 patients with MDS had RUNX1 mutations at initial diagnosis: 6 missense mutations with two of them carrying additional silent mutations, 5 non-sense mutations, 1 silent mutation and 1 frameshift mutation. Six mutants were located in the runt homology domain. One patient with RAEB2 had homozygous mutation, the remainders had heterozygous mutations. At AML transformation, 12 patients retained the identical RUNX1 mutants and one had pattern change, from Met283X to Ser114Pro. Three patients did not have RUNX1 mutations at MDS phase acquired frameshift mutations during AML progression. Of the 27 patients with CMML, 14 had RUNX1 mutations at diagnosis, including 3 missense, 3 non-sense, 1 silent and 7 frameshift mutations; all but one had heterozygous mutations and 9 located in the N-terminal region. Twelve of the 14 CMML patients with RUNX1 mutations at diagnosis carried the same mutations at AML transformation. The remaining 2 had patterns changed, one from Ser268fsX578 to Arg177X and the other from Leu71fsX94 to Arg80Leu. None of the CMML patients acquired RUNX1 mutations during AML evolution. Genotyping analysis with 15 loci of short tandem repeats at 13 different chromosomes showed identities for the 3 paired samples carrying different mutants at two phases of disease. Missense mutations were associated with a higher percentage of bone marrow blasts in MDS. The mutation status, their locations and patterns did not influence outcome of MDS patients. There were no differences in the clinicohematologic features with respect to RUNX1 mutation status in CMML patients who subsequently had AML transformation. We found that CMML patients with C-terminal mutations had a shorter survival than those with N-terminal mutations (p=0.0315). Conclusions. RUNX1 mutations play a role in the development and progression of MDS or CMML in a subset of patients. Pattern changes or acquisition of RUNX1 mutations may occur during AML transformation.

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0257

EXPRESSION OF P53 OR CYTOPLASMIC NUCLEOPHOSMINE ASSOCIATED WITH INCREASED RISK OF DISEASE PROGRESSION IN MYELODYSPLASTIC SYNDROME WITH ISOLATED DEL(5Q)

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Background. Myelodysplastic syndrome (MDS) with isolated del(5q) and <5% marrow blasts has around 10% cumulative risk of leukemic transformation. Lenalidomide effectively improves hemoglobin levels in this category of MDS. However, due to concerns about the observed rate of disease progression the European Medicines Agency (EMEA) refrained from approval of the drug in 2008. It is unclear whether lenalidomide indeed increases the risk of AML or if progression merely reflects the natural course of the disease. A recent case report indicates that p53 and aberrant cytoplasmic nucleophosmine (NPMc) was present in small subpopulations of marrow progenitors at time of diagnosis, and that these clones evolved in conjunction with disease progression during lenalidomide treatment. Also, recent data demonstrated that 7 of 22 (32%) of 5q- patients treated with lenalidomide developed complex karyotypes and 6 of these 7 patients evolved to AML. *Aims.* To evaluate the association of p53 and NPMc expression with outcome in MDS with del(5q). *Methods.* We investigated 32 patients with MDS and del(5q) and less than 10% marrow blasts diagnosed at our department. Twenty-five had del(5q) as single abnormality, 5 had del(5q)+1, and 2 had del(5q) as part of a complex karyotype. Immunohistochemistry was used to detect expression of p53 and NPMc on sections of bone marrow biopsies or clots. The Kaplan-Meier estimate and the logrank test were used for analysis of survival and disease progression. *Results.* The median age was 79 years (range 38-94). The median follow-up was 47 months (interquartile range 25-63) and the median overall survival was 63 months (range 4-146+). Fourteen patients (44%) expressed p53 or NPMc in subpopulations of marrow cells at an early stage of the disease (5 only p53, 5 only NPMc, and 4 both). Nine patients (28%) progressed during the observation period, either by blast increase above 10% and/or by the acquisition of a complex karyotype, and 8 of these subsequently evolved to AML. Seven of 9 patients with disease progression expressed p53 or NPMc before transformation. Merely 2 of 9 patients with transformation had complex karyotypes at diagnosis, and none had del(5q)+1. The 5-year Kaplan-Meier estimate of survival was 63% and 45% (logrank test p=0.52) in patients with or without expression of p53 or NPMc before disease progression, respectively. The 5-year-risk of AML evolution was 12% and 52% (p=0.060), and of blast or cytogenetic progression 7% and 57% (p=0.019; Figure 1), respectively. The dataset was not powered to assess any potential influence of lenalidomide, however, this is currently being evaluated prospectively in a population-based trial within the Nordic MDS Group. *Summary and Conclusions.* The presence of p53 and/or NPMc expression in marrow progenitors at an early stage of MDS with del(5q) significantly correlates with subsequent disease progression in the study cohort.

Leonie Saft and Martin Jädersten contributed equally to this paper.

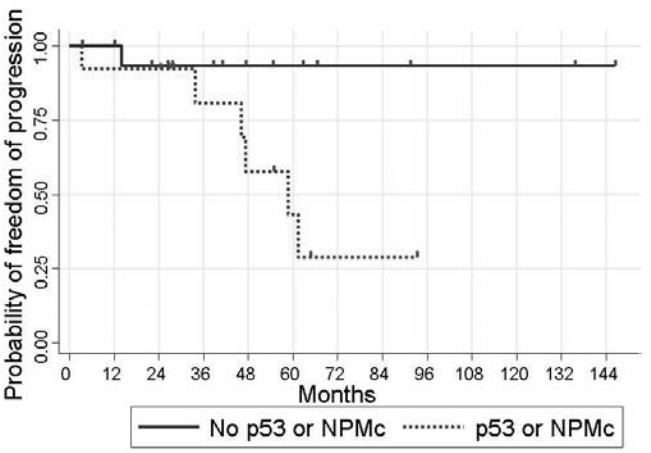

Figure 1. Time to disease progression

0258

DIFFERENT PATTERNS OF TELOMERASE REVERSE TRANSCRIPTASE (HTERT) MRNA VARIANT EXPRESSION PROFILES IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Background. Alternative splicing of telomerase reverse transcriptase (hTERT) mRNA contributes to the regulation of telomerase activity in normal and neoplastic cells. The deleted variant A (Adel), lacking 36nts in the beginning of exon 6, blocks telomerase activity while the B delet-

ed variant (Bdel), lacking exons 7 and 8, exhibits no action in terms of telomerase activation. The expression of hTERT mRNA has been investigated in myeloid malignancies with controversial results. The expression of hTERT mRNA variants has not been explored in patients with myelodysplastic syndromes(MDS). *Aim.* To investigate the expression of hTERT mRNA variant transcripts A+B+ (contained in the full-length product), Adel and Bdel in the bone marrow of MDS patients. Seventeen patients with acute myelogenous leukemia(AML) were used as controls. *Methods.* Bone marrow aspirates from 27 patients with refractory anaemia (RA or RCMD defined as group A), 13 patients with refractory anaemia with excess of blasts (RAEB1 or RAEB2, defined as group B) and 17 AML patients (defined as group C) were studied. Relative expression of the above transcripts was assessed on total RNA with reverse transcription followed by real-time polymerase chain reaction (PCR). Specific primers and probes targeting exons 5-6, exons 8-9 and exons 10-11 were designed in order to distinguish between the different isoforms. The data obtained were analyzed by using Pearson chi-square and Kruskal Wallis statistics. *Results.* hTERT mRNA was detected in 10, 4 and 3 patients of groups A, B and C respectively. The full length product (A+B+) was detected in groups A,and C (2/27, 4/13, 3/17 respectively) (p0.05). Adel isoform was detected in groups A (7/27) and B (2/13) but not in group C (0/17) (pA/C=0.02). Bdel isoform was observed in group A (3/27), B (3/13) and C (2/17). Co-expression of isoforms was observed in 2 patients of A group (Adel/Bdel, Adel/A+B+), 4 patients of group B (1 Adel/A+B+, 1 Bdel/A+B+, 2 Adel/Bdel/A+B+) and two patients of group C (Bdel/A+B+). Relative expression levels of Adel, Bdel, and A+B+ transcripts were not significantly different between patient groups. *Conclusions.* Alternatively spliced hTERT variants either lack a critical reverse-transcriptase motif or produce a non-functional reverse-transcriptase therefore cells may control telomerase activity by switching their hTERT mRNA variant expression profile. Our results indicate that aberrations in hTERT variant profiles, especially regarding the Adel isoform, may be implicated in the pathogenesis and progression of MDS. The increased incidence of Adel positivity of RA or RCMD patients may be connected with a reduction of telomerase activity and thus, contribute to the establishment of chromosomal abnormalities. The presence of the full-length pruduct (A+B) in patients with RAE'-1 or RAEB-2 may be associated with the decreased apoptosis observed in these patients. Overall, our findings address the necessity of discriminating between the different hTERT mRNA variants when investigating hTERT expression in MDS patients.

0259

INCREASED FREQUENCY OF REGULATORY T-CELLS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Background. Regulatory T-cells (Treg) represents a small fraction of peripheral $CD4^+$ T-cells which plays a crucial role in the maintenance of immune tolerance. Moreover, they seem to modulate the susceptibility to and the evolution of several neoplastic and autoimmune diseases. Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal haematologic diseases, characterised by a marked immune dysregulation. Indeed, beside the frequent occurrence of autoimmune manifestations, T-cell clones seem to be directly responsible of the functional inhibition of haematopoietic precursors. *Aims.* We analysed the frequencies of Treg and other T-cell subsets in a cohort of patients with MDS, especially focusing on their possible impact on the disease progression. *Methods.* The frequency of Treg was determined on the peripheral blood of 35 MDS patients (7 RA, 5 RARS, 18 RCMD, 4 RAEB and one 5q- syndrome) and 35 normal controls by flow-cytometry. 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. *Results.* We first showed in our patients a decreased frequency of both $CD3^+$ T-cells (mean 65% vs. 71%, $p<0.05$) and $CD4^+$ T-cells (35% vs. 40%, $p<0.05$) when compared to normal controls, while $CD8^+$ and CD16-56^+ cells were characterized by similar expression levels in patients and controls. We then showed that MDS patients had a higher frequency of Treg (1.51% vs. 1,14%, $p<0.05$) than normal controls. When we compared Treg frequencies in patients belonging to different WHO subclasses, we demonstrated a clear trend towards a higher frequency of Treg in high risk (RCMD and RAEB) than in low risk (RA, RARS and 5q- syndrome) patients (1.71% vs. 1.20%, $p=0.056$). When we stratified patients by IPSS, cytogenetics, blood counts and coexistence of autoimmune phenomena, we could not detect any statistically significant difference. Only transfusion dependence was associated to a reduced frequency of Treg (1.79% vs. 1,12%, $p<0.05$). *Summary and Conclusions.* Our data show that patients with MDS display an increased frequency of $CD4^+CD25^{high}$ $Foxp3^+$ Treg, which is even more pronounced in high risk patients. Such a difference between different patient subgroups may imply an involvement of Treg in modulating disease evolution, thus suggesting that their expansion could favour a progression of MDS towards more aggressive entities. Moreover we may speculate that the lower frequency of Treg in transfusion dependent patients may mirror a reduced control of possible autoreactive T-cells, which are known to be responsible of the inhibition of haematopoietic precursors and, therefore, of an increased need of transfusions.

0260

PHOSPHOINOSITIDE-PHOSPHOLIPASE C BETA1 AS A TARGET OF EPIGENETIC THERAPY IN MDS PATIENTS

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Background. Myelodysplastic syndromes (MDS) are a heterogeneous group of hematological malignancies characterized by an increased although variable risk of evolution in acute myeloid leukemia (AML). Lipid signalling pathways are involved in many important processes, such as cell growth, differentiation and apoptosis. Namely, nuclear phosphoinositide-phospholipase C (PI-PLC) β1 appears as one of the main players of the signal transduction pathways. Our group demonstrated, by fluorescent in situ hybridization analyses, that in MDS patients PI-PLCβ1 can undergo an interstitial mono-allelic deletion, as 35/80 (43.75%) of the MDS patients analyzed showed this cytogenetic alteration. Interestingly, 23/35 (65.7%) of the MDS patients bearing the PI-PLCβ1 mono-allelic deletion evolved into AML, retaining a statistically higher significance as a prognostic factor of evolution into AML. On the other hand, the structure of PI-PLCβ1 promoter, displaying two CpG Islands, showed that also epigenetic mechanisms could be involved in the MDS progression. Azacitidine, an inhibitor of DNA methyltransferases, has proven effective in prolonging survival and delaying evolution into AML. *Aims.* The observation, in a single patient who favourably responded to azacitidine, (Follo *et al.*, Leukemia 2008) that treatment induced an increase in PI-PLCβ1 mRNAs accompanied by a down-regulation of activated Akt, prompted us to further investigate the relationship of this enzyme to demethylating treatment. *Methods.* We studied 14 patients with high risk MDS (IPSS risk: intermediate-2 or high) treated with azacitidine, 6 patients who only received best supportive care, as control group and 10 healthy subjects. 8/14 patients (57,1%) showed a favourable response to azacitidine (Complete Remission: 1 ; Partial Remission: 1; Hematologic Improvement: 6). We analyzed the structure of PI-PLCβ1 promoter and quantified the degree of PI-PLCβ1 methylation during azacitidine administration, and we also studied 6 patients only treated with supportive care, as control group. Results. We report for the first time that not only that PI-PLCβ1 is hyper-methylated in MDS as compared to healthy subjects, but also that the amount of PI-PLCβ1 is linked to azacitidine responsiveness in MDS patients. Following azacitidine treatment, and in correlation with the therapeutic response, PI-PLCβ1 expression increased, whereas PI-PLCβ1 promoter methylation was reduced in responder patients. Moreover, we observed that the decrease of promoter gene methylation may anticipate the hematologic response, given that the variations in PI-PLCβ1 gene expression may occur some cycles prior to the hematologic improvement. *Summary and Conclusions.* To our knowledge, this is the first time that PI-PLCβ1 has been demonstrated to be hyper-methylated in MDS patients. In addition, we showed that PI-PLCβ1 induction, and particularly the nuclear splicing variant PI-PLCβ1b, directly correlated with response to azacitidine. Our findings therefore suggest that PI-PLCβ1 promoter hypermethylation could represent an epigenetic mechanism involved in the pathogenesis of MDS so that PI-PLCβ1 could be a target for demethylating therapy.

0261

HYPOMETHYLATING AGENTS IN MYELODYSPLASTIC SYNDROME - SYSTEMATIC REVIEW AND META-ANALYSIS

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Background. Supportive care for myelodysplastic syndrome (MDS) does not induce complete remission nor does it prevent progression to acute myelogenous leukemia (AML). Allogeneic stem cell transplantation has curative potential but is not applicable for most patients. Hypomethylating agents, e.g. 5-azacitidine and 5-aza 2' deoxyazacitidine (decitabine) have recently been shown to improve outcome of patients with MDS. *Aims.* We aimed to evaluate the effect of hypomethylating agents on overall survival for patients with MDS. *Methods.* Meta-analysis and systematic review of randomized controlled trials comparing treatment with hypomethylating agents to conventional care, e.g., best supportive care or chemotherapy, in patients with MDS. The Cochrane Library, MEDLINE, conference proceedings and references were searched until February 2009. Two reviewers appraised the quality of trials and extracted data. Outcomes assessed were: overall survival, time to AML transformation or death, overall (complete and partial) response and toxicity. Hazard ratios (HR) with 95% confidence intervals (CIs) were estimated and pooled for time to event data using methods described by Palmar *et al.* For dichotomous data, relative risks (RR) were estimated and pooled. *Results.* Our search yielded four trials including 952 patients, one of them as an abstract. These trials examined the effect of 5-azacitidine and decitabine. Most patients in the conventional care arm received best supportive care. Data of three trials counting 782 patients was available for analysis of overall survival. Treatment with hypomethylating agents improved overall survival (HR 0.68, 95% CI 0.57-0.83) (Figure 1). When 5-azacitidine was compared to conventional care, there was an advantage in overall survival in favor of 5-azacitidine (HR 0.56 95% CI 0.44-0.73, 2 trials, 549 patients). Conversely, this survival benefit could not be shown for decitabine (HR 0.88 95% CI 0.66-1.17, 1 trial, 233 patients). There was a significant advantage in favor of hypomethylating agents in time to AML or death (HR 0.69 95% CI 0.58-0.82) and in overall response (RR 4.01 95% CI 2.58-6.25). As expected, a higher rate of grade 3/4 adverse events was observed with the use of hypomethylating agents (RR 1.20 95% CI 1.09-1.32). *Conclusions.* Hypomethylating agents prolong overall survival as well as time to AML or death and improve response. Hypomethylating agents have an important role in the treatment of MDS patients.

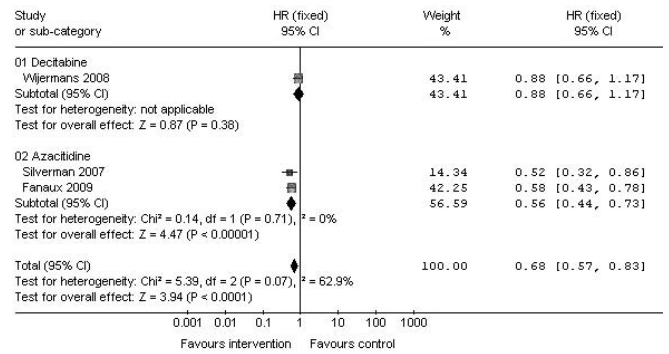

Figure 1. Overall survival, sub-analysis by type of drug

0262

CYTOGENETIC EVOLUTION IN MDS: IS IT PREDICTIVE OF OVERALL SURVIVAL AND RISK OF MDS/AML EVOLUTION?

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In every MDS patient the assessment of the cytogenetic pattern is a mandatory step for an accurate diagnosis and a precise prognostic stratification since it has been revealed that the cytogenetic defects present on clinical diagnosis (primary abnormalities) significantly influence overall survival (OS) and the risk of MDS/AML evolution (Progression-free interval, PFI). In contrast, very few studies have estimated how many patients develop chromosomal abnormalities during the course of the disease (secondary defects) and whether these karyotype defects have any prognostic relevance. These are the goals of the present study which is also aimed at correlating cytogenetic evolution (CE) with FAB/WHO classification and IPSS score, at testing whether the influence of CE on OS and PFI is independent of other prognostic parameters and at establishing which secondary defect is truly responsible of the course of the disease. Our study includes 153 *de novo* MDS patients who were diagnosed at the Division of Haematology, Foundation IRCCS Policlinico San Matteo, Pavia between January 1990 and December 2004. They were 80 males and 73 females; their median age was 60.5 years (range: 49.8-68.3). According to FAB, 27 were classified as RARS, 57 as RA, 58 as RAEB and 11 as RAEB-t; according to WHO classification, 17 were classified as RARS, 21 as RA, 11 as 5q- syndrome, 7 as RCMDS, 27 as RCMD, 27 as RAEB-1, 31 as RAEB-2 and one as unclassifiable MDS. Considering IPSS, 35 patients were low-risk, 58 intermediate-1 risk, 39 intermediate-2 risk and 21 high-risk. Median follow-up was 45.2 months (IQR=23.8-75.7) and median survival was not reached (IQR=34.3-not reached). At the time of the analysis 107 (69.9%) patients had survived and 46 (30%) had died. Disease progression (DP) occurred in a total of 65 (42.4%) patients and median progression-free interval (PFI) was 65.2 months (IQR 16.60-not reached). On clinical diagnosis 94 patients (61.4%) presented primary chromosomal defects. Cytogenetic evolution (CE) occurred in a total of 47 (30.7%) patients according to FAB classification and in 41 (28.9%) according to WHO classification. Since 7 patients according to FAB and 6 according to WHO experienced CE after disease progression they were considered karyotypically stable. CE had a significant influence on OS (p=0.0000) and PFI (p=0.0000) and its impact remained significant even when other variables, including FAB/WHO classification, IPSS score, primary defects, and IPSS cytogenetic categories, were considered. In early MDS the acquirement of a secondary defect significantly effected OS and PFI when patients were diagnosed according to FAB, but it lost its relevance when patients were diagnosed according to WHO classification. Among secondary defects, del(7)(q31q34) was the only one which significantly effected PFI.

0263

ARHGAP21, A PARTNER OF ALPHA-CATENIN, MAY BE ALTERED EXPRESSED IN BONE MARROW CELLS FROM PATIENTS WITH MYELODYSPLATIC SYNDROMES LACKING 5Q DELETION

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Background. The gene encoding α-catenin (CTNNA1) is expressed at a much lower level in leukemia-initiating stem cells from individuals with Acute Myeloid Leukemia (AML) or Myelodysplastic syndrome (MDS) with del(5q). Thus, loss of expression of the α-catenin tumor suppressor in hematopoietic stem cells may provide a growth advantage that contributes to human MDS or AML with del(5q). ARHGAP21 is a partner of α-catenin and a negative regulator of RhoGTPase signaling pathways, which is upregulated during myeloid differentiation. *Aims.* The aim of this work was to evaluate the expression of ARHGAP21, α-catenin and β-catenin in bone marrow cells from MDS patients with del(5q), MDS patients lacking a 5q deletion and healthy donors. *Methods.* CD34$^+$ cells were isolated from bone marrow of 2 MDS patients del(5q), 4 MDS patients, including 3 refractory anemia (RA) and 1 refractory anemia with excess blasts (RAEB), and 4 healthy donors. Total bone marrow cells (BMC) were obtained from 3 MDS patients del(5q), 11 MDS patients, including 9 RA and 2 refractory anemia with ringed sideroblasts (RARS), and 10 control subjects. The National Ethical Committee Board approved the study and informed-written consent was obtained from all subjects. The mRNA expression of ARHGAP21, α-catenin and β-catenin was analyzed by Real-time PCR. Localization of proteins in CD34$^+$ cells was obtained using confocal microscopic analysis. *Results.* Regarding to α and β-catenin, mRNA expression was lower in CD34$^+$ cells from MDS patients del(5q), as compared to MDS and healthy controls (both p<0.05). ARHGAP21 mRNA was higher expressed in total bone marrow cells from MDS patients compared to healthy donors (12.91747±6.93183 vs. 1.185931± 0.341093, p=0.0012). Confocal analysis showed that ARHGAP21 and β-catenin are preferentially localized in the nucleus of CD34$^+$ cells from MDS patients in contrast to the preferential cytoplasmic and membrane localization in MDS del(5q) and healthy donors. α-catenin was mainly

observed in the membrane of the cells. In the patients del(5q) and healthy controls, ARHGAP21 and α-catenin preferentially co-localizated in the cell membrane. *Conclusions.* ARHGAP21 is overexpressed in differentiated cells but not in the progenitors cells of MDS patients. ARHGAP21 and α-catenin are co-localized in hematopoietic progenitor cells, suggesting that ARHGAP21 may be related with α-catenin regulation on those cells. The nuclear localization of ARHGAP21 and β-catenin in MDS patients may suggest an aberrant signalling in the Wnt pathway for β-catenin, or an abnormality of ARHGAP21 and β-catenin themselves, which may be implicated in the MDS pathogenesis. Supported by: FAPESP.

0264

THALIDOMIDE FOR THE TREATMENT OF MYELODYSPLASTIC SYNDROMES: A SYSTEMATIC REVIEW AND META-ANALYSIS OF THE PUBLISHED STUDIES

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Background. Thalidomide has been used to improve the cytopenia of myelodysplastic syndromes (MDS). However, due to the heterogeneity of patient populations treated and of doses/schedules adopted in the different studies so far performed, the efficacy of this drug in MDS remains controversial. Furthermore, some concerns also exist about its tolerability. Indeed, no individual clinical trial has been sufficiently extensive to provide a basis for a decision model to use thalidomide in MDS. *Methods.* In order to define better the role of thalidomide in MDS, we performed a systematic review of all published articles on this topic by using a Pub-Med web-site methodology. Ten phase I-II studies, including a total of 527 MDS patients who had received thalidomide as single agent, were identified and examined. Neither phase III trials, nor previous meta-analyses were found. *Results.* Thalidomide doses widely varied in the different studies, ranging from 50 to 1000 mg/d. The response criteria also were not uniform. Overall, average response rate was 32% (range 9-56%) and 43% (range 16-88%) on intention-to-treat analysis or considering only patients able to receive the drug for 12-16 weeks, respectively. The large majority of responses were erythroid in nature (mostly resulting in transfusion-independence) and were achieved within 2-3 months, without a clear evidence of a dose-response effect. Responses were more frequently observed in patients with lower IPSS risk score and with a recent diagnosis at treatment (<1 year). There was no evidence of a correlation between response to thalidomide and baseline levels of endogenous erythropoietin, transfusion support or prior treatment with epoetins. Cytogenetic response or changes in marrow morphology were only occasionally reported. The duration of response was highly variable, ranging from three months to more than six years. Side effects, mainly peripheral neuropathy, sedation, constipation, and skin rash, were frequent, determining a very high and often early dropout (mean 44%, range 15-67%), even in responders and especially in elderly patients where thalidomide doses >200 mg/d were employed. Despite the fact that no thrombotic prophylaxis was generally adopted, thrombotic events were very rare and exclusively associated with higher doses of the drug. Two studies suggested a possible survival benefit for MDS patients treated with thalidomide. *Conclusions.*Based on available evidences, thalidomide remains a possible therapeutic option for selected MDS patients, if appropriately employed and managed. An early use of individualized doses, starting with 100 mg/d and increasing the dose up to no more than 200 mg/d, if well tolerated, is recommended for at least 12 weeks. Lower doses may be enough in elderly subjects or for maintaining response. Preferable targets appear to be lower risk patients with transfusion-dependent anemia as single cytopenia, who are not candidates for alternative approaches, such as epoetins (high levels of endogenous erythropoietin or prior resistance), lenalidomide (no evidence of the 5q- cytogenetic abnormality) and high dose chemotherapy or hypomethylating agents (no blast excess). The detailed results of the meta-analysis will be presented at the Meeting

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MESENCHYMAL STEM CELLS FROM 5Q- SYNDROME PATIENTS SHOW INCREASED EXPRESSION OF FGF4 AND IL32 IN COMPARISON TO OTHER MDS

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Background. The 5q- syndrome is a particular form of myelodysplastic syndrome (MDS) with unique clinico-biological features. An altered microenvironment has been involved in the pathophysiology of MDS through the abnormal production of several cytokines and growth factors by mesenchymal stem cells (MSC). Recently, our group has shown that that MSC from MDS display several genomic aberrations, as assessed by array-CGH and FISH. (López-Villar *et al.*, Leukemia, 2009). In addition, we observed that 5q- syndrome samples displayed a singular genomic profile, including the gain of some genomic regions. Within these regions, there are some genes related to erythroid differentiation (GDF15 in 19p13.11), megakaryopoiesis (PDGFα in 7p22.1; FGF4 in 11q13.1) and TNFα-mediated apoptosis (IL-32 in 16p13.3). *Aim.* Our aim in the present work was to compare the expression profile of these molecular targets between MSC from 5q- syndrome and MSC from other MDS, which could be related with their differential characteristics. *Methods.* Twenty-one patients with untreated MDS were included. The median age was 74 years (range 54-92). The male/female ratio was 10/11.Diagnosis of MDS was established according to the WHO classification: 5q- syndrome (n=4), refractory anemia (n=2), refractory anemia with ringed sideroblasts (n=4), refractory cytopenia with multilineage dysplasia (RCMD;n=4), RCMD with ringed sideroblasts (n=1), refractory anemia with excess blasts type (RAEB) I (n=1), RAEB-II (n=3) and MDS unclassified (n=2). In addition, four normal bone marrow (BM) samples from healthy donors were assessed as expression control. MSC were isolated and *in vitro* expanded following standard procedures. After the third passage, total RNA was isolated using the phenol/chloroform protocol and subsequently retrotranscribed into cDNA using a commercial kit. The relative quantification of the different genes mentioned above was performed by quantitative PCR using the equation 2-ΔΔCt where ΔCt = Ctgen - CtGADPH and ΔΔCt= ΔCt Sample - ΔCt Control (mean). The Mann-Whitney non-parametric U-test was used to compare gene expressions between healthy donors, 5q- syndrome and other MDS. *Results.* MSC from MDS displayed increased RNA levels of TNFα (median 7.3, range 0.1-1231; p=0.014), PDGFα (median 2.6, range 0.8-5.2; p=0.018) and IL32 (median 2.3, range 0.1-39.2; p=0.05) than healthy MSC. Strikingly, MSC from 5q- syndrome showed higher gene expression of IL32 (median 5.3, range 2.4-39.2 vs. median 1.8, range 0.1-7.6; p=0.024) and FGF4 (median 7.1, range 0.4-14.4 vs. median 0.3, range 0.05-19.3; p=0.045) than MSC from other MDS, while a trend toward higher TNFα gene expression (median 27.8, range 1.1-1231 vs. median 7.2, range 0.1-170; p=0.09) was observed in the former group. A good correlation was found between IL32 and TNFα levels (Spearman correlation=0.592; p<0.001), which supports the fact that both markers may participate in an autoamplification loop that promotes apoptosis in MDS. *Summary.* We conclude that genotypic changes detected in MSC from 5q- syndrome by CGH-array and FISH, affect the expression of some relevant genes. Therefore, the differentiated expression of IL32 and FGF4 seems to promote a pro-apoptotic (TNFα-dependent) and thrombopoietic environment, which may explain the distinctive phenotype (increased anemia and normal/elevated platelet counts) of 5q- syndrome patients.

0266

'MONOCYTOSIS' THAT DEFINES CHRONIC MYELOMONOCYTIC LEUKEMIA INCLUDES A PROPORTION OF IMMATURE GRANULOCYTES WITH A DISTINCT MOLECULAR SIGNATURE

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Background. The accumulation of monocytes in the peripheral blood is the main criterion that defines chronic myelomonocytic leukemia (CMML). In these disorders, a population of myeloid cells is hypersensitive to granulo-monocyte colony stimulating factor (GM-CSF) and a phosphorylation of STAT5 in CD14+ cells exposed to low doses of GM-CSF was recently proposed as their molecular signature. However, monocytes demonstrate important cellular heterogeneity. *Methods.* Clinical findings and laboratory findings were evaluated for 110 CMML patients. *Results.* In 94 of 110 CMML, blood cells usually identified as monocytes include a variable proportion of immature granulocytes (mean 22.8%; range 5-95%). A similar observation was made in juvenile myelomonocytic leukemias. The two cell populations belong to the leukemic clone, as demonstrated by comparative genomic hybridization or N-RAS or PTPN11 sequencing, respectively. They exhibit dramatically distinct immunophenotypes, gene expression patterns, functional properties and differentiation potential and are easily separated by simultaneous analysis of CD14 and CD24 at the surface of peripheral blood mononuclear cells. The phospho-STAT5 activation in response to low doses of GM-CSF is observed in CD14-positive CMML as well as normal monocytes, not in CD14-negative CMML cells. *Conclusions.* These results open new perspectives in the disease pathogenesis analysis and provide a new tool to follow the response to drugs currently developed to treat these diseases.

0267

ISOLATED DEL 20Q DEFINES A SUBGROUP OF MDS PATIENTS WITH LOWER BLAST COUNTS AND MORE FREQUENT THROMBOCYTOPENIA

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Background. Isolated del 20q is common in MDS, and considered of good prognosis, but no large series of MDS with isolated del 20q have been reported. In addition, it has been suggested that MDS with isolated del 20q may be associated with only mild dysplastic features, sometimes causing difficulties to distinguish them from other causes of cytopenias (Br J Haematol. 2007, 139,265). Patients: 64 MDS pts with isolated del 20q diagnosed between 1990 and 2008 in 5 GFM centers were analyzed and compared with 1368 karyotyped MDS without del 20q included in the GFM registry. *Results.* Median age of the 64 MDS with isolated del 20q was 72 (48-92), 41 M, 23 F. They included 34 RA, 8 RARS, 16 RAEB, 4 CMML and 2 RAEB-T. Fifty two percent had <5% of marrow blasts vs 34% of non del 20q MDS (p=0.006). Despite a significantly higher proportion of low and int-1 IPSS risk (90% vs. 78%, p=0.021) pts with isolated del 20q had lower platelet count (mean 156 vs 212 G/l, p=0.014) and higher reticulocyte count (mean 84 vs 52 G/l, p=0.012) than non del 20q pts, but no significant differences in other parameters especially Hb level. When the analysis was restricted to pts with less than 5% marrow blasts, the same differences were seen between isolated del 20q and non del 20q pts. Nineteen percent of pts with isolated del 20q had Hb >12 g/dL and platelets < 100 G/L vs. 8% of non del 20q patients (p=0.007). In those purely thrombocytopenic del 20q pts, dysplastic features were present to the same extent as in non del 20q pts. *Conclusions.* MDS with isolated del 20q are characterized by lower percentage of marrow blasts and lower platelet counts. About 20% of them present with isolated thrombocytopenia and morphologic analysis may help to distinguish MDS from other causes of cytopenias (especially ITP) before cytogenetic results.

Acute myeloid leukemia - Biology I

0268

MODULATION OF MIR-449A EXPRESSION DECREASES CELL VIABILITY, INCREASES APOPTOSIS AND INDUCES DIFFERENTIATION IN EVI1 DEREGULATED LEUKEMIA CELLS

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Background. Chromosomal rearrangements involving the EVI1 gene are a recurrent finding in malignant myeloid disorders. These translocations or inversions contribute to ectopic expression or to the formation of fusion genes involving the EVI1 gene. EVI1 transcriptional activation has been reported in up to 10% of acute myeloid leukemia (AML), and is a prognostic marker of poor outcome. *Aims.* As microRNA (miRNA) deregulation was recently identified as a major contributor to cancer initiation and progression, and since miRNA genes were shown to be directly regulated by activated proto-oncogenes, we aimed to identify miRNAs involved in EVI1 deregulated leukemia. *Methods.* In this study, miRNA profiling was performed on patient samples and on siRNA mediated EVI1 knockdown model systems of the EVI1 rearranged myeloid leukemia cell lines Kasumi-3, UCSD-AML1 and MUTZ-3. Our patient cohort consisted of 38 EVI1 rearranged and overexpressing samples as confirmed by FISH, karyotyping and RT-qPCR, 6 normal bone marrow samples and 2 CD34+ cord blood fractions. A total of 366 miRNAs and 18 small RNA controls were profiled using high-throughput megaplex stem-loop RT-PCR followed by PreAmp pre-amplification and RT-qPCR of individual miRNAs and controls. *Results.* MicroRNA profiling of patient samples allowed identification of a subset of significantly differentially expressed miRNAs (26 up and 27 down, p<0.05) in EVI1 rearranged samples compared to normal bone marrow. Among these, 2 upregulated and 6 downregulated miRNAs were also differentially expressed in the EVI1 knockdown model systems. The expression of three selected differentially expressed miRNAs, i.e. the downregulated miR-449a and upregulated miRs-213 and -107, was reconstituted by electroporation of a precursor miRNA or anti-miRNA molecules, respectively, in the Kasumi-3 and UCSD-AML1 cell lines. A decrease in cell viability and an increase in apoptosis were detected, as compared to the controls, with the strongest effects noticed for miR-449a. Furthermore, loss of *early* myeloid markers and an increase of megakaryocytic and monocytic markers were indicative for an effect on myeloid differentiation. To identify miR-449a target genes responsible for these effects we interrogated different prediction algorithms, and several candidate genes involved in apoptosis, differentiation or cell proliferation are currently under further investigation. *Conclusions.* This report is the first study that identifies miR-449a as an important miRNA involved in EVI1 pathogenesis. Given the poor prognosis of EVI1 rearranged leukemias and currently limited treatment options, the present findings together with the emerging possibilities of miRNA based therapeutics open new perspectives for treatment of this patient subgroup.

0269

RESTORING OF FOXO3 TRANSCRIPTION FACTOR ACTIVITY BY CHEMOTHERAPY INDUCES OF A QUIESCENT STATUS OF LEUKAEMIC PROGENITOR CELLS IN ACUTE MYELOID LEUKAEMIA

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Background. The FoxO transcription factor promotes apoptosis and triggers cell cycle inhibition through multiple mechanisms. It is inactivated by PI3K/Akt induced phosphorylation thus resulting in nuclear exclusion and degradation. Moreover, several data demonstrated an abnormal activation of PI3K/Akt pathway in acute myeloid leukemia (AML) with contrasting prognostic significance. The aim of this study

was to clarify the role of FoxO3 in AML and to investigate alternative pathways eventually responsible for FoxO3 inactivation. Importantly, we explore the effects of FoxO3 re-activation in CD34+ AML cells. *Methods.* Cell cycle was analyzed by FACS in CD34+ cell population as well as the levels of CD47, which has been demonstrated to increased during progression through the cell cycle and stem cell mobilization. Protein amount and localization were analyzed by Western blot and immunofluorescence, the DNA binding activity was measured by EMSA. 35 BM samples from AML patients at diagnosis and in 20 healthy donors were analyzed. Furthermore Spred1, known to be a FoxO3 target gene was quantified by RQ-PCR. We previously described the absence of Spred1 in AML patients and demonstrated that it promotes growth arrest and apoptosis in haematopoietic cells. Finally BM cells were incubated with a PI3K inhibitor LY294002 and the IKK inhibitor PS1145, alone and in combination. Moreover, the t(8;21) positive Kasumi cell line was transfected with pECE-FoxO3 to evaluate FoxO3 effects on cell growth and apoptosis. *Results.* We found that, while FoxO3 in control cells is localized in both nucleus (mean value of intensity of 21.4 2) and cytoplasm (14.6 1.7), it is completely cytoplasmatic in AML cells (18.1 4,6 in cytoplasm vs. 8,2 4 in the nucleus) and enters the nucleus after chemotherapy or *in vitro* incubation with LY29400. Moreover, FoxO3 DNA binding activity in AML patients is completely absent at diagnosis and restored after therapy. Also the mRNA of Spred1 is rather undetectable at diagnosis (2- Ct= 0,009 0,3) and shows normal levels during remission (2- Ct = 2 1,5) or after LY29400 incubation (2- Ct =0,8 0,3). In addition LY294002 and PS1145 treatment results in FoxO3 partial nuclear re-localization while their association induces a complete nuclear shuttle suggesting that both pathways could be implicated in FoxO3 inactivation. The restoring of FoxO3 function by LY29400 in CD34+ cells induces quiescence of this progenitor cell compartment as demonstrated by the comparison of cell cycle kinetics and the decreased expression of CD47 (p=0.01). Finally, FoxO3 overexpression in transfected cells results in a block of proliferation rate (66% of inhibition compared to empty vector transfected cells) and apoptosis induction (35% vs. 12%). *Conclusions.* Taken together these data suggest that FoxO3 inactivation may be crucial for the leukemic progression and demonstrate that also IKK pathway contributes to this effect, providing the rationale for a therapeutic strategy. On the other hand, the re-activation of FoxO3 induces quiescence of the stem cell compartment so providing a mechanism of escape from chemotherapy induced apoptosis.

0270

PREVALENCE OF TET2 MUTATIONS IN *DE NOVO* ACUTE MYELOID LEUKAEMIA

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Background. Recently, it was established that TET2, a member of Ten-eleven translocation family located on the 4q24 locus, is a gene frequently altered in myeloid malignancies (Delhommeau *et al.*, in press). *Methods.* To investigate the prevalence of TET2 mutations in *de novo* AML, we designed a retrospective study of 78 patients enrolled in two clinical centers (Lille and Cochin hospitals) for whom DNA was available at diagnosis and at first complete remission (CR). TET2 events were assessed by direct sequencing of exons 3 to 11. LOH and copy number variations were investigated by SNP arrays (Affymetrix® SNP array 6.0). All the patients are *de novo* AML (excluding APL), the median age was 43 years (14 to 69), the sex ratio M/F was 0.6, the cytological repartition was: 5 AML0, 14 AML1, 24 AML2, 20 AML4, 10AML5, 2AML6 FAB subtypes. Among these patients, 75 had an informative karyotype (14 favorable-risk, 60 intermediate-risk with 35 normal karyotypes, and 1 unfavorable-risk according to the British MRC classification). *Results.* We found a total of 16 anomalies of TET2 gene by direct sequencing: 7 nonsense and 5 missense mutations in conserved domains and 4 missense mutations in non conserved domains. For the latter 4 mutations, we confirmed that the mutations were absent in the CR sample in all 4 cases. Overall, 13/78 (17%) patients presented an acquired TET2 mutations at diagnosis. 3 patients had two anomalies of TET2 detected by direct sequencing, suggesting that the two copies are targeted in 4% of the patients. By SNP analysis, none of the 67 patients analyzed presented a deletion in the TET2 locus and only one presented a large UPD of 4q. The population with TET2 mutations had no difference in age, sex ratio, leukocytes, FAB subtypes, however, regarding the cytogenetic data, no mutation was detected in the MRC unfavorable-risk subgroup, and the majority of the TET2 mutated patients presented a normal karyotype *Conclusion*. In conclusion, in *de novo* AML patients, TET2 alterations are observed at a frequency of 17% in all FAB subtypes, but was preferentially associated with a normal karyotype. With the exception of a few cases, TET2 alterations are point mutations. The association with the others biological and clinical parameters will be presented.

0271

IDENTIFICATION OF FREQUENT GENOMIC ABNORMALITIES IN ADULT ACUTE MYELOID LEUKEMIA PATIENTS USING SINGLE NUCLEOTIDE POLYMORPHISM ARRAYS

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Introduction. Acute myeloid leukemia (AML) is a heterogeneous disease with various chromosomal aberrations. The karyotype at diagnosis provides important prognostic information that influences therapy and outcome of this disease. However, using conventional chromosome banding techniques alone, karyotype abnormalities are detected in only half of all AML cases. Aims.We sought to identify novel genomic regions of interest in normal karyotype AML and to identify novel candidate regions and disease-related genes in patients with complex karyotypes using genome-wide high resolution SNP-array. Patients and Methods. Samples from 57 AML patients with FAB-M0, M1, M2, M3, M4, M5, miscellaneous cytogenetic abnormalities and normal karyotype were examined. Genomic DNA was isolated from mononuclear AML cells and applied to GeneChip® Human Mapping 250K NspI and Genome-Wide Human SNP 6.0 array microarrays (Affymetrix, Santa Clara, CA) following the manufacturer's instructions. Copy number aberrations were scored using the Hidden Markov Model and the segmentation approach available within the Partek software package as well as by visual inspection. All aberrations were calculated with respect to a set of 270 Hapmap normal individuals and a set of samples obtained from acute leukaemia cases in remission in order to reduce the noise of raw copy number data. When available, in order to exclude inherited copy number variants a comparison to paired constitutional DNA and to paired remission DNA was performed. Fluorescence in situ hybridization, quantitative PCR and nucleotide sequencing were used to confirm genomic alterations. Results. A wide spectrum of different genetic lesions (gains/losses) involving complete chromosome arms (del 16q, i(13q10), del 3p, del 7p, monosomy 9) or submicroscopic genomic intervals were identified in a substantial proportion of cases (55%) without differences in the frequency of losses or gains. Focal genetic alterations were detected at the breakpoints of previously cytogenetically identified chromosomal translocations, such as t(2;3)(p22-23)(q26-27) and t(1;11)(p32;q23). Hemizygous deletions were identified at 2q33.3-q34 involving ERBB4 (v-erb-a erythroblastic leukemia viral oncogene homolog 4 avian), at 9p21.3- p21.2 (CDKN2A-2B), at 12p13 (ETV6), at 17q12 (NF1) and 21q21.2 (RUNX1). Most frequent alterations affected the oncogene MYC at 8q24.13 - q24.21 (4.33 Mb), the ABC transporters genes ABCA8, ABCA9, ABCA6, ABCA5, ABCA10 at 17q24, PTPRM at 18p11.31-p11.23 and ERG at 21q22. Other recurring genetic lesions were uncommon and were identified only in single cases. Some lesions affected regions with a single gene, such as: ETAA1, FIGN, STK32B, PRAGMIN, PCM1, GLIS3, MRGPRX1, SESN3, BCL2L14 or lacking annotated genes. Marked differences in the combination of copy number anomalies were identified across the different genetic subtypes of AML. Patients with normal karyotype showed no relevant genetic alterations. Conclusions. These data demonstrated that, in contrast to adult acute lymphoblastic leukaemia (ALL), AML is characterized by relatively few recurring copy

number alterations, and that spectrum of genetic anomalies is significantly associated with AML disease subtype.

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0272

THE PROGNOSTIC ROLE OF C-FLIP IN AML

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Background. Defects in apoptosis contribute to the aggressive clinical phenotype of Acute Myeloid Leukaemia (AML). The anti-apoptotic protein c-FLIP (cellular FLICE-Inhibitory Protein) is a key inhibitor of processing and activation of Caspase 8 at the Death Inducing Signalling Complexes (DISCs). Differential splicing gives rise to long (c-FLIPL) and short (c-FLIPS) splice forms. Clinically relevant prognostic biomarkers are becoming increasingly important in risk stratifying the management of AML patients, particularly within the Cytogenetically-Normal (CN)-AML group. *Aims.* To investigate the prognostic role of c-FLIP gene expression in AML and determine the role of c-FLIP in chemosensitivity. *Methods and Results*. c-FLIP gene expression was assessed in 318 NCRI AML trial samples from the Cardiff and MILE studies which used standard U133 and U133Plus2 Affymetrix' chips. Overall, c-FLIP gene expression showed marked heterogeneity that was independent of WHO subclassification. However, c-FLIP gene expression had a convincing trend to display prognostic importance in the CN-AML subgroup (n=112), where those individuals with > median expression of c-FLIP had a marked trend to worse Overall Survival (OS) compared to those with < median expression levels (Log Rank; Chi Sq (df=1) p=0.08). Moreover, we found that within the NPM1 mutant group of the CN-AML subset (n=42), individuals with a c-FLIP gene expression score > median had a dramatically reduced OS compared to those individuals with scores < median (Log Rank; Chi Sq (df=1), p=0.009). There was also a marked increase in the risk of relapse within the first year in those with > median c-FLIP compared to those with < median c-FLIP expression in the NPM1 mutant group. Further analysis of c-FLIP gene expression via qRT-PCR in an independent cohort of AML samples (n=62) demonstrated that patients with FLIPL mRNA expression > median values had a significantly shorter OS and RFS compared to those with FLIPL mRNA expression < median values (p=0.03 in both cases). The role of c-FLIP in AML apoptosis has been investigated in the OCI, U937, NB-4 and K562 cell line models. c-FLIP gene silencing with a dual splice form targeted small interfering RNA (FT siRNA) alone resulted in enhanced apoptosis in the OCI, U937 and NB-4 cell lines in comparison to a control siRNA. In addition, FT siRNA sensitised all four cell lines to recombinant TRAIL with an increase in apoptotic cells detected by Flow Cytometry and enhanced Poly (ADP-ribose) polymerase (PARP) and Caspase 8 cleavage detected by Western Blot. *Conclusions.* In summary, gene expression profiling revealed a potential prognostic role for c-FLIP expression, which is highly statistically significant in the NPM1 mutant group. The prognostic significance may be related to the expression of the FLIP(L) splice form. Furthermore, as c-FLIP gene silencing modulates the apoptotic threshold and sensitises the cell lines to TRAIL, targeting c-FLIP may therefore be a therapeutic option.

0273

SPECIFIC PATTERNS OF GLOBAL HDAC1 DYSLOCALIZATION IN ACUTE MYELOID LEUKEMIA

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HDACs regulate transcriptional repression and are essential for fundamental cellular processes. HDACs are involved in tumorigenesis and in mediating the function of oncogenic translocation products in specific forms of leukemia and lymphoma. Furthermore, aberrant recruitment of HDAC activity has been reported in cell lines from patients with acute promyelocytic leukemia (PML). The oncogenic transcription factor produced by the fusion of the PML gene and the retinoic acid receptor alpha (RARα) represses gene transcription by association with a corepressor complex containing HDAC activity. The reversal of aberrant gene repression could benefit leukemia therapy and HDAC inhibitors (HDI) are currently in clinical trials for the treatment of leukemia and solid tumors. While these agents show promise, their mechanism(s) of action and selective toxicity against tumor cells have not yet been adequately defined. The altered expression of specific genes by HDAC1 is important for certain biological outcomes, but it is still unknown which genes are altered due to different HDAC1-promoter binding in patients with AML or whether altered chromosomal localization of HDAC1 is a general phenomenon in AML. Using ChIP-chip analyses we provide evidence that HDAC1 deposition patterns are specifically altered in human leukemias. The global HDAC1-chromatin binding patterns differed significantly between subtypes of AML (global test: p=0.04; Inv(16)=3; complex karyotype=5; t(15;17)=6; t(8;21)=4), implicating the importance of specific regulation of HDAC1 chromatin modifications modulated by different translocations. However, beyond the subtype specificity, we wanted to know if a general signature emerged that revealed an AML associated pattern of HDAC1 distribution to gene promoters. We therefore analyzed differences in HDAC1-binding patterns of specimens derived from AML (n=48), ALL (n=11) or $CD34^+$ progenitor cells (n=15) samples. The HDAC1 patterns differed significantly between these three groups (global test p=0.03). A class comparison analysis stringently corrected for multiple testing identified 196 genomic loci (FDR <0.05) that in HDAC1 binding differed between AML and CD34p specimen and 134 genomic loci between ALL and CD34p specimen. Interestingly, within the 196 identified genomic loci between AML and CD34p samples, HDAC1 binding was mainly increased in AML (130 genomic loci high HDAC1 binding vs 66 genomic loci high HDAC1 binding in CD34p). Gene ontology analysis revealed groups of genes to be altered, e.g. genes involved in biological processes as hematopoiesis or the involvement of MAPKK-pathway. Real-time RT-PCR analysis of two regulated genes also indicated higher mRNA levels in AML specimen that correlated with a decreased HDAC1 binding. Interestingly, based on specific HDAC1 binding differences, correct classification of AML, ALL or CD34p was achieved. Classification using leave-one-out cross validation was correct in overall 89% of cases (66/74), with highest sensitivity in AML (96%). Misclassification was low and occurred mainly in ALL samples. HDAC inhibitors have shown promising activity in AML and other diseases. In the current study, we provide evidence that disease specific alterations in HDAC1 localization exist in AML. These findings provide a rationale for the specific action of HDAC-inhibitors in malignant disease.

0274

GENE OVEREXPRESSION AND PROTEIN DELOCALIZATION OF PROTEINASE 3 MAY INCREASE CHEMOSENSITIVITY IN BLAST CELLS FROM CORE BINDING FACTOR LEUKEMIA PATIENTS

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Background. Proteinase 3 (PR3) gene codes for a serine protease with a broad spectrum of proteolytic activity. PR3 is involved in the control of proliferation of myeloid leukemia cells. When abnormally expressed it confers factor-independent growth to hematopoietic cells. The Aim of this study was to investigate the role of PR3 gene in leukemic haematopoiesis. Mathods: We analyzed the expression levels of PR3 by RQ-PCR in 113 BM samples collected from AML patients at diagnosis. The FAB distribution was as follows: M0=5, M1=12, M2=38, M3=12, M4=37, M5=5, M6=4. 19 patients were characterized by t(8;21) and 16 by inv(16). PR3 expression level was also analyzed in 57 BM and 42 PB samples from 88 MDS patients (44 RA, 32 RAEB and 12 secondary-AML) and in 15 BM and 40 PB samples from healthy volunteers. PR3 protein was analyzed by western blot (WB) and its localization determined by immunofluorescence assay using specific antibodies. The transcription factor C/EBPa, which negatively regulates PR3 expression was studied in parallel at the RNA and protein level by RQ-PCR and WB. The DNA binding activity of C/EBPa was investigated by EMSA assay. Gain and loss of function experiments were performed by transfecting COS and 293T cell lines with a plasmid containing the full length PR3 sequence and HL60, Me-1, and Kasumi cell lines with specific shRNA. *Results.* We found that PR3 is significantly overexpressed in AML sam-

ples. The median value of 2-DDCt is 740, (range 15-5043). Interestingly, patients affected by Core Binding Factor leukemias showed significantly higher PR3 values compared to patients with normal karyotypes (NK) (p<0.0002 for t(8;21), p<0.001 for inv16) and lower C/EPBa levels. EMSA assay demonstrated the absence of C/EBPa DNA binding activity in CBF AML cells but not in NK AML. In addition, PR3 overexpression was detected in 60% of RA patients (mean value: 10, range 3-268), and in all the cases of RAEB (mean value 201: range:128-803) and secondary AML (mean value 589, range 207-7131). WB demonstrated the correlation between the mRNA and protein amount. Interestingly, immunofluorescence demonstrated the de-localization of the protein within the nucleous in CBF AML but it is completely cytoplasmatic in leukemic cells with normal karyotype and in MDS. Transfection experiments with PR3 plasmid demonstrated that PR3overexpression results into a significantly increased proliferation and reduced apoptosis. By contrast transfection with shRNA triggers apoptosis and cell growth inhibition. In addition, WB demonstrated that nuclear PR3 is able to cleavage the p65subunit of NF-kB into a p56 isoform which lacks any transcriptional activity as confirmed by EMSA. *Conclusions.* PR3 gene expression and protein are significantly increased in AML and MDS, particularly in CBF leukemias in which the protein is not only increased but also completely delocalized within the nucleous. PR3 overepression My be due to a significant downmodulation of C/EBPa. Ectopic expression of PR3 induces increased proliferation and apoptosis arrest. The abnormal nuclear localization of PR3 in CBF leukemias results into the loss of function of NF-kB thus representing one mechanism of chemo sensitivity in this group of patients.

0275

PROGNOSTIC VALUE OF MINIMAL RESIDUAL DISEASE BY REAL-TIME QUANTITATIVE PCR IN AML WITH CBFB - MYH11 REARRANGEMENT: THE FRENCH EXPERIENCE

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Background. Despite the favorable prognosis of AML with inv(16)/t(16;16) leading to CBFB-MYH11 rearrangement, relapses still occur in 30 to 40% of the cases. With the possible exception of age and receptor tyrosine kinase mutations, no pretreatment-factor can strongly predict risk of relapse.

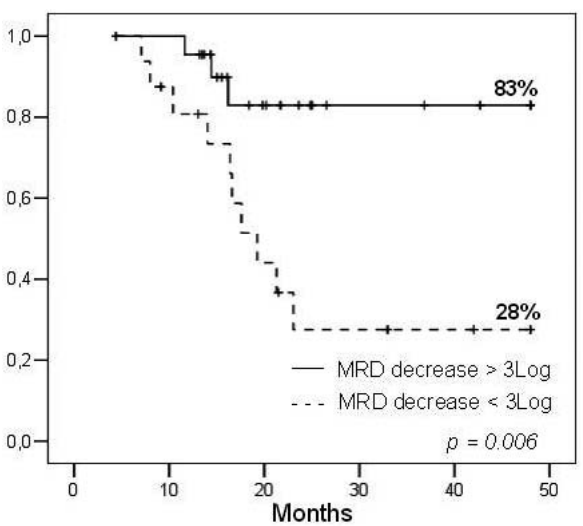

Figure. Kaplan-Meier plot of RFS according to MRD decrease after the first course of consolidation by comparison to the pretreatment level.

Aims and methods. To explore minimal residual disease (MRD) prognostic impact, we monitored CBFB-MYH11 transcript level by real-time quantitative PCR (RQ-PCR) in 59 inv(16)/t(16;16) AML patients included in French multicenter trials . *Results.* Median age was 36 years [4-76]. With a median follow-up of 26.5 months, 3 year-RFS was 63% and 3 year-OS 88%. No initial clinical or hematological characteristic including age, initial WBC or platelets count nor additional trisomy 22 was predictive of relapse. Pretreatment CBFB-MYH11 transcript level had no impact on RFS. After induction therapy, transcript levels >0.5% were significant predictors of poorer 3 year-RFS (35.7% vs. 75.5% if <0.5%, p=0.01). A reduction of transcript level by more than 3 log after first consolidation therapy course (compared to diagnosis) was also predictive of better RFS (83% vs. 28%, p=0.006) and better OS (100% vs. 67%, p=0.017). We did not observe any prognostic impact of mutational profile of the c-KIT, FLT-3 and RAS genes. Impact of the reduction of transcript level appears as an independent prognostic factor in a multivariate analysis. Conclusion. MRD monitoring by RQ-PCR appears to be a major prognostic factor of RFS in AML with CBFB-MYH11 rearrangement which may help therapeutic decisions for consolidation therapy.

0276

PROGNOSTIC VALUE OF MINIMAL RESIDUAL DISEASE MONITORING USING NPM1 MUTATION IN AML

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Background. Mutations of the NPM1 gene can be found in 35% of AML patients, mainly those with normal karyotype. They are mostly frameshift mutations (type A or B), that are usually stable at relapse, allowing their use to monitor minimal residual disease (MRD) during follow-up by real-time quantitative PCR (RQ-PCR). *Aims.*We retrospectively analyzed our AML patients with type A or B NPM1 mutation at diagnosis for whom blood or bone marrow (BM) MRD was assessed sequentially during follow-up, in order to identify the timing of MRD assessment with greatest prognostic value. *Patients and Methods.* 47 *de novo* AML with NPM1 mutation who had reached complete remission with intensive chemotherapy, M/F: 24/23; median age: 51y, median initial WBC 33.0G/L, karyotype normal, abnormal, and failure in 36, 5 and 6 pts resp. 42 and 5 pts had type A and type B NPM1 mutation. 17/45 pts (38%) had FLT3-ITD, and 8/45 (18%) a FLT3-D835 or I836 point mutations. Post-remission treatment (tx) included HD-araC in 23 pts, and allo-SCT in 8 pts. With a median follow-up of 12 months, 11 pts have relapsed. RQ-PCR was performed on cDNA from BM and/or blood samples. The MRD level (ratio of mutated NPM1/104 ABL copies), and log reductions were analysed as continuous variables, with stratification on NPM1 mutation type to account for differences in RQ-PCR sensitivities (10/104 and 100/104 for type A and B mutations, resp.). *Results.* 387 (197 BM and 190 blood) samples (median 4, range 2-13 per pt) were analysed. Median NPM1 MRD levels were 24.65 in blood, and 10.08 in BM samples at diagnosis, with no significant correlation to age, WBC, FAB, cytogenetics, NPM1 mutation type, or FLT3 status. MRD decreased by a median of 3.4 and 3.7 log10 after induction in BM and blood resp., and further decreased by a median of 1.0 and 0.6 log10 levels after post-remission tx, resp. During the follow-up, 78% and 87% pts reached undetectable MRD in BM and blood resp. In univariate Cox analysis, only FLT3-ITD status (p=0.02), WBC (p=0.053), MRD level at diagnosis in blood (p=0.03), but not in BM were associated with DFS. Post induction MRD levels in the blood (p=0.04) and BM (p=0.03) were correlated to DFS whereas at the end of tx, only the MRD level in BM (p=0.02) was correlated to DFS. In multivariate analysis, only post-induction BM MRD level retained a statistical significance independent of WBC and FLT3-ITD status (p=0.03). *Conclusions.* MRD monitoring in AML pts with type A or B NPM1 mutation is correlated to the relapse risk, but analysis seems more reliable on BM samples. The BM MRD level after induction therapy yields prognostic value independent of usual variables, including WBC and FLT3-ITD. Accrual to this cohort is continuing.

0277

THE AMP-KINASE AGONIST METFORMIN HAS TUMOR SUPPRESSOR ACTIVITY IN ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) is a clonal hematological disease characterized by the deregulation of multiple signaling pathways that contributes to cell proliferation and survival. The characterization of targets whose inhibition impairs AML growth advantage is therefore under active investigation. The mammalian Target Of Rapamycin Complex 1 (mTORC1) pathway is frequently activated in cancers including AML, and usually stimulates the expression of highly oncogenic proteins through the control of cap-dependent mRNA translation. However, we showed that allosteric mTORC1 inhibitors (rapamycin and derivate) fails to repress mRNA translation in AML, due to a rapamycin-resistant phosphorylation of the translation regulator 4E-BP1 on S65. Indeed, when phosphorylated on multiple residues, 4E-BP1 facilitates the assembly of active translation initiating complexes (eIF4F) and subsequent mRNA translation. It is noteworthy that the phosphorylation of 4E-BP1 on the N-terminal residues T37 and T46 is absolutely required for its phosphorylation on S65 residue, which is critical for translation activation. *Aims and Methods.* We studied the potential of AMPK as an inducible negative regulator of mTORC1 activity in AML biology. Experiments were done in 20 primary AML samples obtained from patients treated in varying studies of the GOELAMs French group after informed consent according to the declaration of Helsinki. MTORC1 and AMPK signaling were studied by immunoblot. Cap-dependent mRNA translation was studied by 7m-GTP pulldown assay, polysome analysis, [3H]leucine pulse assay and immunoblot. AML blast cells survival was assessed by methylcellulosis cultures and annexin V fixation in flow cytometry. *Results.* We show here in primary AML cells that mTOR inhibition by siRNA or by a catalytic inhibitor, PI-103, inhibited 4E-BP1 phosphorylation on T37/46, in contrast to rapamycin. Thus, a rapamycin-resistant activity of mTOR is involved in 4E-BP1 priming phosphorylation and represents a target to achieve inhibition of cap-dependent mRNA translation in AML. The AMP Kinase (AMPK) pathway is an inducible repressor of mTOR activity and constitutes a central regulator for mRNA translation process. AMPK is activated by metabolic stresses that either inhibit ATP production or stimulate ATP consumption and represents thereby the main cellular energy sensor. We tested 3 pharmacological AMPK agonists, metformin, AICAR and the A-769662 compound. Overall, only metformin specifically induced AMPK activity in AML. When tested in primary AML cells, metformin-induced AMPK activation led to a complete inhibition of all 4E-BP1 phosphorylation events that we reported to be rapamycin-resistant. Moreover, metformin markedly repressed cap-dependent mRNA translation in AML: this compound inhibited the assembly of the eIF4F translation initiating complex, impaired polysomes formation and decreased the amounts of polysome-bound c-Myc mRNA in AML cells. As a result, total protein synthesis was dramatically reduced in metformin-treated AML cells, as well as the expression of highly oncogenic proteins regulated at the translation-initiation level (c-Myc, Cyclin D1, Bcl-xL). Significantly, AMPK activation impaired the survival of primary AML cells, with a favourable therapeutic index when compared to the effects of metformin on normal $CD34^+$ hematopoietic progenitors. *Conclusions.* The activation of the AMPK tumor suppressor pathway represents a promising perspective in AML therapy and could be further explored in clinical trials.

0278

SUPPRESSOR OF CYTOKINE SIGNALING 1 (SOCS1) IS INDUCED BY FLT3-ITD IN HEMATOPOIETIC STEM AND PROGENITOR CELLS AND COOPERATES IN LEUKEMOGENESIS

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FLT3 (FMS-like tyrosine kinase 3) is a receptor tyrosine kinase expressed on early hematopoietic progenitors. Internal tandem duplications are frequently found mutations in the juxtamembrane domain of FLT3 (so called FLT3-ITD), lead to constitutive activation of the receptor and the STAT5 signaling pathway. Retroviral expression of FLT3-ITD in primary murine hematopoietic stem and progenitor cells leads to expression of SOCS (suppressor of cytokine signaling) genes, which are known STAT5 target genes. As SOCS proteins are known to inhibit growth of hematopoietic stem and progenitor cells, we investigated their role in FLT3-ITD-mediated transformation. In the presence of pro-proliferative cytokines and growth factors, colony growth was severely impaired in SOCS1-expressing primary bone marrow cells, while this inhibition was reversed by co-expression of FLT3-ITD, suggesting resistance of FLT3-ITD to SOCS1. Importantly, when colony assays were performed in the presence of anti-proliferative interferonγ (IFNγ), the SOCS1 co-expression protected FLT3-ITD from the IFNγ growth inhibitory effects when compared to FLT3-ITD alone. In a murine bone marrow transplantation model, the co-expression of SOCS1 and FLT3-ITD led to the rapid development of a myeloproliferative disease or acute lymphoblastic leukemia with significantly faster onset of disease and shorter median survival compared to FLT3-ITD. Together, these data demonstrate that in the context of the oncogene FLT3-ITD, SOCS1 promotes colony growth and leukemogenesis, while if SOCS1 is expressed alone it maintains its role as a negative regulator of cytokine signaling. Our data support the model that the expression of SOCS proteins in hematopoietic stem and progenitor cells may shield FLT3-ITD-expressing cells from cytokine control and that impaired cytokine signaling contributes to FLT3-ITD-mediated leukemogenesis.

0279

A SINGLE 8-COLOR FLOW-CYTOMETRIC IMMUNOSTAINING ALLOWS DELINEATION OF BOTH TYPICAL MYELOID AND LYMPHOID ACUTE LEUKEMIA AND UNDIFFERENCIATED/IMMATURE ACUTE LEUKEMIA (ON BEHALF OF THE EUROFLOW CONSORTIUM)

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Background. The development of standardized 8-color flow cytometry (FC) combined with bio-informatic merging of data from multiple tubes allows multiparameter evaluation of surface and intracellular proteins at the single cell level, while maintaining all the advantages of FC diagnostics (rapidity, evaluation of cellular heterogeneity, application to minimal residual disease, etc). *Aims.* Within the European EuroFlow program, we designed an 8-color acute leukemia screening tube (ALST) for fast and efficient evaluation of patients suspected of acute leukemia in order to choose more detailed immunophenotyping panels. *Methods.* 157 acute leukemia samples (105 BM, 45 PB, 7 others) were analyzed with the ALST tube by FC in 8 EuroFlow centers, using standard operating procedures which provided intercenter reproducibility. In parallel, conventional procedures for lineage assessment of blast cells were used at each centre. ALST data was collected and analysed centrally using the Infinicyt software (Cytognos®). The blast cell population was identified bio-informatically, extracted from each sample and virtually merged in a common database with the blast cell populations from all other samples. Within this database, each leukemic sample was represented by its median expression for each individual ALST antibody, and the results from all 157 samples underwent unsupervised discrimination by principal component analysis (PCA) using automated software tools. *Results.* Using a pair-wise PCA-based comparison, 152/157 AL could be clearly separated into 3 well defined clusters (89 B-Cell Precursor-ALL, 27 T-ALL and 36 AML), with no mis-classification compared to classical, more extensive FC panels. Five cases (3%) clustered together in between the typical T-ALL and AML clusters, in keeping with an undifferenciated T/Myeloid maturation arrest. These included 3 AML and 2 undifferen-

tiated AL. No intermediate Myeloid or clusters were observed. No center dependent effect on clustering was observed, in keeping with the use of highly standard operating flow cytometric procedures. *Summary and Conclusions.* This ALST FC strategy linked to Infinicyt analysis allows efficient, fast and accurate lineage orientation of acute leukemia samples and multicenter, multiparametric analysis of patient data. In addition, this approach allowed identification of a subgroup of T/Myeloid AL which should be further analysed in both T-ALL and AML panels. Multicenter identification of these relatively rare samples will allow analysis of their clinical and biological characteristics and, if appropriate, their individualization for specific therapy. In conclusion, we propose an easy, efficient and highly sensitive flow-based tool to screen for cell lineage in acute leukemias which contributes to optimise health resources for further FC panels, allow multicenter comparisons and may help in identifying new subgroups of patients.

0280

EXPRESSION, FUNCTION AND REGULATION OF AUTOTAXIN, A RELEVANT MOTILITY AND SURVIVAL FACTOR, IN FLT3-ITD POSITIVE ACUTE MYELOID LEUKEMIA AND PRIMARY HEMATOPOIETIC STEM CELLS

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Genetic aberrations of the FLT3 receptor gene are among the most common mutations of adult patients with acute myeloid leukemia (AML) and result in constitutive activation of several downstream pathways including the RAS/ERK, PI3K and STAT5-signaling pathway. With an incidence of approximately 25%, the internal tandem duplication (ITD) mutation of the juxtamembrane coding sequence represents the most frequent abnormality of the FLT3 receptor gene and is associated with inferior prognosis. We have recently demonstrated by microarray analysis that leukemic samples of patients with FLT3-ITD mutations have significantly upregulated expression levels of Autotaxin (ATX). The ATX protein acts as a secreted lysophospholipase D (lysoPLD) through generating lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC). LPA has several important functions in cell migration and proliferation and acts via G-protein coupled receptors. It has been shown that ATX represents an aberrantly expressed motility and growth factor in a variety of cancer cells. However data on ATX in myeloid leukemias and especially in AML are missing. To study more deeply the role of ATX in human leukemic cells, we stably expressed two alternatively spliced transcripts in several human leukemic cell lines without detectable levels of endogenous ATX. The expression of ATX transcripts was confirmed at both mRNA and protein levels by RT-PCR and Western blotting in several leukemic cell lines as well as in human primary progenitors. Transwell migration assays in the presence of LPC or LPA were performed to study effects of ATX on cell motility. Proliferation and clonogenic potential were investigated using MTT and colony forming assays. High ATX expression was found primarily in malignant cells. In normal cells, highest ATX mRNA expression was found in purified CD34+ cells. Stable overexpression of FLT3-ITD in OCI-AML3 cells induced an increased ATX expression (up to 6 fold). Vice versa, inhibition of FLT3-ITD by sublethal doses of PKC412 in MV4-11 cells resulted in a significant reduction of ATX expression down to 10% of the initial expression level. PKC412 treatment also resulted in a complete loss of LPC induced specific migratory capacity in MV4-11 cells. Moreover, we could show that the Jun N-terminal kinase (JNK) is an important mediator between FLT3 and ATX. Specific inhibition of JNK resulted in a reduction of ATX expression levels and subsequently in a complete loss of LPC mediated chemotaxis. The transduction of ATX increased the colony-forming capacity by 75% and significantly increased the short term proliferation. LPA increased chemotaxis in human leukemic cell lines and human $CD34^+$ progenitors in a dose dependent manner and induced significantly higher migratory rates by at least 50%. LPC induced chemotaxis by 80-200% only in cells with high expression of endogenous or exogenous ATX, demonstrating the autocrine activity of ATX. This LPC/LPA induced chemotaxis could be blocked by pertussis toxin (PTX) and Ki16425 demonstrating the involvement of PTX/Ki16425 sensitive LPA1 receptors in MV4-11 cells. Our data suggest that the production of bioactive LPA through ATX is involved in controlling proliferation and migration of haematopoietic stem cells and its deregulation may contribute to the pathogenesis of AML.

0281

A MOLECUALR CIRCUITRY COMPRISING MICRORNA-223, C/EBPA AND E2F1 IN GRANULOPOIESIS AND IN ACUTE MYELOID LEUKEMIA

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MicroRNAs are novel regulators of gene expression and have shown to possess significant roles in various biological processes. Recent findings suggest deregulation of microRNAs as a hall mark of many cancers including leukemia. During granulopoiesis, microRNA-223 (miR-223) is upregulated by the transcription factor CCAAT enhancer binding protein α (C/EBPα). Mice deficient for miR-223 display defects in granulopoiesis pointing out the importance of miR-223 during granulopoiesis. Emerging studies suggest that deregulation of C/EBPα is a critical step in the development of acute myeloid leukemia (AML). Inhibition of E2F1, the master regulator of cell cycle progression by C/EBPα is pivotal for granulopoiesis. How ever, the mechanism with which C/EBPα inhibit E2F1 in granulopoiesis is poorly understood. Computational analysis suggests that E2F1 could be a putative target of miR-223. By luciferase assay using 3'UTR of E2F1, we show that E2F1 is a potential target of miR-223. Bone marrow cells isolated from miR-223 null mice shows accumulation of E2F1 protein as observed by Western blot analysis. Meanwhile, overexpression of miR-223 leads to downregulation of E2F1 protein levels. Proliferation assays as well as cell cycle analysis demonstrate that miR-223 blocks cell cycle progression in myeloid cells. We observed that miR-223 is downregulated in different subtypes of AML as analysed by quantitative Real-Time RT-PCR. It has been reported that E2F1 is able to block granulocytic differentiation. We demonstrate that E2F1 inhibits the microRNA-223 promoter activity through its transactivation domain as shown by promoter assays. Chromatin immunoprecipitation assays show that E2F1 binds to miR-223 promoter and this binding is reversed during granulocytic differentiation. Furthermore, overexpression of E2F1 down regulates the expression of miR-223, suggesting E2F1 as a transcriptional repressor of the miR-223 gene. Recent studies demonstrate that disruption of E2F1 inhibition by C/EBPα leads to leukemia, pointing out the significance of E2F1 inhibition in the development of AML. Our data support a circuitry comprising miR-223, C/EBPα and E2F1 as major components of the granulocyte differentiation programme, which is deregulated in AML. Manipulation of miR-223 could be therapeutically relevant in AML subtypes in which E2F1 inhibition is deregulated.

0282

IMMUNOPHENOTYPIC CHARACTERIZATION OF $CD34^+/CD38^-$ AML PROGENITOR CELLS AND THEIR RESPONSE TO VARIOUS DRUGS

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Background. In acute myeloid leukemia (AML), the malignant clone is organized hierarchically with i) more mature cells programmed to undergo apoptosis after a variable number of cell divisions and ii) immature cells that self-renew and repopulate NOD/SCID mice with leukemias. In most AML subtypes, leukemic stem cells supposedly reside within the $CD34^+/CD38^-$ fraction of the leukemic clone. Although AML stem cells are a logic target of therapy little is known so far about the regulation of growth and survival of these cells. *Aims.*We examined the expression of cytokine receptors (SCFR/KIT, IL-3Ra, GM-CSFRa, IL-3/GM-CSFRß, G-CSFR, M-CSFR, TGFßR/endoglin, EPOR, TPOR/MPL, FLT3, VEGFR/KDR) and of other molecular targets and markers (CD33, CD44, CD133) on $CD34^+/CD38^-$ cells in patients with AML (n=30), and determined responses to cytokines, conventional antileukemic drugs (ARA-C, fludarabine), and targeted drugs including Gemtuzumab/Ozogamicin (GO=Mylotarg®) in these cells. *Methods.* Apoptosis was analyzed by combined staining for surface markers and AnnexinV. In a group of patients, $CD34^+/CD38^-$ cells were purified to homogeneity by cell sorting and examined for 3H-thymidine uptake. *Results.* AML stem cells were found to display a variable pattern of cytokine receptors and other surface targets. In fact, only the IL-3R and CD44 were expressed consistently on AML stem cells in all donors. In most patients, at least a subset of AML stem cells also co-expressed the SCFR/KIT, G-CSFR, TGFßR, FLT3, CD33, and CD133. By contrast, AML stem cells in most donors were found to lack substantial amounts of the GM-CSFR, M-CSFR, EPOR, TPOR, and VEGFR/KDR. When cultured in RPMI-1640 medium and 10% FCS, about

5-15% of the CD34+/CD38- cells and about 10-40% of the more mature CD34+ AML cells were found to undergo spontaneous apoptosis within 48 hours. Spontaneous apoptosis was prevented by exposure to SCF, IL-3, or G-CSF, but not by EPO. In most donors, ARA-C (5 μM), fludarabine (5 μM), and clofarabine (5 μM) and GO/Mylotarg (1 μg/mL) were found to promote apoptosis in CD34+/CD38- cells and more mature AML cells. The effects of ARA-C, fludarabine, and clofarabine were dose-dependent. Moreover, we were able to show that ARA-C (2 μM) and fludarabine (2 μM) and also ARA-C (2 μM) and clofarabine (2 μM) cooperate with each other in producing apoptosis in CD34+/CD38- and CD34+/CD38+ AML cells. 3H-thymidine uptake experiments performed on purified CD34+/CD38- AML stem cells confirmed growth-inhibitory drug effects. In particular, ARA-C, fludarabine, and clofarabine were found to inhibit cytokine-induced 3H-thymidine incorporation into sorted AML stem cells in all samples analyzed. *Conclusions.* Together, our data show that multi-color flow cytometry and combined staining for surface markers and AnnexinV is a powerful approach to determine apoptosis-reverting effects of cytokines and apoptosis-inducing effects of anti-leukemic drugs in immature CD34+/CD38- AML cells. Using this assay, it should be possible to identify combinations of targeted and/or conventional drugs eliminating maximal numbers of leukemic stem cells in AML.

0283

RETROVIRAL INTEGRATION MUTAGENESIS IN MICE AS A PLATFORM FOR IDENTIFICATION OF CRITICAL TUMOR SUPPRESSOR GENES IN HUMAN MYELOID DISORDERS

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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 genetic defects underlying these diseases are still unknown in many patients. New genome-wide methods (e.g. gene expression profiling, SNP/CGH analysis and methylation profiling) are currently being used to identify new genes that are deregulated in these diseases. However, because for instance multiple genes are located at regions affected by LOH and many genes have aberrant expression levels in AMLs, it is difficult to identify the key players that contribute to the pathogenesis of these disorders using these approaches. Other approaches, like retroviral integration mutagenesis in mice have been proven powerful to identify new genes relevant for human myeloid malignancies. *Aims.* We aimed to develop a new approach to retroviral integration mutagenesis, by using methylated DNA immunoprecipitation (MeDIP), inverse PCR and promoter array hybridisation, to specifically identify new candidate TSG in mice. These candidate TSG should serve as an important basis for detection of new TSG in human myeloid malignancies, by enabling a candidate gene approach for analysing large datasets. *Methods.* Tumor samples of a previous retroviral integration mutagenesis screen were used. Genomic DNA of 6 tumors, induced by Graffi 1.4 murine leukemia virus, was isolated and digested. Methylated fragments were immunoprecipitated using MeDIP, followed by inverse PCR to amplify fragments flanking methylated viral integrations (mVIS). Amplified fragments were labeled, fragmented and hybridized on murine promoter DNA arrays. We developed new algorithms to detect mVIS after promoter array hybridization, using strict criteria based on probe intensities and restriction sites employed in the inverse PCR procedure. CEAS (Cis-regulatory element annotation system) was used to identify the genes flanking the mVIS. *Results.* We identified a total of 891 mVIS in 6 different tumors. Within 10.000 base pairs downstream of these mVIS, 708 genes were found. Twenty-four genes were present in more than one tumor and are referred to as common mVIS (mCIS) genes. Using HomoloGene (NCBI) based approaches the human orthologues of 619 mVIS and 20 mCIS genes were identified. Interestingly, when looking into the AML expression data of 532 de novo AML patients, a highly significant downregulation of these 619 mVIS ($p<0.001$) and the 20 mCIS orthologues ($p<0.05$) was seen in comparison with genes not detected in our screen. The mCIS genes include SHIP1, a well known phosphatase with tumor suppressor activity in human AML. Other mCIS genes are involved in signalling, transcription, chromatin remodelling, apoptosis and DNA repair. Five genes show extensive promoter hypermethylation in more than 5% of AML patients and/or decreased expression levels in a subset of AML patient samples. *Conclusion.* By using a new approach to retroviral integration mutagenesis in mice, we are able to detect genes that interestingly show a highly significant downregulation of expression in a large AML cohort and/or hypermethylation in a subset of AML patient samples. These findings clearly show the high potential of this new method for detection of new TSG in human myeloid malignancies.

Acute myeloid leukemia - Biology II

0284

THE FAVORABLE IMPACT OF CEBPA MUTATIONS IN PATIENTS WITH ACUTE MYELOID LEUKEMIA IS ONLY OBSERVED IN THE ABSENCE OF ASSOCIATED CYTOGENETIC ABNORMALITIES AND FLT3 INTERNAL DUPLICATION (FLT3-ITD) (FOR THE ACUTE LEUKEMIA FRENCH ASSOCIATION ALFA GROUP)

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Background. CEBPA mutations are associated with a favorable outcome in younger patients with newly-diagnosed acute myeloid leukemia (AML). This was confirmed by other groups but mainly in patients with cytogenetically normal (CN) AML. The impact of cytogenetic abnormalities and bad-prognosis FLT3 internal tandem duplication (FLT3-ITD) on CEBPA mutated favorable outcome is not clear. Moreover, it has been recently reported that Double CEBPA mutations, but not single CEBPA mutations was associated with a favorable outcome. *Methods.* Here, we analyzed the impact of associated cytogenetic abnormalities, FLT3-ITD and CEBPa single or double mutation status in 53 patients with CEBPA+ *de novo* AML treated in the ALFA trials (26 CN-AML without FLT3-ITD, 10 CN-AML with FLT3-ITD, 17 abnormal karyotypes AML). CEBPa mutations screening was performed by direct sequencing. Gene expression profiling was performed on a subset of 15 of these CEBPA+ AML (8 AML with CEBPa double mutations and 7 with single mutation) using a CEBPA+ AML prediction model obtained from a previous AML cohort. *Results*. We found that only AML with a normal karyotype and no FLT3-ITD displayed the expected favorable outcome. In this context, relapse-free, disease-free, and overall survival were significantly longer than in corresponding patients without CEBPA mutation (p=0.035, 0.016, and 0.047 respectively). This was not observed in the context of an abnormal karyotype or associated FLT3-ITD. In univariate analysis, single CEBPa mutation compared to double mutation did not significantly influenced CR rate, RFS, or DFS, but a trend towards a shorter OS was observed compared to patients with double mutation (36 vs. 60% at 5 years, p=0.09). Furthermore, prediction model based on gene expression profiling detected CEBPA+ AML with a great specificity (no false positive) but a poor sensibility (8/15 false negative). Strikingly, 7/8 false negative were CEBPA+ AML with single mutations, suggesting a significative difference in gene expression profile between single or double mutations. This heterogeneity could explain the trend towards a shorter OS in single mutation CEBPA+ AML. *Conclusion.* These results suggest that CEBPA mutations are predictive of a favorable outcome mostly in patients with a normal karyotype and no FLT3-ITD. The difference between double or single CEBPA mutations has to be confirmed on a larger cohort.

0285

DIFFERENTIAL EXPRESSION OF NATURAL CYTOTOXICITY RECEPTORS (NCRS) IN CD56-POSITIVE ACUTE MYELOID LEUKEMIAS

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Background. A subset of acute myeloid leukemias is characterized by the coexpression of CD56, a canonical natural killer (NK) cell marker. This raises the issue whether this is a random, leukemia associated phenomenon or whether it constitutes evidence for a NK cell origin of CD56 positive AML. *Aims.* Detailed immunophenotypic characterization of CD56 positive AMLs in order to detect a subgroup with enhanced expression of multiple NK- cell specific markers compatible with a NK-cell lineage derivation. *Methods.* We used multi-color flow-cytometry to analyse the expression of NK-cell specific markers on CD56 positive acute myeloid leukemic blasts and compared it with its respective expression on mature NK cells within the same sample. Bone marrow aspirates of 130 patients with newly diagnosed CD56 positive AML were characterized by their expression of the NCRs NKp30, NKp44 and

NKp46. These samples were also analyzed in detail for their expression of myeloid (CD13, CD33, CD65, CD15, MPO, LF), monocytic (CD14, CD64, CD4), B-lymphoid (CD19, CD20, CD22, CD79a), T-lymphoid (CD3, CD2, CD5, CD7, CD4, CD8) and progenitor (CD34, CD133, TdT, CD117) markers. Additionally, bone marrow samples of 52 patients were analyzed with nine color flow cytometry for the expression of the selected NK-cell markers CD94 and CD122. *Results.* Blast cells from all patients were negative for CD3 and positive for CD13 and/or CD33. The percentage of CD56-positive blasts varied between 21% and 98% with a mean of 59% for the whole group. Of the 50 female and 80 male patients with CD56-positive AML, 11.5% (n=15) were positive for at least one of the NCRs. The NCR-positive patients revealed a significantly lower expression (quantified as mean percentage of positive blast cells) than the NCR negative patients for the lymphoid/monocytic marker CD4 (23% vs. 42%), for the myelomonocytic markers CD14 (7% vs. 20%) and CD65 (23% vs. 43%), for the B-lineage marker CD79a (9% vs. 26%) and for the progenitor marker CD133 (3% vs. 33%). The investigation of additional NK-cell markers performed so far, revealed in 4 patients a strong expression of CD122 and in one patient a strong expression of CD94 on the blast cell population. *Conclusions.* These results indicate a subgroup of CD56-positive AML defined by the positivity of NCRs. The negativity for CD3 and the lower expression of other antigens related to the lymphoid and myelomonocytic lineage together with the strong positivity for NK cell specific markers are compatible with a NK-cell origin of this leukemia subgroup. Whether this represents a clinically relevant subset remains to be determined.

0286

COOPERATING MUTATIONS IN *DE NOVO* AML WITH MLL-PARTIAL TANDEM DUPLICATION AT DIAGNOSIS AND RELAPSE

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Background. Two-hit model of leukemogenesis has been proposed for AML. Cooperating mutations in *de novo* AML with MLL-partial tandem duplication (MLL-PTD) at diagnosis and relapse have not been systematically investigated. *Aims*. We analyzed cooperating mutations on diagnosis and relapse samples in *de novo* AML with MLL-PTD to determine the roles of class I mutations including receptor tyrosine kinases/JAK2/Ras signaling pathways and class II mutations including RUNX1 and CEBPa, as well as NPM1, P53 and WT1 mutations in the development and relapse of AML with MLL-PTD. *Patients and methods.* Eighty-eight patients were diagnosed with MLL-PTD which was screened by Southern blot analysis or RT-PCR then confirmed by real-time quantitative PCR. Mutational analyses were performed by DNA/cDNA PCR with GeneScan analysis for FLT3/ITD, PCR-RFLP followed by direct sequencing for FLT3/TKD, DNA/cDNA PCR and direct sequencing for c-KIT, c-FMS, N-Ras, K-Ras, PTPN11, RUNX1, CEBPa, NPM1, P53 and WT1 mutations, and allele-specific PCR for JAK2V617F. Results. In AML patients with MLL-PTD at diagnosis, 56 patients (64%) had class I mutations including 38 FLT3-ITD, 12 FLT3-TKD, 4 c-FMS (1 silent), 1 JAK2V617F, 2 N-Ras, and 7 PTPN11 (1 silent) mutations; 24 patients (27%) had class II mutations consisting of 23 RUNX1 and 3 CEBPa. In addition, P53 and WT1 mutations were detected in 1 and 4 patients, respectively. None had c-KIT, K-Ras or NPM1 mutations at diagnosis. Taken together, 63 patients (72%) with MLL-PTD AML had cooperating mutations. Eighteen patients had relapse samples available for comparative analysis. None of the 18 paired samples had c-KIT, c-FMS, JAK2V617F, N-Ras, NPM1, P53 or WT1 mutations. For class I mutations, 7 patients had FLT3-ITD mutations at both diagnosis and relapse, 1 lost and none gained the mutations at relapse; 2 retained FLT3-TKD, 2 lost and none acquired FLT3-TKD mutations at relapse; 1 gained K-Ras mutation at relapse; 1 retained the PTPN11 mutation and another 2 lost PTPN11 mutations at relapse. For class II mutations, of the 6 patients who harbored RUNX1 mutations at diagnosis, all relapsed with the identical mutants; another one patient acquired RUNX1 mutation at relapse. Additional 2 patients acquired CEBPa mutations at relapse. All the mutations detected at diagnosis were not present in the complete remission samples, indicating these mutations were leukemia-specific. There were no differences in overall survival or event-free survival between MLL-PTD AML patients with cooperating mutations and those without mutations of FLT3-ITD, FLT3-TKD, PTPN11 or RUNX1 genes. Conclusions. Cooperating mutations were detected in more than 70% of MLL-PTD AML at diagnosis. Most of those carrying FLT3-ITD or RUNX1 mutations retained the identical mutations at relapse; loss or gain of class I or class II mutations might occur in relapse.

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0287

NUP98 FUSION PROTEINS. NEW INSIGHTS INTO SUB-CELLULAR LOCALIZATION AND INTERACTION WITH NUCLEO-CYTOPLASMIC TRAFFIC ELEMENTS

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Background. Translocations involving 11p15/NUP98 were reported in myeloid leukaemia, myelodysplasias, chronic myeloid leukemia in blast crisis and acute T-cell lymphoblastic leukemia. A *promiscuous* gene with 24 fusion partners cloned to date, NUP98 belongs to the nucleoporin family, a class of proteins constituting the nuclear pore complex (NPC) which regulate nuclear-cytoplasmic traffic. Since abnormal nucleo-cytoplasmic traffic results in oncoprotein mislocation which may contribute to neoplastic transformation, we explored NUP98 fusion protein cellular localization in patients with hematological malignancies. *Aim.* To elucidate mechanisms underlying NUP98 fusions by investigating sublocalization of NUP98 and its fusions to HEX, LOC348801, HOXA9 and PMX1 and interactions with proteins involved in Nuclear Cytoplasmic Trafficking. *Methods.* Plasmids: pEGFP-NUP98, pEGFP-HHEX, pEGFP-NUP98/HHEX, pEGFP-LOC348801, pEGFP-NUP98/HHEX (ΔGLFG/GLEBS), pEGFP-NUP98/HHEX(ΔHD), pEGFP-NUP98/LOC348801(iso1), pEGFP-NUP98/LOC348801(iso2), pDsRed-NPM1wt, pEGFP-HOXA9, pEGFP-PMX1. *Cell transfection*. Hela cell lines were cultured in MEM medium supplemented with 10% FBS and 1% glutamine, seeded on glass coverslips, transfected using Lipofectamine 2000 (Invitrogen) and treated with Leptomycin (LMB) (5ng/mL for 6 hours). Immunofluorescence: Glass coverslips were fixed in 4% paraformaldehyde pH=7.4 and flipped on to standard glass slides with Mowiol mounting medium. Confocal microscopy (Zeiss LSM 510) analyzed protein sub-cellular localization, after deconvolution and 3D confocal image reconstruction. *Results and Comments*. EGFP-NUP98 was found in the nuclear rim and as small dots in nucleoplasm and nucleoli. Co-transfection experiments with DsRed-NPM1 showed NUP98 localized in the nucleolar fibrillar compartment. NUP98 and hCRM1 colocalized strongly in nucleoplasmic and nucleolar dots. LMB treatment, which impairs hCRM1 binding with nuclear export sequences, resulted in a diffused nucleoplasmic staining pattern and disappearance of dots, confirming the NUP98-karyopherin interaction played a functional role in nucleo-cytoplasmic traffic. EGFP-HHEX diffusely stained nucleoplasm excluding nucleoli. EGFP-LOC348801 diffusely stained nucleoplasm and cytoplasm. NUP98 fusions: EGFP-NUP98/HHEX , containing GLEBS domain and 9 GLFG repeats of NUP98 fused to the HEX homeodomain, gave a microspeckled pattern in nucleoplasm, sparing nucleoli. EGFP-NUP98/HHEX(ΔGLFG/GLEBS), lacking 4 GLFG repeats and GLEBS domain, provided a speckle-free nucleus diffuse distribution pattern like EGFP-HHEX. EGFP-NUP98/HHEX(ΔHD), lacking the homeodomain, gave a dot pattern in nucleoplasm and nucleoli like EGFP-NUP98. LMB treatment of EGFP-NUP98/HHEX(ΔHD), like EGFP-NUP98, dissolved nucleolar dots, suggesting the NUP98 N-terminal maintains its interaction with hCRM1 when fused to HHEX. NUP98/HOXA9 and NUP98/PMX1, yielded speckles like NUP98/HHEX. EGFP-NUP98/LOC348801(iso1) and EGFP-NUP98/LOC348801(iso2) showed speckles in nucleus, sparing nucleoli. Both isoforms were found in nucleoli when co-transfected with pDsRed-NPM1wt, a CRM1-binding protein. NPM1 co-transfection did not localize NUP98-HEX, NUP98-HOXA9, NUP98-PMX1 in nucleolus. *Conclusions.* NUP98 and partner determine the fusion protein subcellular localization. NUP98 interacts with the hCRM1 system through its N-terminal portion which is maintained in all fusions. In NUP98-Homeobox fusions, the homeodomain prevents the NUP98 localization in the nucleolus.

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0288

FLOW CYTOMETRIC PROTEIN EXPRESSION PROFILING AS A POTENTIAL APPROACH FOR DEVELOPING AN ALTERNATIVE CLASSIFICATION OF ACUTE MYELOID LEUKAEMIA

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Background. In patients with acute myeloid leukaemia (AML), a multidisciplinary approach needs to be employed in order to make a complete assessment of the disease, which includes the diagnosis, classification, prognosis and stratification of the treatment. However, these complimentary efforts provide substantial prognostic and therapeutic information in about half of the patients and the current classification system does not fully reflect the molecular heterogeneity of the disease. Thus, treatment stratification is difficult, especially, for patients with a normal karyotype. *Aim.* To determine the association between antigen expression and cytogenetics in patients with AML. *Methods.* Antigen fluorescence intensity of 12 cellular antigens from samples of peripheral blood or bone marrow in 246 patients was determined. The determination of positive antigen expression of the blast cell populations was carried out qualitatively by visual approach and the measurement of the fluorescence intensity of 12 cellular antigens by geometric mean then was obtained. The geometric mean of the fluorescence intensity of the 12 cellular antigens exhibited by the positive blast cell populations and 10 selected of cytogenetic abnormalities of the patients were computational analysed using *DNA-Chip Analyzer* (dChip) software to determine the significant hierarchical clusters of the fluorescence intensity in relation to cytogenetic findings. *Results.* Unsupervised hierarchical clustering analysis identified 6 significant (p<0.001) clusters or groups of patients with AML on the basis of antigen fluorescence intensity using geometric mean associated with 6 chromosomal abnormalities; t(8;21), t(15;17), inv(16), normal karyotype, complex karyotype and +8. *Conclusions.* Hierarchical clustering of geometric mean of antigen fluorescence intensity might have the potential to allow a robust, comprehensive, clinically significance and cost-effective classification of AML that includes previously identified genetically defined subgroups and a novel cluster with an intermediate prognosis of normal karyotype.

0289

GENOME PROFILING OF NORMAL KARYOTYPE AML WITH HIGH DENSITY ARRAY COMPARATIVE GENOME HYBRIDISATION

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Introduction. AML is the most common myeloid leukemia affecting 3.8 people per 100000. Slightly more than half of the AML patients (55%), harbour at least one chromosomal abnormality, which is detectable by cytogenetics. There is a clear correlation between cytogenetic findings and survival and, or response to therapy. However, for the 45% of the AML patients with no cytogenetic findings (Normal Karyotype, NK), there is a need for discovering the molecular triggers that lead to leukemiogenesis. *Aims.*To screen the genome of AML NK patients with aCGH to identify common aberrations that might have a role in disease onset and/or progression. *Methods.* We analysed the genomes of 26 NK AML and the genomes of 10 healthy individuals with a customised Agilent 244k high density array. The custom aCGH chip that we constructed has been enriched with probes for 236 genomic loci that were previously shown to be implicated in leukemias. These include all the known tyrosine kinases and other signalling genes and transcription factors. *Results.* We have been able to identify a set of 147 probes that differentiate normal individuals from NK AML and in addition this probe set has shown to have a high predictive value in distinguishing between the two groups (Figure 1). We have failed to identify such a probe set that can distinguish between the different NK AML subgroups according to their mutation status, but we were able to detect a total of 47 loci with an aberrant copy number in more than 2 AML patients and that has not been detected in any of the normal neutrophil samples. These loci include genes that after Gene Ontology EASE analysis reveal their involvement in pathways previously shown to be involved in AML, 10 protein kinases and 28 genes involved in cell differentiation. Among these are deletions of hsa-mir-1233 in 5 patients, deletion of EPHA3 in 4 patients and deletions of PIAS4 in 3 patients. *Conclusions.* Normal karyotype AML is a heterogeneous group according to genomic copy number changes. However, we are able to identify *hits* in pathways that are involved in cell differentiation and cell signalling.

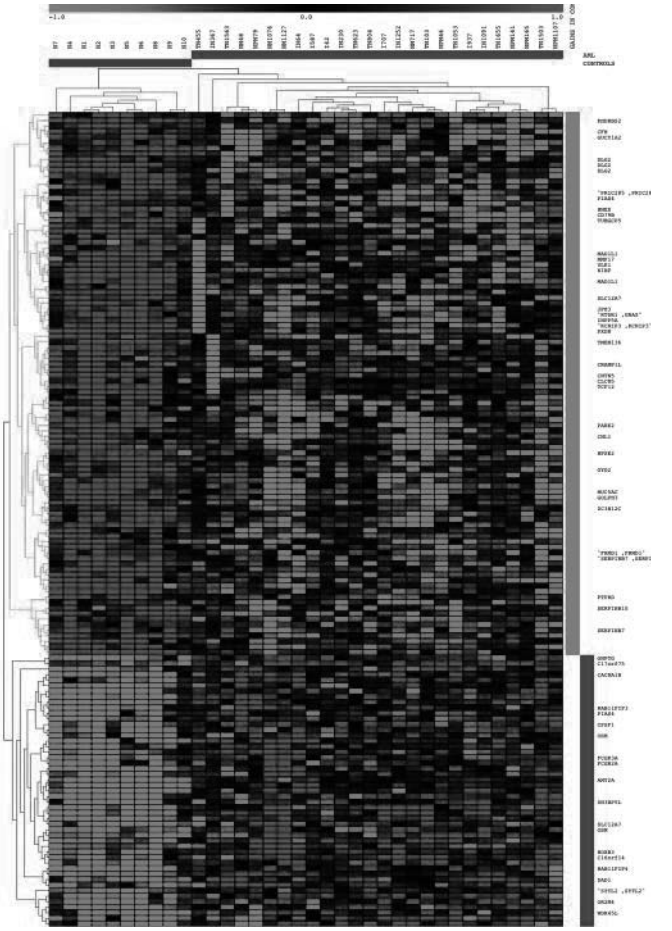

Figure 1. Heatmap of 147 significant genes as iden.

0290

BOTH SEPT2 AND MLL ARE DOWN-REGULATED IN MLL-SEPT2 THERAPY-RELATED MYELOID NEOPLASIA

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Background. A relevant role of septins in leukemogenesis has been uncovered by their involvement as fusion partners in MLL-related leukemia. Recently, we have established the MLL-SEPT2 gene fusion as the molecular abnormality subjacent to the translocation t(2;11)(q37;q23) in therapy-related acute myeloid leukemia. *Aims.*In this work we quantified MLL and SEPT2 gene expression in 58 acute myeloid leukemia patients selected to represent the major AML genetic subgroups, as well as in all three cases of MLL-SEPT2-associated myeloid neoplasms so far described in the literature. *Methods.* Cytogenetics, fluorescence in situ hybridization (FISH) and molecular studies (RT-PCR, qRT-PCR and qMSP) were used to characterize 58 acute myeloid leukemia patients (AML) at diagnosis selected to represent the major AML genetic subgroups: CBFB-MYH11 (n=13), PML-RARA (n=12); RUNX1-RUNX1T1 (n=12), normal karyotype (n=11), and MLL gene fusions other than MLL-SEPT2 (n=10). We also studied all three MLL-SEPT2 myeloid neoplasia cases reported in the literature, namely two AML patients and a t-MDS patient. *Results.* When compared with normal controls, we found a 12.8-fold reduction of wild-type SEPT2 and MLL-SEPT2 combined expression in cases with the MLL-SEPT2 gene fusion (p=0.007), which is accompanied by a 12.4-fold down-regulation of wild-type MLL and MLL-SEPT2 combined expression (p=0.028). The down-regulation of SEPT2 in MLL-SEPT2 myeloid neoplasias was statistically significant when compared with all other leukemia genetic subgroups (including those with other MLL gene fusions). In addition, MLL expression was also down-regulat-

ed in the group of MLL fusions other than MLL-SEPT2, when compared with the normal control group (p=0.023). *Conclusions.* We found a significant down-regulation of both SEPT2 and MLL in MLL-SEPT2 myeloid neoplasias. In addition, we also found that MLL is under-expressed in AML patients with MLL fusions other than MLL-SEPT2.

0291

METHYLATION ANALYSIS OF THE SNRPN AND MEG3 IMPRINTED GENES IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. MEG3 and SNRPN represent imprinted genes, maternally and paternally expressed respectively. MEG3 is involved in the pathogenesis of chromosome 14 uniparental disomy and SNRPN in Prader-Will syndrome. *Aim.* As both genes have been demonstrated to possess tumor suppressor activity, we investigated whether promoter hypermethylation of the differentially methylated region (DMR) of the specific genes occurs in acute myeloid leukemia (AML) patients. *Methods.* We studied 42 patients, 26 males and 16 females, with newly diagnosed AML. Median age was 62.85 years (range 35-91). Cytogenetic analysis was not performed in 12 cases and 30 patients were classified according to the SWOG cytogenetic prognostic system in good, intermediate, and poor prognosis karyotypes. DNA methylation pattern was determined by methylation-specific PCR of bone marrow samples previously subjected to bisulphite modification, according to preestablished protocols. Twelve subjects, who had undergone bone marrow aspiration for investigation of thrombocytopenia, and were proven to have no hematological or solid tumour malignancy, uniparental disomy 14 or Prader-Willi syndrome, served as controls. *Results.* The normal pattern of the MEG3 gene consists of 2 alleles, namely one corresponding to the methylated paternal allele, and one corresponding to the unmethylated maternal allele, while the normal pattern of the SNRPN gene consists of a methylated maternal chromosome and an unmethylated paternal chromosome. No abnormal methylation pattern was observed in any of the control group subjects. We found that alterations of the DMR (presence of the methylated allele only) of the MEG3 and SNRPN genes were present in 20 (47.62%) and 21 (50%) patients respectively, while 14 (33.33%) patients presented abnormal methylation of both genes. Aberrant methylation of the MEG3 gene was associated with a poorer overall survival (HR=1.98; p=0.047), while methylation of the SNRPN gene was not (HR=0.94; p=0.87). When both genes were hypermethylated there was a trend for reduced overall survival (HR=1.76; p=0.18). No significant association between WHO subtype or SWOG risk system with either gene was observed. *Summary and Conclusions.* MEG3 and SNRPN aberrant methylation has been observed in various types of solid tumors. Our findings suggest that these genes are abnormally methylated in AML patients, and methylation of MEG3 confers worse overall prognosis. The role of these imprinted genes in the pathogenesis of AML merits further investigation.

0292

GENE EXPRESSION PROFILE MIGHT PREDICT PROGNOSIS IN PATIENTS WITH INTERMEDIATE-RISK ACUTE MYELOID LEUKEMIA LACKING NPM1 AND FLT3 MUTATIONS

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Background. In the heterogeneous category of acute myeloid leukemia (AML) patients with intermediate-risk cytogenetics (IR-AML), mutations of NPM1 gene (NPM1mut) and FLT3 internal tandem duplication (FLT3-ITD) segregate subgroups with different prognosis. Nonetheless, approximately 40% of IR-AML cases lack both mutations and their biological and prognostic background is mostly unknown. *Aim.* To elucidate if gene expression profiling (GEP) might contribute to undercover biological subtypes and prognostic markers within the subgroup of patients with IR-AML. *Methods.* Forty patients (age: 52, 17-68; gender: 27 M/13F) diagnosed with IR-AML (normal karyotype 73%) during interval 1996-2008 who achieved complete remission (CR) after intensive chemotherapy were included in the study, excluding those patients who underwent allogeneic hematopoietic stem-cell transplantation in first CR. Global gene expression was examined with oligonucleotide HGU133 Plus 2.0 arrays (Affymetrix), and gene expression measures were normalized using RMA methodology from the Affy package (Bioconductor project). Unsupervised and supervised comparisons were performed using TM4 Microarrays Software Suite (Saeed AI *et al.*, 2003) and Limma software (Bioconductor). Supervised analysis was based on patients outcome, comparing the gene signature of long-term responders (i.e. CR>2 years, n=25; FAV) with that of patients presenting with early relapse (n=15; UNFAV). Those genes with a significance value <0.005 and log fold change >0.5 were selected. NPM1mut and FLT3-ITD were screened as previously described. *Results.* Fourteen patients harbored a favorable genotype (i.e., NPM1mut/FLT3-ITDneg, 35%), 7 patients had FLT3-ITD (4 of them with concomitant NPM1mut), and the remaining 19 patients (48%) lacked both mutations. The molecular category (NPM1wt/FLT3-ITDneg vs. other) predicted outcome, with a 5-year LFS of 92% vs. 49.5% (p=0.01). On unsupervised GEP analysis, patients grouped in two major arms, which differed according to the presence of NPM1 mutations (77% vs. 31%, p=0.02) but not in terms of prognosis. Under supervised condition, 53 probe sets corresponding to 47 genes were differentially expressed in prognostic subgroups of patients; among these, homeobox genes HOXA4 and HOXB2, and LMNA were found to be overexpressed in the FAV subgroup, and genes such as ENG, PLSCR1, CSF3R, and RETN were overexpressed in the UNFAV subset. Moreover, when the analysis was restricted to patients lacking both NPM1mut and FLT3-ITD, 258 genes were differentially expressed according to outcome. Thus, several genes involved in apoptosis (CFLAR, FAS, DAPP1, BRCA1) and cell cycle control (CDC7, CDC2, CDC5) were highly expressed in the UNFAV subset. Of note, 19 genes correlated with patient outcome in both the overall series and the NPM1wt/FLT3-ITDneg subgroup. In contrast to the characteristic signature of NPM1mut patients, expression of HOX genes was low in the NPM1wt/FLT3-ITDneg population and did not show significant variation depending on prognosis. *Conclusions.* NPM1 and FLT3-ITD mutations are major determinants of prognosis in AML with intermediate cytogenetics. Nonetheless, the analysis of underlying gene signature might add relevant information for predicting the outcome of patients lacking both molecular markers.

0293

SURVIVIN GENE EXPRESSION SIGNIFICANTLY CORRELATES WITH CLINICAL OUTCOME IN PATIENTS WITH ACUTE MYELOID LEUKEMIA IN ASSOCIATION WITH MDR1/P-GLYCOPROTEIN OVEREXPRESSION AND FLT3-ITD

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Background. Adult acute myeloid leukemia (AML) remains a very heterogeneous group with highly variable individual prognosis. It is generally accepted that some molecular markers could help to refine stratification. In AML, FLT3 is the most frequently mutated gene, with internal tandem duplications of the juxtamembrane (Flt3/ITD) accounting for up to 30%, and demonstrated to play a crucial role in driving proliferation and survival of the leukemic clone. Besides, one of the major reasons of induction chemotherapy failure is attributed to the expression of genes and proteins responsible for multidrug resistance and apoptosis. The aim of the study was to evaluate the influence of biomarkers with reported individual prognostic value and a potential to lead to targeted therapy on results of chemotherapy in patients with AML. We focused on the IAP-family gene survivin expression, MDR1/P-glycoprotein (P-gp) ATP-binding cassette transporter, as well as on Flt3/ITD. *Materials and Methods.* The study was performed in 56 patients (mean 53 years, ranged 19-79) with newly diagnosed AML according to the WHO criteria, as follows: AML with recurrent cytogenetic abnormalities - 10; AML with myelodysplasia related changes - 6; therapy-related AML - 2; AML not otherwise specified - 39. The expression of survivin gene and Flt3-ITD in leukemic cells was detected by RT-PCR. MDR1-Pgp expression was evaluated by flow cytometry. *Results.* Our studies showed lack of survivin gene expression in 24 patients (42.8%), intermediate - in 16 pts (28.6%), and high - in 16 pts (28.6%). No correlation was found with the main demographic and laboratory parameters. However, survivin overexpression was observed in only 1 out of 9 patients (11.1%) with cytogenetic/molecular markers associated with favorable prognosis as defined by WHO (AML1-ETO, CBFb-MYH11, PML-RARA), compared

to 31/47 (66%) in the remaining cases [p=0.009]. Complete remission was achieved in as few as 6/31 (19.4%) patients with high levels of survivin mRNA compared to 12/21 (57%) negative cases [p=0.008]. In addition, overexpression was significantly associated with shorter overall survival (OS) - mean 69 weeks, compared to negative cases - mean 224 weeks [log rank test, p=0.009]. The analysis failed to reveal any correlation between survivin gene expression and the presence of Flt3-ITD or high levels of Pgp. However, there was a significant difference in the OS of patients with lack of both survivin and P-gp - 2-years relative survival of 100% with a mean not reached after a follow up of 77 weeks [range 25-198]; expression of one of the biomarkers - 2-years survival of 56% with a mean of 103 weeks; or both - 1-year survival of 23%, mean of 44 weeks [log rank test, p=0.01]. Similarly, patients that had neither survivin nor Flt3-ITD showed best OS - 2-years survival of 73%, mean of 224 weeks. OS was the shortest in the double positive group with a mean of 26 weeks only [log rank test, p=0.002]. *Conclusions.* Our data showed that the apoptosis-related gene survivin is significantly associated with poor therapy outcome in AML. However, the simultaneous involvement of multiple molecular mechanisms is common in AML and has a progressively worse adverse effect on prognosis. After confirmation on larger group of patients our findings may have important significance in defining of prognosis in AML and individualization of therapy, eventually with molecularly targeted modulators.

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0294

GENE TARGETS OF ETV6-NTRK3 FUSION

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Background. A recurrent chromosome abnormality, t(12;15)(p13;q25) which fuses ETV6 with NTRK3 uniquely occurs in both solid and hematologic tumors and, though of singular interest, has yet to be characterized in any detail. *Aims.*We describe the characterization of t(12;15) in a cell line (AP-1060) recently established from a patient with acute promyelocytic leukemia. In particular, we set out to identify potential downstream targets of this unique rearrangement. *Methods.* FISH using tilepath clones; rapid amplification of c-DNA ends (RACE); microarray transcriptional profiling; reverse transcriptase quantitative (RQ)-PCR; sequencing technology. *Results.* FISH revealed t(12;15)(p13;q25) with ETV6 rearrangement. 3´-RACE revealed NTRK3 involvement which was supported by FISH using fosmid clones. RT-PCR confirmed ETV6-NTRK3 fusion transcripts. Sequencing revealed the presence of both ETV6 exon-4 / NTKR3 exon-14, and ETV6 exon-2 / exon-18 of NTKR3 transcripts - the former dominating. Comparative transcriptional profiling of AP-1060 and control leukemia myeloid leukemia cells showed upregulation of RAS-MAPK and PI3K-AKT related genes, highlighting the involvement of both signaling pathways via ETV6-NTRK3. Several additional genes were conspicuously expressed by AP-1060 cells to serve as candidate ETV6-NTRK3 targets. Since growth and proliferation of AP-1060 cells was maximally sensitive to protein tyrosine kinase inhibitor (PTKi) treatment when compared to other hematopoietic cell lines, pharmacologic modulation of conspicuously expressed genes by PTKi was used to identify potential targets. Three candidates target genes thus emerged: CTHRC1 and IL32 (upregulated), and the MDS-EVI1 fusion transcript (downregulated). *Conclusions.* We identified CTHRC1, IL32 and MDS-EVI1 as potential targets of leukemogenic ETV6-NTRK3 growth signaling. Pharmacologically unmodulated but conspicuously expressed genes were preferentially stem cell in character highlighting this setting for t(12;15) formation in AP-1060 cells.

0295

A HYPOTHETICAL-MATHEMATICAL MODEL OF ACUTE MYELOID LEUKEMIA PATHOGENESIS

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Background. Acute myeloid leukemia (AML) is defined by the expansion of a mutated stem cell clone, with the inhibition of surrounding normal clones. AML is in fact a very heterogeneous disease, suggesting various pathogenetic pathways for the different subtypes. *Aim.* To devise a simple theoretical model resembling hematopoiesis, to identify the possible general pathways through which the robustness of the hematopoiesis system can fail, leading to leukemia, and to give mathematical interpretation in term of dynamic systems. *Methods.* We used mathematical modeling with the help of a dynamic system inspired by Mackey--Grass and Dingli--Michor and basic results of stability of nonlinear systems. Numerical simulations with Maple 11 are made for clarifying the dynamic behavior of the system.

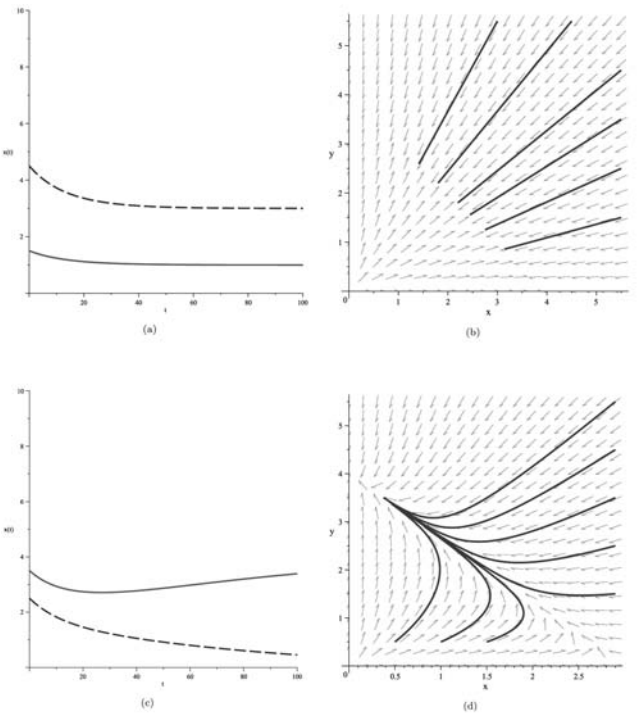

Figure 1.

Results. Theoretically, hematopoiesis can be seen as an evolutionary tree, starting with one cell that undergoes divisions during the expansion phase, afterwards losing cells during the contraction phase. During divisions, offspring cells acquire *variations*, which can be normal or abnormal. We postulate that if an abnormal variation exists in >25% of the cells, a leukemic pattern occurs. This may develop if: (A1) The abnormal variation occurs early, during the first divisions; (A2) The variation confers exceptional proliferative capacity; (B) A sizable proportion of the normal clones are destroyed and a previously non-significant abnormal clone gains relative dominance over a depleted environment; (C) The abnormal, variation confers relative immortality, rendering it significant during the contraction phase. Combinations of these pathways enhance the leukemic risk. A simple mathematical model expressed by a competitive differential system is used in order to descibe the dynamic of normal and leukemic cells populations, to characterize normal and leukemic states and to explain the above basic cellular processes generating leukemic patterns. The model parameters are growth, death and microenvironment sensibility rates of cells, and basic perturbations of the hematopoiesis system appear as alterations of one of the normal kinetic parameters. Estimations of the *aggressivity* and *immortality* of abnormal clones are also given. The Figure 1 presents in (a) the transitory situation from normal to leukemic hematopoiesis which is characterized by the coexistence of the two cells populations, x (normal cells - broken line) and y (leukemic cells - solid line) under a constant proportion depending on the initial concentrations. As a result, in (b), the orbits [x,y] of the dynamic system are semi-lines (rays) and there exist infinitely many stable equilibria of the biological motion. In (c), the time series plots of x and y and their behavior as time increases, is presented in case of leukemic hematopoiesis. Normal cell population x approaches zero, while leukemic population y tends to a saturation value D. Correspondingly, it is shown in (d), that in the xy-phase plane, the orbits are moving to the unique asymptotically stable equilibrium [0,D] (no normal cells, leukemic cells only). *Conclusions.* Our model shows that there are a finite number of pathways in which a hematopoiesis-like system can become leukemic. Such a model may have applicability in plotting therapeutic strategies in individual AML patients.

0296

PROGNOSTIC VALUE OF IMMUNOPHENOTYPE IN AML PATIENTS WITH NPM1 MUTATION

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Background. Nucleophosmin (NMP1) gene mutations are fundamentally detected on AML with normal karyotype, and it is considered a good prognostic factor when not associated to FLT3-ITD mutations. Some studies have already tried to find an association between NMP1 mutations and the presence of several antigenic surface markers. In this way, a low incidence of CD34 has been reported and recent studies have also found specific immunophenotype patterns (positive CD34/CD7/DR) that could identify subgroups of NMP1 patients with a worse outcome. *Aim.* To analyse the incidence and prognostic relevance of CD34/CD7//DR surface markers in a group of NMP1 positive patients and relate it to FLT3-ITD mutations. *Methods.* Forty two non promyelocytic AML patients (24 men and 18 women), all positive for the NMP1 mutation, were retrospectively analysed. According to FAB criteria, there were 1 M0, 6 M1, 7 M2, 6 M4, 10 M5, 9 RAEB and 3 non labelled cases. Ages ranged from 20 to 88 years, with an average of 60. Thirty of them were treated according to a conventional induction to remission chemotherapy protocol (7+3) and subsequent consolidation therapy either with chemotherapy or autologous/ allogenic SCT depending on the different prognostic groups. The remaining patients did not receive any treatment at all. The risk estimation of the different groups as established according to their immunophenotype and the presence of mutations in FLT3-ITD was calculated using Pearson's chi-squared. *Results.* Forty one percent of the patients belonged in the M4/M5 subtypes, and 33.3% in the M1/M2. 84% of the NMP1 positive patients had normal karyotypes. Mutations in FLT3-ITD were detected in 23.8% of the cases. Analyzable patients according to data from their immunophenotypes were, 11 CD34 positive (34.3%), 19 DR positive (59.4%) and 8 CD7 (25%) positive. When time to complete remission and/or relapse were analysed together with each specific surface marker, no significant findings were obtained. Patients FLT3-ITD positive had a higher chance of being CD7 positive and carried a higher risk of relapse when compared to the group as a whole ($p<0.0001$), but not to the FLT3-ITD positive CD7 negative group. *Conclusions.* 1. A low incidence of CD34 and CD7 was found among the NMP1 positive patients as described previously in the literature. The incidence of DR was higher when compared to other reports, and was most frequently associated to FAB M4/M5 subtypes. 2. In contrast to what has been published, no significant differences were found as to response to treatment, and risk of relapse depending on the presence of CD34, CD7 and DR. 3. Patients that had NMP1 and FLT3-ITD mutations had a significantly higher risk of relapse and a higher chance of being CD7 positive when compared to those that only portrayed NMP1 as a sole genetic finding. This higher risk of relapse cannot be linked to CD7, since this risk was similar on both groups: FLT3-ITD/CD7 positive and FLT3-ITD/CD7 negative. 4. In the group studied, the presence of CD7 by itself, which has been suggested as a poor prognostic factor, can not be considered as such.

0297

FLT3 ANTIGEN EXPRESSION IN ACUTE LEUKAEMIA USING FLOW CYTOMETRY

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Background. The FMS-like tyrosine kinase (FLT3) is expressed in normal early myeloid and lymphoid progenitors and in many types of leukaemia. FLT3 mutations are expressed in a smaller percentage of acute leukaemias and correlate with worse outcomes. Traditionally FLT3 expression and mutation has been detected using PCR techniques followed by gel electrophoresis. To our knowledge, we are the first centre to routinely use flow cytometry (FC) analysis to measure FLT3 expression for the diagnosis of acute leukaemia. *Aims.* 1) To investigate the patterns of FLT3 expression in all acute leukaemias by FC and compare this to data with standard methods. 2) To identify whether any specific trends of FLT3 expression can aid diagnosis and classification of acute leukaemia. *Methods.* A retrospective analysis was carried out on peripheral blood or bone marrow samples on 414 patients (300 AML, 90 ALL, 25 bi/triphenotypic according to EGIL scoring) who were diagnosed with acute leukaemia between January 2003 and October 2008. Surface expression of FLT3 was measured on a Coulter Epics XL flow cytometer until spring 2006 and then on a BD Canto II flow cytometer using an IgG1 monoclonal antibody (4G8 clone; BD Pharmingen). *Results.* Using a threshold of ≥20%, 84.4% of AML and 77.8% of ALL patients were positive for FLT 3. However, a cut-off of ≥30% was found to be more discriminatory: analysis of the AML FAB groups revealed 72.5% of patients with M0-M6 subtypes were FLT3$^+$ in contrast to 0% of patients with M7 (0/3 cases); ALL subtypes showed 89.3% of B-ALL (Pro B-ALL, common B- ALL & pre B- ALL) were FLT3$^+$ compared to 6.3% of T-ALL (1/16 cases); 60% of all ambiguous lineage leukaemias were FLT3$^+$ whereas only 12.5% ofT lymphoid leukaemias were FLT3$^+$. This compares to 100%, 75% and 66% FLT3 positivity for Myeloi trilineage and myeloid/T respectively. The fluorescence intensity (FI) of FLT3 expression did not aid separation of different leukaemia subtypes, with a range from weak to moderate FI and the majority of cases showing weak expression. *Summary and Conclusions.* This data supports the use of flow cytometry for detecting FLT3 antigen expression in acute leukaemia. The distribution of FLT3 positivity in the acute leukaemias by FC correlates closely with that reported using molecular techniques. The observed trends indicate the utility of FLT3 detection in the immunophenotyping panel for acute leukaemia. In particular, FLT3 negativity provides supportive data in the diagnosis of M7 AML and T lineage diseases using a cut-off of 30%.

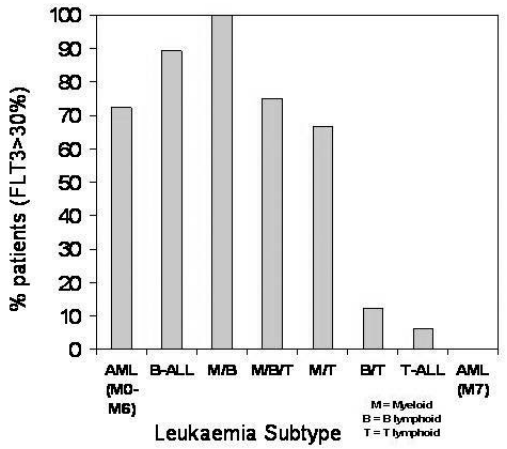

Figure. FLT3 positivity by leukaemia subtype.

0298

QUANTITATIVE ASSESSMENT OF WT1 EXPRESSION: A USEFUL TOOL FOR MONITORING MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIA PATIENTS

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Introduction. Wilms' tumor 1 (WT1) gene encodes a transcriptional factor important for normal cellular development and cell survival. Recently, the study of WT1's involvement in malignant cells, including haematological neoplasias, has revealed a potential role as an oncogene. Moreover, its overexpression in leukemias has been used as a molecular marker for the detection of minimal residual disease (MRD). *Aims.*Our study aims to verify if quantitative assessment of the WT1 transcript amount can be used as a marker for monitoring MRD and whether WT1 levels may be predictable of clinical outcome. *Patients and Methods.* WT1 amount was determined in BM and PBL samples of patients with AML lacking a molecular marker for MRD detection, and in normal control. We also analyzed WT1 levels at sequential time intervals during follow-up. Samples from 45 AML patients with miscellaneous cytogenetic abnormalities and normal karyotype at diagnosis (36 BM, 9 PBL) and 16 healthy control (4 BM, 12 PBL) were examined for WT1 expression. A total of 201 specimens of AML patients at diagnosis and during follow-up (181 BM, 20 PBL) were analyzed for quantitative assessment of the WT1 transcript amount. RQ-PCR reaction and fluorescence measurements were made on the ABI PRISM 7900 HT Sequence Detection System (PE Applied Byosystems). *Results.* In healthy controls, the median value of WT1 levels in BM

and PBL was 23,08 (range 0-176.70) and 0 (range 0-8,50), respectively. Among all the samples of AML patients at diagnosis and during follow-up, BM and PBL specimens showed a median of 276 (range 0-42.553,33) and 540,47 (range 2.18-25.696) respectively. At diagnosis BM and PBL samples had a median value of 3816,50 (range 7.97-23.094,10) and 7397 (range 6.00-25.696), respectively and overexpression of WT1 was observed in 92% of the cases. In 4 patients bearing an additional molecular marker, like AML1-ETO, PML-RARα or CBFβ-MYH11, we found a complete parallelism between the behaviour of WT1 transcript and the fusion genes. Clinically, the haematological relapse was correlated with the increase of WT1 expression. Moreover, we observed that the persistence of high level of WT1 transcript after induction chemotherapy was associated with imminent relapse. *Conclusions.* 1) WT1 expression may be useful for detection of MRD in AML patients lacking additional molecular markers; 2) there is a good correlation to other follow-up markers; 3) normal and abnormal WT1 transcript level may be able to detect patients at high risk of treatment failure after induction therapy, and quantitative analysis during follow-up can predict imminent relapse.

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0299

THE DELETION OF THE PROLINE-RICH REGION OF HOXB4 IS ASSOCIATED WITH MYELOID LEUKEMIA IN A MOUSE MODEL

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Background. HOXB4 belongs to the family of homeobox transcription factors, which play a key role in hematopoietic development. The expression of HOXB4 induces a significant increase of long-term repopulating stem cells (SC) in human and mouse models, without inducing malignant transformation. So far the underlying mechanisms of the SC amplificatory impact of HOXB4 are poorly understood. *Aim.* In an attempt to understand the unique characteristics of HOXB4, we performed a mutational study by deleting its proline-rich region, which has been described to act as a transcriptional activation domain in many other proteins, like non-homeobox genes (e.g. p53, AP2) and other homeobox genes (e.g. HOXD4 and HOXA13). *Methods.* We performed *in vitro* and *in vivo* experiments transducing murine 5-FU enriched HSCs with the pMSCV-IRES-GFP based retroviral vector harbouring the HOXB4 wild-type (wt) and several mutants, including a Δproline HOXB4 mutant (ΔP), where the proline-rich sequence (50% P) between the amino acidic positions 71-120 in the exon 1 is deleted. *Results.* In previous experiments, when HOXB4-ΔPro (n=14) was over expressed in 5-FU enriched progenitor cells from BM, we reported a significant decrease (75fold, $p<0.05$) of the 12 days Δ-CFU-S frequency, in comparison to the HOXB4-wt (n=5), while it still generated significantly more Δ-CFU-S in comparison to the GFP control (n=11) (35fold, $p<0.0003$). Furthermore, we performed the CRU assay by transplanting lethally irradiated C3HxC57Bl/PeB mice with serial dilutions of 5-FU isolated bone marrow progenitor cells, in order to evaluate the effect of the HOXB4-ΔPro on the competitive repopulating unit frequency. At the 16th week post transplantation we reported no significant difference in the CRU frequency between the mice receiving HOXB4wt (CRU 1/834, n=18) and the mice receiving HOXB4-Δpro (CRU 1/413, n=18) expressing transplants. However, in mice transplanted with HOXB4wt (n=12) 45.3% of the circulating cells belonged to the transduced compartment compared to 19.2% in the HOXB4-Δpro group (n=13) ($p<0.006$), whereas the lineage distribution within the transduced compartment did not differ between both experimental arms 16 wks post transplant. Of note and in contrast to HOXB4wt, mice engrafted with HOXB4-ΔPro BM cells (n=9) developed myeloproliferation with a significant increase of Mac-1 and Gr-1 positive cells over time in the PB (29% Gr1 and 43% Mac1 wk 4-16 compared to 71.2% Gr1 and 86.6% Mac1 week 36-56 wk, $p<0.004$). The HOXB4-ΔPro mice developed acute myeloid leukemia without maturation, as confirmed by immunohistochemical analysis after a median latency time of 279 days (n=9), while the mice transplanted with HOXB4wt expressing BM cells did not develop disease after an observation for more than 466 days (n=5, $p<0.05$). The AML in HOXB4-ΔPro mice was readily transplantable (66.5 days for 2nd Tx, n=6; 43 days for 3rd Tx, n=4) ($p<0.05$ compared to 1st recipients). In order to investigate the proviral integration pattern in the transplanted mice, we performed LM-PCR. In more than five HOXB4-Δpro mice we did not find recurrent integration sites. *Conclusions.* Taken together our results demonstrate that the N-terminal proline-rich region of HOXB4 has an important function for the stem cell amplifying function of HOXB4 and that loss of this domain converts HOXB4 in a leukemogenic gene.

Acute myeloid leukemia - Clinical I

0300

CONTINUOUS SEQUENTIAL INFUSION OF FLUDARABINE AND CYTARABINE FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA SECONDARY TO MYELODYSPLASTIC SYNDROME

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Background. Acute myeloid leukemia (AML) secondary to myelodysplastic sydrome (MDS) is characterized by poor prognosis, namely in older patients. The combination of fludarabine (F) with cytarabine (ARA-C) +/- G-CSF was proven as effective in patients with poor risk AML. *Aims.* To investigate in a phase II trial the efficacy and toxicity of a regimen including F + ARA-C administered as sequential continuous infusion (CI-FLA) in a series of 57 untreated patients aged more than 60 years, in whom AML arose after a previously diagnosed MDS (5 RA, 20 RAEB1 and 32 RAEB2. *Methods.* F at loading dose of 10 mg/sqm over 15 min at day 0, and after three hours and half ARA-C at a loading dose of 390 mg/sqm over 3 hours were given; at the end, F at 20 mg/sqm/ci/24 hours for a total of 72 hours and ARA-C at 1440 mg/sqm/ci/24 hours for a total of 96 hours were started. G-CSF was added at day +15 at a dose of 5 microg/kg. Patients achieving CR were programmed to receive an additional reduced course of CI-FLA, followed by G-CSF given at 10 microg/kg from day 15 in order to mobilize $CD34^+$ cells and perform autologous stem cell transplantation (ASCT). Between June 2001 and October 2008, 57 patients underwent the therapeutic program. Median age was 67 years (range 61-81). Cytogenetic analysis was successful in 50 patients out of 57 (87%) and showed normal karyotype (classified as intermediate) in 33 patients (66%), while 17 patients (34%) had different chromosomal abnormalities and were classified as unfavourable according to MRC criteria. Finally, 18 patients (31%) were affected by one or more concomitant diseases requiring specific treatment. *Results.* Overall, 40 patients (70%) achieved CR, all but one following one course of CI-FLA. There were 8 induction deaths (14%), while 9 patients (15%) were refractory to induction treatment. The median number of days to neutrophil $>0.5\times10^9$/L and platelet $>20\times10^9$/L was 19 (7-34) and 20 (9-38), respectively. Documented infections occurred in 7 cases (12%). Thirty-two patients (80% of remitters) were eligible for the programmed consolidation course. Twenty-nine patients were monitorized for the mobilization of $CD34^+$ cells, collection being successful in 19 of them (66%). Median number of $CD34^+$ cells/kg collected was 6.8×10^6 (2.5-40.3), median number of apheresis being 2 (1-2). Eleven patients (20% of the whole population) received autologous stem cell transplantation (ASCT). Median disease free survival (DFS) and overall survival were 9 and 10 months, respectively. Survival at 5 years is projected to 15%. The only parameter significantly related to DFS duration was the presence of unfavorable cytogenetics (i.e. complex karyotype or chromosome 5 and or 7 aberrations). In particular, DFS was 15 months for patients with diploid karyotype as opposed to 7 months for those with adverse one (p:0.01). *Summary and conclusions*. CI-FLA is effective and well-tolerated in elderly patients with AML secondary to previously diagnosed MDS. Therapeutic results are encouraging as to CR achievement and ASCT feasibility and compare favorably with conventional anthracycline/ARA-C based therapy. Best results are achievable in the subgroup of patients with diploid karyotype.

0301

INFLUENCE OF HIGH EXPRESSION OF SMAC/DIABLO PROTEIN ON CLINICAL OUTCOME IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Smac/DIABLO (second mitochondrial derived activator of caspase/direct IAP binding protein with low pI) is the best known antagonist of inhibitor of apoptosis proteins (IAPs) family. Smac/DIABLO was shown to trigger both external and internal apoptosis pathways in acute leukemia cell lines. The role and prognostic significance of Smac/DIABLO protein is not clearly determined in acute myeloid leukemia (AML) patients. *Aims.* The main objective of this study was to verify whether expression of Smac/DIABLO protein has a prognos-

tic impact on response to induction chemotherapy and overall survival (OS) of adult patients with AML. *Material and Methods.* Intracellular expression of Smac/DIABLO protein using multi-color flow cytometry was examined in leukemic blasts isolated from bone marrow or peripheral blood of 71 *de novo* AML patients (median age 54 years, range 28-81). The isotype controls were performed for all measurements. Protein expression was assessed by percentage of Smac/DIABLO positive cells. In parallel, immunocytochemistry was preformed to confirm the Smac/DIABLO expression. *Results.* Fifty six out of 71 AML patients received standard induction chemotherapy with daunorubicine and cytarabine (Ara-C) (3+7) and 15/71 were treated with low dose Ara-C. Fifty nine (47%) of all patients achieved complete remission (CR). The intracellular expression of Smac/DIABLO protein ranged from 0 to 99,8% of leukemic blasts. A cut-off point of the upper quartile was used to divide patients into *high-expressers* and l*ow-expressers* of investigated protein. It was found that CR rate in the *high-expressers* group was significantly higher than in the *low-expressers* (76% vs. 28% respectively; $p<0.01$). The median time of the follow up reached 7.2 months (range 0.2-63.4). The high expression of Smac/DIABLO was associated with significantly better (OS) in AML patients ($p<0.01$; Figure 1).

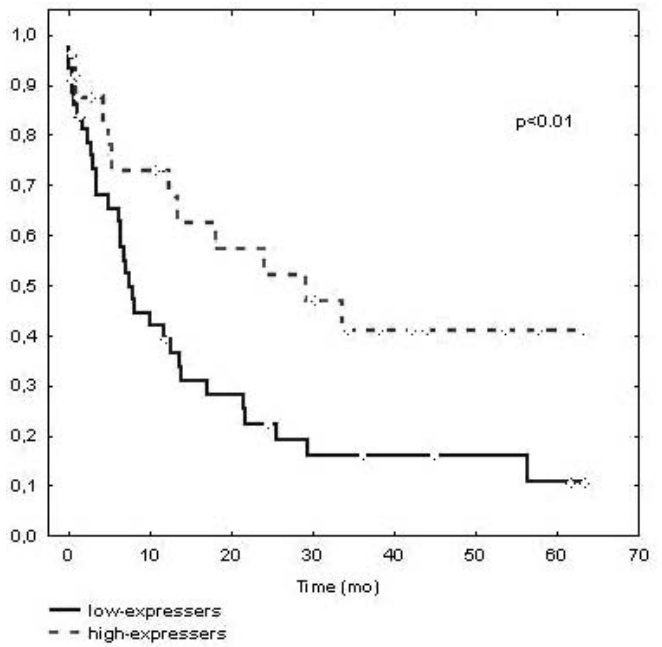

Figure 1.

Additionally, we observed that patients with good and intermediate karyotype according to SWOG classification showed significantly higher expression of Smac/DIABLO protein compare to poor risk group (median 74.7% vs. 37.7% respectively; $p<0.01$). *Conclusions.* These data, for the first time indicate that Smac/DIABLO protein expression is associated with higher sensitivity to standard chemotherapy, favorable karyotype and longer OS in AML patients. Further investigations evaluating the relationship between Smac/DIABLO as well as the other pro- and anti-apoptotic proteins should be undertaken to better demonstrate its prognostic and potentially therapeutic value.

0302

A PHASE 2 STUDY OF VORELOXIN AS SINGLE AGENT THERAPY FOR ELDERLY PATIENTS WITH NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA (AML)

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Background. Voreloxin is a naphthyridine analog that intercalates DNA and inhibits topoisomerase II, inducing apoptosis. Interim results of REVEAL-1, a Phase 2 study of single agent voreloxin in newly diagnosed elderly AML patients, are reported. *Aims.*Overall remission rate (CR + CRp), duration of remission, overall and leukemia-free survival, 30-day all-cause mortality. *Methods.* Phase 2 study of 3 voreloxin schedules (approximately 30 patients/schedule): A, 72 mg/m^{2}qw x 3 or B, 72 mg/m^{2}qw x 2 or C, 72 mg/m^2/dose on D1 and D4. Eligibility: newly diagnosed AML (*de novo* or secondary AML), patients age ≥60 and ≥1 additional adverse risk factor (age ≥70, secondary AML, intermediate or unfavorable cytogenetics, or PS 2). PK were evaluated in a patient subset in cycle 1. Ex vivo sensitivity of patient BMA to voreloxin was evaluated by CellTiter-Glo® proliferation assay. *Results.* A (29) and B (35) are fully enrolled. A. Demographics: 66% male, 35% female; median age 75 (range: 61-89); ECOG PS: 28% PS 0, 62% PS 1; 10% PS 2. 28% had AHD and cytogenetics were intermediate in 48%, unfavorable in 45%, and unknown in 7%. Eleven patients achieved a CR (9) or CRp (2) for an overall remission rate of 38%. The 30-day all-cause mortality rate was 17%. Infection was the most common cause of early mortality. Most frequent common Grade 3 or higher non-hematologic AEs (≥10%) included mucosal inflammation, and pneumonia. B. Preliminary data: Demographics: 63% male, 37% female; median age 76 (range: 61-87); ECOG PS: 21% PS 0, 65% PS 1; 14% PS 2. 22% had AHD and cytogenetics were favorable in 3%, intermediate in 22%, unfavorable in 44%, and unknown in 31%. Nine patients have achieved a CR (7) or CRp (2) thus far; and 3 patients are too early to evaluate. All CR and CRp patients remain in remission and so median duration cannot be estimated. The 30-day all-cause mortality rate is now 9% (3 of 34). Tolerability has improved over Schedule A. Grade 3 or higher non-hematologic AEs (≥10%) reported in Schedule B vs Schedule A: mucosal inflammation (7% vs 24%), and pneumonia (19% vs. 24%). The change in dosing schedule and the addition of recommendations for infection prophylaxis has resulted in a reduction in early mortality while preserving clinical activity. C is enrolling to explore the more dose intense schedule. Voreloxin PK were similar in this frontline population and in a Phase 1 in relapsed/refractory AML. Ex-vivo sensitivity did not predict clinical response. *Conclusions.* In REVEAL-1, voreloxin demonstrates clinical activity with 2 dosing schedules in previously untreated elderly (age ≥ 60) patients with AML who are unlikely to benefit from standard chemotherapy. CR + CRp rate was 38% (11 of 29 patients) for 3 weekly voreloxin doses (A). Early results from 2 weekly voreloxin doses (B) show 9 CR + CRp, with 3 patients pending, and improved tolerability. Enrollment to C, voreloxin dosed D1 and D4, is ongoing.

0303

LONG-TERM MOLECULAR COMPLETE REMISSION WITH PULSED ATRA AS SINGLE AGENT IN PML-RAR-ALPHA-POSITIVE ACUTE PROMYELOCYTIC LEUKEMIA

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Background. All-trans retinoic acid (ATRA), alone or combined with chemotherapy (CHT) is widely used in the treatment of acute promyelocytic leukemia (APL). If used alone, ATRA results in a substantial pro-

portion of complete remissions (CR). However, the continuous administration of ATRA as single therapy almost invariably leads to relapse in a short period of time (months). Current state-of-the-art treatments for molecular relapses include simultaneous administration of ATRA either with anthracycline-based chemotherapy or with arsenic trioxide (ATO). In addition, intensification with autologous or allogeneic stem cell transplantation (SCT) is often recommended in patients with persistent or recurrent disease. *Aims.* Basing on the pharmacokinetic evidence that acquired resistance to ATRA is frequently suppressed by the intermittent use of the drug, we designed the so called "pulsed" ATRA regimen for APL patients who were either molecularly refractory after combined ATRA/CHT treatment, or relapsed, or untreated, but not eligible for the combination treatment. *Methods.* Herein, we report on the long term follow up results of a cohort of pilot study. Eight patients were treated with ATRA (45 mg/m^2/day) for 15 days. After 2 weeks of rest, the treatment was then prolonged continuously for 1 week every 2 weeks. Molecular analysis was performed by qualitative and quantitative reverse transcription-polymerase chain reaction (RT-PCR). *Results*. All patients achieved a molecular CR after pulsed ATRA administration. Notably, none of the four patients treated at diagnosis relapsed. Conversely, 3/5 relapsed/refractory ones did present with further relapses (after 5, 20 and 24 months, respectively); two of them died, while one achieved a third molecular CR after allo-SCT. The remaining two are still CR after 8 and 11 months follow-up, respectively. Overall, 6/8 patients are alive and in continuous molecular CR, being the median relapse free survival 76 months (range, 11-111) and the median overall survival 78 months (range, 11-113). The toxicity was indeed mild; two cases of mucositis (grade II and III respectively) were recorded. *Summary and Conclusions.*In conclusion, though the relatively low number of cases, pulsed ATRA appeared to be particularly effective in previously untreated frail APL patients, who achieved long term remission and possibly cure. Indeed, to the best of our knowledge, this is the first evidence of persistent molecular CR in APL patients treated with ATRA alone. Interestingly, also relapsed/refractory cases did have some benefit; however, it was temporary, suggesting that this approach could be eventually used only as a possible *bridge* to allo-SCT. Certainly, confirmation in a larger cohort of patients is now warranted.

0304

A LUMINESCENT WHOLE CELL BACTERIAL BIOSENSOR FOR RAPID PRE-SCREENING OF CHEMOTHERAPY EFFICACY

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Background. Patients with Acute Myeloid Leukaemia (AML) are routinely given cytosine arabinoside (Ara-C) chemotherapy, without any pre-screening, although up to 70-percent of patients fail to respond. This is mainly due to non-uptake of Ara-C or lack of conversion to the active form (Ara-CTP). Current methods to determine drug sensitivity of AML are time-consuming and unsuitable for routine screening. *Aims.*We have constructed a bioluminescent reporter strain of Escherichia coli that determines patient sensitivity to Ara-C within hours, which could allow screening of patient blood or bone marrow before administering chemotherapy. *Methods.* Ara-C has no effect on E. coli, which lacks deoxycytidine kinase (dCK) and deaminates Ara-C into Ara-U using cytidine deaminase (cdd). A cdd-deficient strain of E. coli was constructed and transformed with the human dCK gene under the control of the lac promoter. Expression of dCK, inducible with IPTG, rendered E. coli sensitive to Ara-C. The bacterial biosensor was made self-bioluminescent by introduction of a plasmid carrying the lux CDABE operon. *Results.* Following incubation with Ara-C (25 μM), significant increases in bacterial light output were detected from the lysate of an AML cell line known to be sensitive to Ara-C (KG-1a), compared to untreated controls. Lysate from a cell line (THP-1) known to deaminate Ara-C through over production of cytidine deaminase showed no significant increase in light production in the presence of Ara-C. Peak light output could be detected 5 hours after the start of the assay. This biosensor is currently under evaluation using clinical samples obtained with informed consent, to evaluate the uptake and metabolism of Ara-C by leukaemic blast cells, and shows 100-percent correlation with patient outcomes in over thirty blood samples examined to date. *Conclusions.* This technology could indicate the sensitivity of AML cells to Ara-C as well as other chemotherapeutic agents used in combination with Ara-C, such as anthracyclines, giving a chemosensitivity profile to clinicians and customising treatment for individual patients.

0305

INDUCTION INTENSIFIED REGIMENS INCLUDING FLUDARABINE OR GEMTUZUMAB-OZOGAMICIN FOR ACUTE MYELOID LEUKEMIA PATIENTS: COMPARISON BY RESPONSE AND FOLLOW-UP

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Background. Conventional induction treatment in young Acute Myeloid Leukemia (AML) patients (≤60 years old) is still represented by the association of antracycline and cytarabine, which offers a complete remission (CR) rate not inferior to 63%. Since many non-randomized trials have recently demonstrated the superiority of intensified regimens, the present gold standard therapy includes the addition of at least a third drug to the classic 3/7 schedule. *Aim of the study*. We evaluated the safety profile and the efficacy of two four-drugs induction schedules, adding either fludarabine (25 mg/sqm days 1-5) or mylotarg (3 mg/sqm day 6) to idarubicin (6 mg/sqm days 1, 3, 5), cytarabine (1 g/sqm days 1-5), etoposide (100 mg/sqm days 1-5) (FLAIE and MyAIE, respectively). *Methods.* Sixty-six consecutive AML patients were enrolled either in the FLAIE (N=44, from 2002 to 2005) or in the MyAIE (N=22, from 2005 to April 2007) schedule, with similar clinical and biological characteristics. The median age was 45 and 48 years, respectively. According to kariotype, WBC count and FLT3 status, seventy and sixty-four percent of cases, respectively, were considered at high risk. Consolidation therapy consisted of 2 cycles of ID-AraC and da. *Results*. The complete remission rate was 75% and 59% for FLAIE and MyAIE, respectively (p=NS). Death during treatment rates were 5% and 0. After 1 consolidation course the overall CR rate was 80% and 73%. After a similar median follow up, 27 months (1-62) and 21 months (5-42) respectively, 41% of patients are alive in CR in the FLAIE group (12 SCT and 3 ASCT) and 64% in the MyAIE group (7 SCT and 4 ASCT) (p=n.s.; Chi-square, Fisher's exact test). Toxicity was comparable in the two regimens. The median time to ANC recovery (>1.0×10^9/L) was 31 and 23 days for FLAIE and MyAIE, respectively. The median time to PLT recovery (>100×10^9/L) was 28 and 24 days, respectively. The median time of neutropenic fever episodes for patients was 1 and 1.4 in the 2 groups, respectively. Grade III/IV GI toxicities occurred in 11% and 22% of cases, respectively. *Conclusions*. These data showed that four-drugs intensified induction therapy is a feasible approach in young AML patients. Because of the limited number of patients involved in this study, we showed no differences between these new therapeutic approaches, including fludarabine or mylotarg in addition to conventional chemotherapy. Nevertheless, when compared with previously tested ICE and FLAI regimens, these schedules seemed to be not more effective and with similar toxicities profiles. Future analyses and randomized trials will define their possible and definite role in AML treatment.

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0306

PROGNOSTIC SIGNIFICANCE OF FLT3/ITD MUTATION AND ABCB1 GENE POLYMORPHISMS IN CORE BINDING FACTOR (CBF)-ACUTE MYELOID LEUKEMIA (AML)

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Background. Although CBF-AML is associated with a relatively favorable prognosis, only approximately half of these patients are cured with standard therapy. Flt3/ITDs and NPM1 mutations are generally well known to be associated with clinical outcomes in AML patients. However, these mutations are reported to be rarely found in cases with CBF-

AML and it remains a matter of debate as to whether these mutations play an independent role in the prognosis of CBF-AML. MDR1/Pgp, the gene product of MDR1, is recognized as an important class of proteins for regulating pharmacokinetics. Several reports showed the effects of ABCB1 (mutidrug resistance 1 gene, MDR1, PgP) genotypes on pharmacotherapy in various malignancies including AML. *Aims.* To assess the prevalence and the prognostic role of Flt3/ITDs and ABCB1 gene polymorphisms in CBF-AML. *Methods.* Flt3/ITD, NPM1 mutation status and ABCB1 gene polymorphisms (SNP numbers: rs1055302, rs1002205, rs4148750, rs7779562, rs6980101, rs1922242, rs2235013, rs4728702, rs1922240, rs1922241, rs4148734, rs6950978, rs10256836, rs1202172, rs17327442, rs7802773, rs13229143, rs4148732, rs1978095, rs10264856) were evaluated by performing DNA polymerase chain reaction assays on BM samples obtained at initial diagnosis from the CBF-AML patients. DNA sequencing and GeneScan analysis was performed to confirm genotyping results. *Results.* Seventy-five CBF non-transplant AML patients who received intensive induction and consolidation chemotherapy including cytarabine were evaluated. Fifty-five (73.3%) were AML with t(8;21)(q22;q22) and twenty (26.7%) were AML with inv(16)(p13q22). The median age of patients was 38 years (range, 17-69 years). Twelve patients (16.0%) demonstrated the aberrant Flt3/ITD mutations. There was no statistically significant difference in age, gender, leukocyte count, hemoglobin level, platelet count and percentage of peripheral or bone marrow blasts, cytogenetics between the patients with or without Flt3/ITD. No NPM1 mutation was found in these patients. In univariate analysis, there was no significant difference in complete response (CR) rate (Flt3/ITD+: 100% vs. Flt3/ITD-: 98.4%, p=0.658). However, the presence of Flt3/ITD was associated with higher relapse rate in these patients (Flt3/ITD+: 75.0% vs. Flt3/ITD-: 13.3%, p=0.000). There was a significant shorter leukemic-free survival (LFS) in patients with Flt3/ITD compared to those without Flt3/ITD (1yr LFS; 13.9±11.9% vs. 57.7%±12.7%, p=0.024). On the other hands, there was significant difference in CR rate with ABCB1 gene polymorphisms types such as rs6980101 (genotype C/C: 65.0% vs. C/T: 100%, p=0.026), rs10256836 (G/G: 86.4% vs. G/C: 50.0%, p=0.037), rs17327442 (T/T : 84.6% vs. T/A: :40.0%, p=0.029) and rs4148732 (A/A: 91.3% vs. A/G: 50.0%, p=0.017). Whereas, there was no significant difference in relapse rate, LFS and overall survival between homo- and heterozygote groups in these polymorphsims. *Conclusions.* This study revealed that ABCB1 SNPs can affect CR rate of CBF-AML patients. Therefore, a stratified treatment plan in remission induction chemotherapy such as augmentation or addition of other chemotherapeutic agents may be warranted for the CBF-AML harvoring such ABCB1 polymophrisms. We also demonstrates that the presence of Flt3/ITD mutation is associated with significantly higher relapse rate and shorter LFS in CBF-AML, indicating the need for improved therapeutic approaches including stem cell transplantation as a post-remission therapy in CBF-AML with Flt3/ITD mutations.

0307

LAROMUSTINE INDUCES REMISSIONS IN ELDERLY POOR RISK AML PATIENTS WITH ADVERSE CYTOGENETICS AND DEMONSTRATES SIGNIFICANTLY IMPROVED SURVIVAL IN A HISTORICAL COMPARISON WITH PATIENTS TREATED IN THE UK NCRI AML14 TRIAL

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Background. Among older patients with AML a considerable number are unlikely to benefit from intensive chemotherapy due to known poor risk factors. These patients have typically been treated with either best supportive care (BSC) typically involving hydroxyurea, or low dose Ara-C (LDAC). It has been shown that LDAC is superior to BSC in such patients;[1] however no remissions were induced in patients with adverse cytogenetics as defined by Grimwade (1998). Laromustine (Onrigin') is a novel sulfonylhydrazine alkylating agent which preferentially targets the O6 position of guanine resulting in DNA cross-links. Laromustine has shown clinical activity in patients with *de novo* AML and high risk MDS.[3] A confirmatory phase II study of single agent laromustine was conducted in previously untreated patients ≥60 years old with *de novo* AML, prospectively considered likely to be unfit for intensive chemotherapy. Patients had at least one poor risk factor, defined by age ≥70, performance status 2, unfavorable cytogenetics, or cardiac, pulmonary or hepatic dysfunction. Eighty-five patients received induction therapy with 600 mg/m^2 laromustine. *Aims.* To investigate outcomes for laromustine patients compared with a matched cohort from the NCRI AML14 trial. *Methods.* The 85 patients treated as part of this phase II study were combined with 55 patients fulfilling the same eligibility criteria from an earlier study of laromustine to form a cohort of 140 patients. A retrospective non-randomised comparison was performed between this cohort and 121 patients satisfying the same entry criteria treated in the UK NCRI AML 14 trial with either BSC or LDAC. Outcomes for patients with intermediate and adverse risk cytogenetics, according to the refined MRC criteria were performed.[4] *Results.* Patients in AML14 were slightly older than those treated with laromustine (median age 75 vs. 73), and tended to have higher white blood cell counts. Other important risk factors such as performance status and cytogenetics were similar between the groups. Overall 51/140 (36%) laromustine patients exhibited a response (CR/CRp) compared to 14/61 (23%) LDAC and 1/60 (2%) BSC patients. Importantly, 8/34 (24%) patients with adverse cytogenetics according to the refined criteria exhibited a response. LDAC or BSC did not result in remissions in patients with adverse cytogenetics. In the group of patients with adverse cytogenetics, laromustine also demonstrated significantly longer survival than LDAC (1 year OS 12% vs. 0%, HR 0.44, 95% CI 0.20-0.95), with borderline significant interaction between treatment effect and cytogenetics (p=0.05). There was significant benefit compared to BSC in all cytogenetic groups. *Conclusions.* Despite the well-known limitations of retrospective comparisons, laromustine induces more remissions in patients with adverse cytogenetics than LDAC or BSC. The achievement of remissions after laromustine therapy translates into significantly improved overall survival in patients that are unlikely to benefit from intensive chemotherapy.

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0308

RASGRP1/APTX RATIO IS A STRONG BIOMARKER WHICH PREDICTS RESPONSE TO THERAPY WITH TIPIFARNIB PLUS BORTEZOMIB IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) is characterized by a wide genetic heterogeneity, for which the identification of targeted-treatments for specific subset of patients is strongly required. A recent study based on microarray profiling identified RASGRP1/APTX gene expression ratio a selection criteria for predict responders to Tipifarnib (Zarnestra, Z), a farnesyltransferase inhibitor with demonstrated activity in AML patients. Interestingly, Z and Bortezomib (Velcade, V) appeared synergistic in AML cell lines; therefore, we designed a phase I/II study with Z and V in newly diagnosed, resistant or refractory AML elderly patients, unfit for conventional chemotherapy approach. *Aim.* In order to identify biomarkers to predict clinical response to each drugs, we performed a gene dosage analysis of candidate biomarkers, to assess the correlation between RASGRP1/APTX ratio and treatment response. *Methods.* We analyzed gene expression profile of several suggested genes predictive of response to Z (RASGRP1, APTX, SCAP2, AKAP13, AHR1, GAPDH-control gene) either on bone marrow (173 samples) or on peripheral blood (76 samples) of 126 patients with AML. *Results.* By quantitative RT-PCR assay of analyzed genes, we confirmed two candidates biomarkers RASGRP1 and APTX, identified by genes expression profile (Raponi *et al.*, Blood 2008). Their ratio was evaluated using the following formula: 2-DDCt(RASGRP1) / 2-DDCt(APTX) . Median value of RASGRP1 expression on BM was 2.70 (range 0.01-209.8) evaluated on 126 samples and 6.60 (range 0.18-444.16) on PBL, analyzed on 68 specimens. Median value of APTX expression on BM was 1.38 (range 0.01-80.6) evaluated on 126 samples and 1.24 (range 0.04-85.32) on PBL, evaluated on 68 samples. Median value of RASGRP1/APTX ratio was 2.02 (range 0.01-120.60) and 4.98 (range 0.12-102.4) on BM and PBL, respectively. Fifty one patients out of 68 were enrolled, treated and evaluable for treatment response (median age 72, 33 male/35 female). It is noteworthy

that the 7 patients who obtained a CR have a median RASGRP1/APTX ratio at screening of 15.29 (range 2.83-19.8) on BM samples and 29.03 (range 25.65-35.45) on PBL. On the contrary, among patients (40 pts) who were non responsive to therapy the median value of RASGRP1/APTX ratio at screening was 2.19 (range 0.46-32.7) on 31 BM samples and 4.90 (range 0.99-27.1) on 19 PBL specimens (p=0.002). *Conclusion.* RASGRP1/APTX ratio may be suggested to be performed for screening and selection to target clinical trial with Tipifarnib in AML.

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0309

BODY MASS INDEX IS AN INDEPENDENT PREDICTOR OF DIFFERENTIATION SYNDROME IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA

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Differentiation syndrome (DS) can be a life-threatening complication in patients with acute promyelocytic leukemia (APL) undergoing induction therapy with all-trans retinoic acid (ATRA).Several factors have been associated with an increased risk of developing DS. The observation of several obese patients who developed DS led us to investigate this possible association in APL patients treated with an AIDA regimen. Between August 2004 and December 2008, 39 patients with APL genetically confirmed by t (15,17) and/or PML/RARA were treated with the PETHEMA LPA 99 protocol. Obese patients received adjusted doses of chemotherapy and ATRA on the basis of an adjusted Ideal Body Weight [IBW] (IBW+0.25 x [actual weight-IBW]). Seventeen patients were male and 22 female with a median age of 26 yrs (range,4-64).The median WBC 6.4×10^9/L (range,0.6-123.3). According to PETHEMA/GIMEMA risk stratification, 41% of patients were intermediate risk and 59% high risk. DS was observed in 11 patients with a median onset time of 12 days (range,3-23) and median WBC of 29×10^9/L (range,1.2-82.7). Five patients of 11 with DS had a BMI ≥35. All patients presenting WBC count $>10\times10^9$/L were on prednisone prophylaxis (1mg/kg from d1 to d15). In univariate analysis and then confirmed in multivariate analysis, we identified BMI ≥35 as an independent factor associated with both DS (p<0.0001, p=0.001) and ATRA-related complications (p=0.023, p=0.038) in addition to age >40 yrs and WBC $\geq20\times10^9$/L. As far as we know, this the first study that identifies high BMI as independent predictor of DS. Potential mechanisms implicated in the association between obesity and risk of developing DS should be investigated further.

0310

A PHASE I STUDY WITH CP-4055 AND IDARUBICIN IN PATIENTS WITH REFRACTORY/RELAPSED AML

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Background. CP-4055 (elacytarabine [pINN]) is a novel cytotoxic nucleoside analogue. While CP-4055 has similar mechanisms of action to cytarabine, it is, unlike cytarabine, independent of nucleoside transporters for cellular uptake. *Aims.*Determination of safety, MTD, and recommended phase II dose (RP2D) of CP-4055 in patients with refractory/relapsed AML receiving CP-4055 in combination with idarubicin. *Methods.* Adult pts from whom informed consent was obtained received CP-4055 as continuous infusion over 24 h (CIV) in a d1-5 q3w schedule. The CP-4055 starting dose was 1150 mg/m^2/d in combination with a fixed dose idarubicin 12 mg/m^2/d in a d2-4 q3w schedule. *Results.* 15 pts (9 male and 6 female) with a median age of 51 years (range 21-76) were enrolled. All patients presented with refractory/relapsed AML. Six pts had received one and nine pts two or more previous chemotherapeutic regimens. Four pts (2 male, 2 female) were enrolled at the first dose level, 1150 mg/m^2/d and this was determined the MTD. Dose limiting toxicities (DLTs) were typhilitis and hand-foot syndrome. Eleven pts were enrolled at the dose level below MTD, 1000 mg/m^2/d. RP2D was determined at 1000 mg/m^2/d. One patient died on d19 due to sepsis. Related adverse events of CTCAE grade 3-4 (preliminary data) were typhilitis, hand-foot syndrome, tumour lysis syndrome, nausea, thrombocytopenia, leukopenia, neutropenia (ANC), klebsiella infection, all events reported only once. Clinical activity, 3 CR and 1 CRp, was recorded at the RP2D. All patients recorded with CR had previous therapy regimen(s) including ara-C. Two patients moved on to transplant. *Summary and Conclusions.* Toxicity was manageable when CP-4055 was administered CIV d1-5 q3w in combination with idarubicin. The recommended dose for CP-4055 was determined to be 1000 mg/m^2/d. Clinical activity, 3 CR and 1 CRp, was recorded. Efficacy and safety of this novel combination will be further elucidated in Phase II studies.

0311

CNS MANIFESTION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA SCHEDULED FOR ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Transplantation centres in Europe use heterogenic strategies concerning intrathecal (IT) prophylaxis as part of the conditioning regimen before allogeneic stem cell transplantation. An EBMT survey in 2005 (Ruutu *et al.*, BMT, 35: p.121) argued, that total body irradiation and busulfan both treat effectively the central nervous system (CNS) and did not recommend on IT prophylaxis due to lack of prospective data, neither pre- nor posttransplant. However, it remained unclear whether regular evaluations of the cerebospinal fluid (CSF) are performed in patients with acute myeloid leukaemia, who are scheduled for allogeneic transplantation at all and how the incidence of CNS involvement prior to allo-HSCT can be rated. This might be particularly important, because nowadays more AML patients suffering from refractory disease are treated and the more frequent use of fludarabine based reduced-intensity conditioning regimens leads to a reduced penetration of active cytotoxic drugs into the CNS. Here, we report on our prospective analysis evaluating the CSFs of 199 AML patients receiving an allogeneic HSCT at our centre in the years from 1997 to 2008. *Patients and Methods.* Between 1997 and 2008, 240 adult patients with AML received an allo-HSCT at our institution. The median age was 49 years at the time of transplant. Within this group, cerebrospinal fluids of 199 adult AML patients were examined for cell counts and if abnormal results were seen cytologically thereafter. A small fraction of patients did not get a CNS evaluation due to a high number of blasts in the peripheral blood, acquired coagulation disorders or technical problems. We did not use a routine flow-cytometry. All patients gave written informed consent. *Results.* In total, we identified 16/199 (8%) patients with cytologically proven CSF involvement with the half of the patients CNS expressing an AML FAB M4 or AML FABM5 phenotype. The cell counts at the timepoint of diagnosis ranged from normal to 1497/3 cells per µL CSF. Normal cell counts did not exclude CNS disease. In 8/16 of the patients the CNS disease was not expected, since they were clinically asymptomatic, and CNS involvement would not have been detected without a routinely scheduled examination. In a subgroup analysis, patients with refractory disease (11/52) had a significant higher risk for CNS disease when compared to patients responding to prior systemic therapy (21% versus 3.4%; p=0.0008, two sided Fisher's exact test). All patients received a prophylactic dose of 12 mg methotrexate during the diagnostic lumbar puncture. Patients with CNS involvement underwent a compartment directed approach with repeated courses of intrathecal chemotherapy or radiation of the neuro-axis after engraftment. *Conclusions.* In refractory AML patients scheduled for allogeneic hematopoietic stem cell transplantation, we found an unexpected high proportion of CNS involvement (21%). Since there were patients with CNS disease who were asymptomatic and had normal CSF cell counts, we emphasize on routine morphological CSF evaluations before starting conditioning in order to optimize posttransplant treatment strategies.

0312

FLAIE (FLUDARABINE, CYTARABINE, IDARUBICIN AND ETOPOSIDE) AS INDUCTION CHEMOTHERAPY OF ADULT ACUTE MYELOID LEUKEMIA. A PILOT STUDY REPORT

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The primary goal of this prospective phase II was to evaluate the efficacy and the safety profile of FLAIE as induction chemotherapy regimen in previously untreated AML. Fifty consecutive AML patients were included between 2003 and 2005. All patients were younger than 65 with a median age of 51 years (range 21-63). The M/F ratio was 19/31, and 37/50 (74%) of patients were poor-risk at diagnosis. The induction regimen (FLAIE) included Fludarabine (25 mg/sqm), Ara-C (2 g/sqm), Etoposide 100 mg/sqm on days 1-5, Idarubicin (6 mg/sqm) on days 1, 3, and 5. Patients were evaluated for response rate and treatment-related adverse events; Overall Survival and Relapse Free Survival were also reported. After induction with FLAIE, Complete Remission (CR) occurred in 63% of patients (31 of 49 evaluable cases); three patients (6%) achieved Partial Remission and 15 patients (31%) were resistant. There were two cases of death during induction (DDI 4%). The hematological and extra-hematological toxicity of FLAIE was acceptable. Infections occurred in 29/50 (58%) of patients including 18 episodes of bacteremia and 15 cases of pneumonia. Oral mucositis grade II-III WHO was reported in 11/50 (22%) of patients. Median time to neutrophil (> 1×10^9/L) and platelet (>50×10^9/L) recovery was 24 (range 18-42) and 26 days (range 20-45), respectively. Supportive treatment: 13 RBC units (range 10-28) and 9 PLT units (range 5-15). G-CSF was required in 22/50 (44%) of cases. After a median follow-up of 22 months (range 1-68), 19/50 (38%) patients are alive (19/19 in CR). The probability of 4-year OS and RFS were 36 and 30%. Allogeneic and autologus HSCT was performed in 36 (72%) and 4 (8%) of patients, respectively. These results suggest that the addition of Etoposide to FLAI scheme does not improve efficacy of FLAI alone in AML patients younger than 65 years. The FLAIE regimen appeared to have acceptable toxicity, but its efficacy was comparable with that of standard induction regimens.

0313

INDUCTION THERAPY WITH '3+7' CHEMOTHERAPY PLUS ATRA FOLLOWED BY CONSOLIDATIONS WITH THREE COURSES OF IDARUBICIN ALONE AND MAINTENANCE THERAPY WITH ATRA IN NEWLY DIAGNOSED ACUTE PROMYELOCYTIC LEUKEMIA HAS AN EXCELLENT LEUKEMIA-FREE SURVIVAL BUT MINIMAL TOXICITY IN LOW AND INTERMEDIATE RISK GROU

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Backgrounds. Currently, there are many efforts to design risk-adapted strategies according to predicted risk of relapse by modulating treatment intensity in newly diagnosed acute promyelocytic leukemia (APL) and this seems to be an efficient approach to minimize treatment-related morbidity and mortality (TRM) while maintain the potential in cure for each relapse-risk group. *Aims*. We performed a prospective multicenter trial in patients with newly diagnosed APL to examine the efficacy of our treatment protocol (induction therapy with "3+7" chemotherapy plus all-trans retinoic acid (ATRA) followed by consolidations with three courses of idarubicin alone and maintenance therapy with ATRA) and to compare this between the different risk groups. *Methods.* Eighty six patients with previously untreated *de novo* APL were enrolled in the multicenter AML-2000 trial after informed consent was obtained from January 2000 to July 2007. For remission induction therapy, patients received oral ATRA (45 mg/m^2/d, maintained until CR) combined with idarubicin (12 mg/m^2/d, D1-D3) plus cytarabine (Ara-C, 100 mg/m^2/d, D1-D7). After CR achievement, they received 3 monthly consolidation courses consisting of idarubicin (12 mg/m^2/d, D1-D3) alone and maintenance therapy with ATRA (45 mg/m^2/d, D1-D15, every 2 month) alone was continued for 2 years. Total patients was divided to low-risk, intermediate-risk and high-risk groups according to a predictive model for relapse risk (Sanz score) based on pretreatment WBC and platelet count and the treatment outcomes were compared in the different risk groups. *Results.* The median age of total patients was 40 years old (range; 6-80) and median follow-up was 27 months (range; 1-90). The distribution of patients in the 3 risk groups was as follows ; 28 (32.6%) patients in low-risk, 40 (46.5%) in intermediate-risk and 18 (20.9%) in high-risk. Overall, CR was achieved in 78 (90.7%) of 86 patients. The CR rate according risk groups was 96.4% in low-risk, 87.5% in intermediate-risk, and 88.9% in high-risk group and there was no significant difference between the different risk groups. During induction therapy, 48 (55.8%) patients experienced grade 3-4 treatment-related toxicity (TRT), mostly fever and infection (38.8% of all patients) and 6 (7.0%) patients died of treatment-related complications. During 3 consolidation courses, 25 (29.1%) of 78 patients experienced grade 3-4 TRT in 1st course, 27 (36.0%) of 75 patients in 2nd course, and 14 (28.0%) of 50 patients in 3rd course. Overall, 3 (3.5%) patients died of treatment-related complications in CR. The incidence of TRT and treatment-reated mortality (TRM) during induction or consolidation therapy showed no significant difference between the different risk groups. The relapse occurred in 6 (7.0%) patients; 2 cases in intermediate-risk and 4 cases in high-risk. 5 patients among them relapsed during consolidation courses and the other patient relapsed during maintenance therapy. The overall survival (OS) and leukemia-free survival (LFS) rate at 7 years in all of patients was 76.7% and 83.5%, respectively. The OS rate at 7 years was 92.9% in low-risk, 78.6% in intermediate-risk and 53.6% in high-risk group and the LFS rate at 7 years was 96.4%, 83.4% and 62.2% respectively, showing the significant difference between 3 different risk groups. *Conclusions.* This study indicates that our protocol composed of induction therapy with "3+7" chemotherapy plus ATRA followed by consolidations with three courses of idarubicin alone and maintenance therapy with ATRA yields a high CR rate and low relapse rate but minimal acceptable toxicity despite of adding Ara-C in induction therapy, leading to excellent outcome in LFS and OS, in low and intermediate risk group. Meanwhile, considering that in high-risk group, the relapse rate was significantly higher than other risk groups and most of the relapses occurred in the middle of consolidation courses. This study suggests that our consolidation therapy composed of anthracycline alone is not enough to minimize risk of relapse in high-risk group in contrast with the low- and intermediate-risk groups. More intensive consolidation therapy combined with Ara-C or ATRA or hematopoietic stem cell transplantation in first CR or the combination of arsenic trioxide in front-line therapy may be considered in the patients with high-risk of relapse.

0314

MORPHOLOGIC AND MOLECULAR CHARACTERIZATION OF LEUKEMIC TUMORS IN THE BREAST: IMPLICATIONS FOR RESISTANCE TO CHEMOTHERAPY

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Background. We have previously demonstrated in a review of 153 cases that leukemic tumors in the breast, occurring in both AML and ALL, are mainly reported in women under 50, before, during, or up to 8 years after chemotherapy or transplant (Leuk Lymphoma 12/06). The occurrence of leukemic localization in the breast constitutes a poor prognostic factor independent of FAB classification, white blood cell count, and karyotype. Treatment with conventional anti-leukemic drugs rarely eradicates the disease, which was noted to subsequently follow a path of extramedullary soft-tissue progression, in many cases in ipsi- and contralateral breasts and pelvic organs, regardless of marrow remission status. Moreover, only 8% of cases of marrow leukemia with breast involvement at any time remain disease-free at 3 years. Evidence suggests that proliferation of leukemia in the premenopausal breast responsible for relapse may occur more frequently than is reported and it appears to be resistant to standard chemotherapy protocols. Thus, elucidation of the biologic behavior of these leukemic cells is critical to understand their intrinsic resistance to chemotherapy. Such an understanding holds the potential for the development of novel and effective therapeutic strategies. *Aim.* The aim of the present study was to characterize morphologic and molecular features of a series of leukemic breast tumors. *Methods.* Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded, tissue sections of 14 cases contributed by authors of case reports, on an IRB-approved protocol. Sections of 5-µm thickness were deparaffinized, dehydrated and stained with H&E for an evaluation of the morphologic characteristics of the leukemic and epithelial cell populations. To evaluate the presence of aberrant signaling pathways, selected sections were subjected to immunohistochemistry using standard techniques. Primary antibodies consisted of anti-pERK, anti-pAKT, anti-p53, anti-pEGFR, estrogen and progesterone receptors, and cytokeratin 18. For negative controls, the primary antibodies were replaced by a non-specific IgG. Visualization of antigen-antibody complexes, after incubation with the appropriate secondary antibodies, was performed with a streptavidin-peroxidase staining kit following manufacturer's instructions. Slides were counterstained with H&E prior to microscopic evaluation. *Results.* The lesions were characterized by dense infiltration by leukemic blasts with marked fibrosis and distortion of normal breast architecture. Leukemic cells were negative for estrogen and progesterone receptors, pEGFR, and cytokeratin 18, confirming the tumors were not of epithelial origin. Leukemic cells showed a strong immunoreactivity for phosphorylated ERK in 77% of cases. There was strong immunoreactivity to phosphorylated AKT in 62%. Importantly, the adjacent normal tissues were negative. p53 was wild-type in all cases. *Conclusions.* Our results show that leukemic breast tumors are characterized by increased immunoreactivity for activated ERK and AKT compared to adjacent normal tissue. These results suggest that resistance of leukemia growing as extramedullary tumors, often in a pattern of hormonally-active sites, is associated with activation of the PI3K/AKT and ERK pathways, known to be correlated with cell proliferation, resistance to apoptosis, and resistance to chemotherapy. Thus, targeting the ERK/AKT pathways could provide, in combination with marrow-directed therapies, a novel approach to overcome the chemoresistance of these tumors.

Chronic myeloid leukemia - Biology

0315

FINAL RESULTS. RESPONSE PREDICTION IN IMATINIB-TREATED CML BY EXPRESSION PROFILING

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Background. CML treatment in era of 1st and 2nd generation TKIs is guided by monitoring cytogenetic and molecular therapy response. The next step ahead towards a further personalized medicine would be response prediction prior to treatment. Although, expression profiling was proven to allow this for other entities, data from previous studies in CML are conflicting, mostly because of the different technical platforms and rather small patient populations used. *Aims.*This profiling study aimed to establish a robust gene set for imatinib response prediction. Ideally, the resulting gene set should consist of few enough genes to be cost effectively and routinely used with inexpensive technologies like real time PCR. *Methods.* Blood samples from at total of 135 pretreated CML patients with early or late chronic phase disease were collected prior to and six weeks after treatment with either 400 mg/day for 12 months or 800 mg/day for 6 months followed by 6 months of 400 mg imatinib therapy. Cytogenetic response and molecular response were monitored. Whole blood samples preserved by PAX gene technology were further processed and evaluated centrally at the expression profiling core facility at Innsbruck Medical University. For expression profiling samples were subjected to hemoglobin mRNA reduction before target preparation for hybridization to Affymetrix hGU133 Plus 2 genechips detecting ~47000 transcripts thereby allowing a whole genome profiling approach. Genes expressed differentially between responders and non-responders prior to and after 6 weeks of treatment were determined. For response prediction we used linear discriminatory analysis (LDA) provided in Bioconductor software packages for the open source statistical language *R*. A 1000- or 100-fold crossvalidation was performed using a robust leave-10-out setting. Additionally imatinib response was analyzed according to molecular response at 12 months and where available based on intra-patient comparisons of samples prior to and after 6 weeks of treatment. For condensing the resulting gene sets we applied recursive feature elimination (RFE). *Results.* Preliminary results presented at EHA 2008 revealed a set of 40 genes which allowed a mean correct prediction of 84.2% corresponding to positive and negative predictive values of 0.89 and 0.78, respectively. Here we present the final analysis of all evaluable patients and subgroups and additional Gene Set Enrichment Analyses based on candidate genes sets from the literature. *Conclusions.* Intra-patient comparisons and subgroup analysis allowed to refine candidate selection and resulted in a imatinib response predicting gene set based on this -so far largest- prospective multicenter gene expression profiling study.

0316

CD34+ OBTAINED FROM HIGH SOKAL RISK CHRONIC MYELOID LEUKEMIA PATIENTS EXPRESSES GENE PROFILES SIGNIFICANTLY DIFFERENT FROM CD34+ OBTAINED FROM LOW AND INTERMEDIATE SOKAL RISK PATIENTS

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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 chronic myeloid leukemia (CML) pts 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 expresses gene profiles (GEP) experiments aimed at the identification of genes and pathways able to predict and/or to elucidate the disease course of CP-CML pts at the onset of the disease. *Patients and Methods.* The study was per-

formed on highly enriched CD34+ cells from peripheral blood obtained from pts with untreated CML in CP. Overall, 27 pts were included in the present 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-value threshold = 0.01). Hierarchical clustering was performed with TIGR MeV. *Results.* In the initial part of the study, the first 14 CML pts (the *training set*) were successfully assayed for global GEP and microarray data were used to define genes differentially expressed in high (6 pts) vs. low (8 pts) Sokal risk pts, thus identifying 89 probes set; clustering of their GEP showed an homogeneous pattern in high Sokal risk pts, where up-regulated genes are mainly related to the positive regulation of immune response (CR1, UBASH3A, EREG, C4B) and to the induction of apoptosis (CD38, TNFRSF25, Apoe, TIMP3) , whereas down-regulated genes are mainly involved in the negative regulation of metabolic processes. Of note, among the most significantly up-regulated genes are ABCC4, an ATP-binding cassette (ABC) transporter and LAT, which is phosphorylated by ZAP-70/Syk protein tyrosine kinases following activation of the T-cell antigen receptor (TCR) signal transduction pathway. Among the most significantly down-regulated genes is PLCB1, which we recently described as being deleted in myelodysplastic syndromes and in myeloid acute leukemia. In the second part of the study, the 89 probes set differentially expressed in the training set were tested on an independent test set of 13 CML pts, including 4 high, 4 intermediate and 5 low Sokal risk pts. The test set GEP clustering displayed the same trend observed in the training set and, while low and intermediate risk pts resulted quite scattered, high risk pts clustered together. *Conclusions.* Overall, our data suggests that the expression at diagnosis of a particular array of genes might drive the evolutive risk of CML pts.

0317

EVALUATION OF THE PROTEASOMAL EFFECTS OF TYROSINE KINASE INHIBITORS AND BORTEZOMIB IN CHRONIC MYELOID LEUKAEMIA

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Background. Chronic myeloid leukaemia (CML) harbours cells which are resistant to the tyrosine kinase inhibitors imatinib and dasatinib irrespective of treatment duration. Proteasomes degrade intracellular protein through their catalytic β 1 (chymotrypsin-like activity), β 2 (trypsin-like activity), and β 5 (caspase-like activity) subunits. We have previously shown that BCR-ABL inhibition reduces proteasome proteolytic activity. The proteasome inhibitor bortezomib induces apoptosis in leukaemic cells by inhibiting the chymotrypsin-like activity of the β 5 subunit, and is a potential therapy in CML. *Aims.* The first aim of this study was to determine how therapeutically achievable concentrations of bortezomib affect proteasome expression and function in BCR-ABL positive cells. The second aim was to elucidate the mechanism by which imatinib or dasatinib reduce proteasome activity in BCR-ABL positive cells. *Methods.* Bone marrow aspirates were performed on patients with newly diagnosed CML. Mononuclear cells were obtained by Ficoll separation and were incubated for 24 hours with 0, 5, 10, and 20 nM bortezomib to examine the effect of proteasome inhibition on proteasome expression and activity. BCR-ABL is maximally inhibited by 1 μM imatinib or 1nM dasatinib, and cells were incubated for 24 hours with these concentrations to investigate the effect of tyrosine kinase inhibition on proteasome expression and activity. Three specific fluorogenic substrates, Suc-Leu-Leu-Val-Tyr-AMC, Z-Ala-Ala-Arg-AMC, and Z-Leu-Leu-Glu-AMC were used to measure the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome respectively. Proteasome subunit expression was measured by real-time PCR and Western blotting. *Results.* There was a concentration dependent reduction in each of the proteasome activities with increasing concentrations of bortezomib. All three proteolytic activities were significantly reduced after 24 hours incubation with 20nM bortezomib ($p<0.05$; n=4). At 10nM and 20nM bortezomib there was a significant increase in the RNA expression of the β 1 and β 5 subunits and increased expression of the β 2 subunit at 20 nM bortezomib ($p<0.02$; n=3). Western Blotting demonstrated that all three concentrations of bortezomib increased proteasome subunit levels ($p>0.05$; n=3). Incubation with imatinib or dasatinib for 24 hours did not alter proteasome proteolytic activity significantly ($p>0.05$; n=4). RNA expression of the β 5 subunit was significantly reduced by imatinib or dasatinib, and β 1 subunit expression was reduced by dasatinib alone ($p<0.05$; n=4). Using Western Blotting, proteasome subunit levels were reduced by both imatinib and dasatinib ($p>0.05$; n=3). *Conclusion*. Bortezomib reduced proteasome proteolytic activity in BCR-ABL positive cells. These cells compensated for proteasome inhibition by increasing proteasome expression. Imatinib and dasatinib reduced proteasome expression, but this did not affect proteasome proteolytic activity at 24 hours. These novel findings suggest that by reducing proteasome expression and ultimately proteolytic activity, tyrosine kinase inhibitors may target the toxic effects of bortezomib to BCR-ABL positive cells.

0318

DELETIONS OF THE DERIVATIVE CHROMOSOME 9 DO NOT INFLUENCE THE OUTCOME OF CHRONIC MYELOID LEUKEMIA IN EARLY CHRONIC PHASE TREATED WITH IMATINIB MESYLATE: A GIMEMA CML WP ANALYSIS OF 521 CONSECUTIVE PATIENTS

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Background. Deletions adjacent to the breakpoint on derivative chromosome 9 [der(9)] have been reported in a subset of Chronic Myeloyd Leukemia (CML) patients and have been associated with an adverse outcome with conventional drugs and α-interferon. Huntly *et al.* (Blood 2003) suggest that der(9) deletions are associated with lower response rates and shorter time to progression. Quintas-Cardama *et al.* (Blood 2005) did not find any difference related with der(9) deletions. In these 2 studies, some patients received imatinib in early CP (51 and 152, respectively) while many patients (224 and 168, respectively) were treated in late CP. European LeukemiaNet (ELN) recommendations include der(9) deletions among the *warnings*, requiring a careful monitoring of the patient. *Aim.* To investigate the prognostic value of der(9) deletions, we performed an analysis by age groups of 3 concurrent clinical trials of the GIMEMA CML Working Party (Clin Trials Gov. NCT00514488, NCT00510926 and the observational trial CML/023). *Methods.* 559 consecutive CML patients in early CP have been enrolled from January, 2004 to January, 2007. The presence of a der(9) deletion has been investigated by FISH in 521/559 at enrollment: 60 (12%) showed a der(9) deletion and 461 (88%) did not. The 2 groups, with/without deletions, were comparable (no significant difference in age, Sokal risk, imatinib dose). Median observation time is currently 42 (1-64) months. FISH analysis of bone marrow cells at diagnosis was performed using BCR/ABL extra-signal, D-FISH or dual-color dual-fusion probes. Response monitoring was based on conventional cytogenetic examination every 6 months and quantitative molecular (Q-PCR) evaluations (PB) after 3, 6 and 12 months on imatinib (every 6 months thereafter). Definitions: Complete Cytogenetic Response (CCgR): 0% Ph+. Major Molecular Response (MMR): BCR-ABL/ABL ratio <0.1%IS . Events (ELN criteria for *failure*): no CHR at 6 months, no CgR at 6 months, no PCgR at 1 year, no CCgR at 18 months, loss of CHR, loss of CCgR, progression and death. *Results.* Overall, the cumulative incidence of CCgR and MMR was 92%/91% and 88%/87% in patients with/without der(9) deletions. The probability of Event Free Survival, Progression Free Survival and Overall Survival was 82%/80%, 89%/90 and 93%/91 respectively. No difference was statistically significant. *Conclusions.* When investigated by FISH, the presence of der(9) deletions do not constitute a poor prognostic factor in early CP CML patients treated with imatinib: the cytogenetic and molecular response rates in the 2 groups, with and without der(9) deletions, are superimposable. No differences in outcome have been observed. This long-term outcome evaluation suggests that a redefinition of ELN *warnings* would be advisable.

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0319

PRESENCE OF BCR-ABL POSITIVE CELLS AND DIAGNOSIS OF CHRONIC MYELOID LEUKEMIA IN IMMUNE SUPPRESSED ORGAN TRANSPLANT RECIPIENTS

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Background. Spatial proximity of specific chromosomes during the cell cycle may predispose to leukemogenic translocations, including t(9;22), t(15;17) and t(8;21). In patients with an impaired immune surveillance, cells carrying any of these alterations may become phenotypically relevant. *Aims.*Therefore, immunosuppressed solid organ recipients represent an optimal population to address the frequency of mRNA products (BCR-ABL, PML-RARα or AML1-ETO) of these translocations. *Methods.* Blood leukocytes were studied in 201 individuals (100 organ recipients and 101 control individuals) for the presence of BCR-ABL, PML-RARα and AML1-ETO transcripts by a nested reverse transcriptase-polymerase chain reaction assay, routinely used in our institution. *Results.* In 5/100 immunosuppressed patients (5%), at least one out of two rt-PCR products was BCR-ABL positive while all controls were negative. In two of these five patients a 12-month follow-up sample was available and was tested BCR-ABL negative. These findings are extended by three cases of solid organ transplant recipients who developed chronic myeloid leukemia (CML) in a total of 2088 transplantations in nine years suggesting a higher incidence of CML in these patients. A fourth case of CML in an immunosuppressed solid organ recipient occured in another centre. No individual was positive for PML-RARα or AML1-ETO transcripts. Aside from immunosuppression, DNA damage caused by immunosuppressant drugs such as azathioprine or 6-mercaptopurine may be an additional factor in the occurrence of bcr-abl transcripts in non-leukemic patients. However, *in vitro* exposure of BCR-ABL negative leukemic cell lines to azathioprine or 6-mercaptopurine did not generate BCR-ABL transcripts. *Conclusions.* This suggests that immune suppression, but not treatment-associated DNA damage may contribute to the presence of BCR-ABL transcripts or CML in suppressed organ transplant (SOT) recipients.

0320

THE ROLE OF THE FANCONI ANEMIA D2 (FANCD2) PROTEIN IN BCR/ABL-MEDIATED LEUKEMOGENESIS

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Background. BCR/ABL oncogenic tyrosine kinase transforms hematopoietic stem cells, also can increase DNA damage and compromise the fidelity of DNA repair causing genomic instability. We have shown that BCR/ABL-mediated leukemogenesis is associated with the elevation of reactive oxygen species (ROS), which may increase the number of oxidative DNA lesions including DNA double-strand breaks (DSBs). BCR/ABL-positive leukemia cells appear to accumulate an excess of ROS-induced DSBs, which may cause apoptosis if not repaired. We reported before that homologous recombination repair (HRR), involving RAD51 protein, plays a pivotal role in the response of BCR/ABL-positive leukemia cells to numerous DSBs induced by ROS. Fanconi D2 protein (FANCD2), a member of the Fanconi protein family, is monoubiquitinated on K561 by FANCL ubiquitinase and phosphorylated by ATM on S222 in response to DSBs. The K561 monoubiquitinated form of FANCD2 (FANCD2-Ub) interacts with RAD51 to facilitate HRR, and phosphorylation of FANCD2 on S222 is important for activation of S phase checkpoint. *Aims.* Determining the role of Fanconi D2 protein in genetic instability of chronic myeloid leukemia (CML) cells. *Methods.* Mo7e human myeloid GM-CSF-dependent and PD20 lymphoblast cells were used (wt and BCR/ABL-transformed). CD34$^+$ CML stem/progenitor cells from chronic phase (CML-CP) blast crisis (CML-BC) and CD34$^+$ cells from healthy donors were used after receiving informed consent. FANCD2$^{-/-}$ and wild type cells were from murine bone marrow. Vitamin E (VE) or N-acetylcysteine (NAC) were used as antioxidants. *Results.* We detected an increased amount of FANCD2-Ub in BCR/ABL-positive leukemia cell line and CD34$^+$ CML-CP and CML-BC cells in comparison to normal counterparts. This effect was not associated with up-regulation of FANCD2 ubiquitinase FANCL or down-regulation of FANCD2 deubiquitinase USP1, but was reversed after inhibition of BCR/ABL kinase with imatinib and reduction of ROS with antioxidants. Therefore we postulate that BCR/ABL kinase-dependent ROS-induced FANCD2-Ub may play a role in leukemic transformation. This hypothesis is supported by impaired transformation potential of BCR/ABL kinase in FANCD2$^{-/-}$ cells in comparison to wild-type counterparts. Restoration of the expression of FANCD2 protein in FANCD2$^{-/-}$ cells rescued the transforming potential of BCR/ABL kinase. In addition, expression of BCR/ABL kinase, but not the kinase-deficient K1172R mutant, inhibited the proliferation rate of FANCD2$^{-/-}$. The growth defect of BCR/ABL-positive FANCD2$^{-/-}$ cells was accompanied by delayed leukemogenesis in SCID mice. Growth potential of BCR/ABL-positive FANCD2$^{-/-}$ could be rescued by co-expression of FANCD2 wild-type and S222A mutant, but not the K561R mutant. This observation supports our hypothesis that K561 monoubiquitination, but not S222 phosphorylation of FANCD2 might play an important role in BCR/ABL-mediated transformation. Elevated levels of ROS-mediated DSBs in BCR/ABL-positive FANCD2$^{-/-}$ cells did not cause any significant changes in cell cycle distribution, but resulted in discrete but persistent apoptosis. Scavenging ROS by antioxidants reduced the number of DSBs and eliminated the growth defect in BCR/ABL-positive FANCD2$^{-/-}$ cells (Figure 1) without affecting their wild-type counterparts . *Summary and conclusions* We hypothesize that FANCD2-Ub, but not FANCD2-phosphoS222 may play an important role in BCR/ABL-dependent leukemogenesis and transition from chronic phase to the fatal blast crisis in CML, probably due to its ability to interact with RAD51 and facilitate HRR of the numerous ROS-induced DSBs.

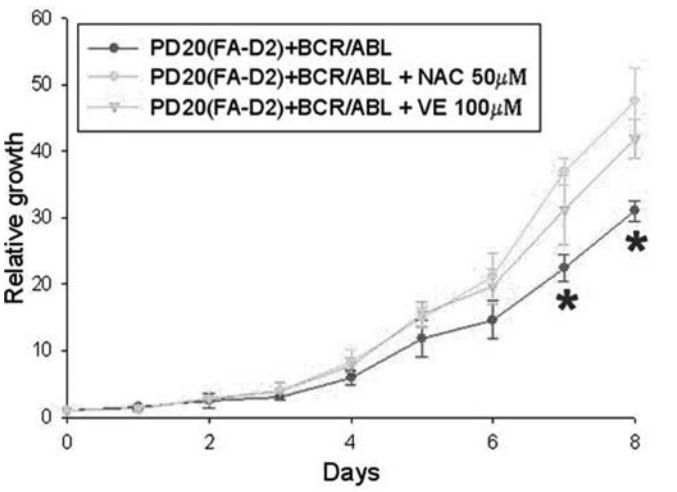

Figure 1.

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BCR-ABL ONCOPROTEIN MODULATES MIRNA INVOLVED IN APOPTOSIS REGULATION

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Background. Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of the Philadelphia (Ph) chromosome and Bcr-Abl oncoprotein. Bcr-Abl is a tyrosine kinase (TK) which presents a deregulated activity associated with proliferation and accumulation of myeloid cells and their precursors. Much attention has been focused on the development of novel therapies based on mechanistic understanding of Bcr-Abl signaling in CML cells. Imatinib (IM), a tyro-

sine-kinase inhibitor (TKI), became the first choice drug in chronic phase CML. Despite of a promising IM clinical response, approximately 20-25% presents resistance caused by the appearance of clones expressing mutant forms of Bcr-Abl. Second-generation TKIs like Dasatinib and Nilotinib have provided new therapeutic option for the patients resistant to IM. These observations emphasize the importance of better elucidate the CML physiopathology. *Aims.*To determine miRNA global expression in HL60.Bcr-Abl and to associate them with apoptosis resistance. *Methods.* A concentration of 107 of the promyelocytic leukemia cell line (HL-60) and HL-60 transfected with the plasmid pSRαMSVp185bcr-abl tkneo which contains bcr-abl was submitted to RNA extraction by TrizolTM method and reverse transcription was performed with specified primers RT stem loop (Applied ByosistemsTM) for each miRNA. TaqManTM MicroRNA Assays Human Panel Early Access Kit (Applied ByosistemsTM) was used to miRNA global expression assay at ABIPRISM 7500 Real Time PCR (Applied BiosystemsTM). The miRNAs differentially expressed in HL60 and HL60.Bcr-Abl was analyzed by the fold change and was considered those genes with a fold change above 2.5. miRanda method was used to identify the miRNAs targets genes, the biological processes and the pathways which miRNAs are involved were determined by Gene Ontology and Kegg or Biocarta, respectively. *Results.* HL-60.Bcr-Abl in comparison to HL-60 presented fourteen up-regulated miRNA (miR-145, miR-96, let-7e, miR-16, miR-21, miR-26a, miR-130b, miR-132, miR-324-5p, miR-130a, miR-15b, miR-30e, miR-26b and miR-326) and twenty-three down-regulated (miR-15a, miR-29c, miR-152, miR-103, miR-23a, miR-198, miR-214, miR-133a, miR-106, miR-181c, miR-141, miR-150, miR-98, miR-125b, miR-142-3p, miR-200a, miR-155, let-7d, miR-302d, miR-204, miR-99a, miR-203 and miR-221). miR-145 was the more expressed miRNA with a fold change of 325.96 and some miR expression was absent in HL-60.Bcr-Abl. The majority of these miRNAs have as targets genes important for cell toxicity, apoptosis regulation, JAK-STAT, AKT, mitochondrial and caspases signaling pathways. *Conclusions.* Bcr-Abl may modulate several miRNAs which is responsible for cell apoptosis resistance phenotype. The results obtained could contribute for the description of the new molecules which could help to elucidate the resistance by TKI, CML progression and apoptosis resistance.

0322

BOSUTINIB (SKI-606) COOPERATES WITH DASATINIB IN COUNTERACTING GROWTH AND VIABILITY OF CML CELLS CARRYING THE T315I MUTANT OF BCR/ABL

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Chronic myeloid leukemia (CML) is a stem cell disease defined by the Philadelphia chromosome and the related chromosome translocation, t(9;22). The ABL tyrosine kinase inhibitor (TKI) imatinib is effective in most patients and considered standard first line therapy. However, not all patients show a long-lasting response. Treatment failure is usually associated with the occurrence of imatinib-resistant BCR/ABL mutations. For these patients, novel TKI such as dasatinib represent alternative treatment options. Still, however, not all patients respond to these drugs, especially when leukemic cells bear the BCR/ABL mutant T315I that confers resistance against most TKI. Bosutinib is a novel multi-kinase inhibitor that has been described to act growth-inhibitory in BCR/ABL-transformed leukemic cells. In the present study, we examined the effects of bosutinib alone or in combination with dasatinib on growth and survival of primary CML cells (chronic phase CML, n=5, accelerated phase, n=1) and various CML cell lines. Bosutinib was found to inhibit 3H-thymidine uptake and thus proliferation in imatinib-sensitive and imatinib-resistant K562 cells in a dose-dependent manner, with comparable IC_{50} values (10-100 nM). Moreover, bosutinib was found to inhibit the growth of primary CML cells and Ba/F3 cells bearing various imatinib-resistant mutants of BCR/ABL, except T315I (IC50>1 μM). The growth-inhibitory effects of bosutinib were found to be associated with cell cycle arrest and signs of apoptosis. Dasatinib showed similar effects, but again did not block the growth of leukemic cells bearing BCR/ABL T315I. Unexpectedly, however, we found that bosutinib and dasatinib synergize with each other in producing growth inhibition in primary CML cells exhibiting BCR/ABL T315I at pharmacologic concentrations (0.01-1 μM). Clear synergistic effects were also observed in imatinib-sensitive and imatinib-resistant K562 cells as well as in Ba/F3 cells bearing BCR/ABL T315I. We also performed multiplexed kinase assays as well as chemical proteomics analysis and mass spectrometry using K562 cells and primary CML cells and coupleable dasatinib and bosutinib analogues. In these experiments, dasatinib and bosutinib were found to express an overlapping but non-identical profile of target kinases. As expected, both drugs were found to bind to wt ABL, SRC kinases, and TEC-family kinases including BTK. Targets preferentially bound and inhibited by bosutinib were STE20s, the FES/FER family, CAMKIIG, PYK2 and TBK1. We were also able to confirm that the dasatinib-targets KIT and PDGFRA are not recognized by bosutinib. Interestingly, whereas wt ABL (IC_{50}<0.5 nM) and most of the ABL mutants tested (H396P, M351T, Q252H, and Y253F) were all completely inhibited by both drugs at 1 μM in the kinase assay, the ABL T315I mutant was inhibited by bosutinib (IC_{50}=26 nM) almost 70 times more potently than by dasatinib. Together, these data show that bosutinib and dasatinib synergize with each other in producing antileukemic effects on CML cells including leukemic cells expressing BCR/ABL T315I. These synergistic effects may be explained by differential target kinase profiles and by the fact that bosutinib retains some activity against the T315I mutant.

0323

EFFECT OF IMATINIB MESYLATE ON SERUM SOLUBLE VASCULAR ENDOTHELIAL GROWTH RECEPTOR-R2 AND MICROVESSEL DENSITY IN BCR-ABL POSITIVE CHRONIC MYELOID LEUKEMIA

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Background and Purpose of the study. Angiogenic markers like serum vascular endothelial growth factor/receptors (sVEGF/sVEGF-R1 and R2) and Micro vessel density (MVD), as prognostic factors have been studied in chronic myeloid leukemia (CML). The degree of angiogenesis adversely affects the clinical and hematological prognostic indicators. VEGF mediates its role in angiogenesis through interaction of cellular and soluble receptors namely VEGF-R1 and R-2. Studies have shown that bone marrow cells in CML patients express very high levels of VEGF-R1 and R2 on their surface 1. Though the effect of Imatinib mesylate on VEGF and MVD in CML has been studied, such an effect on both cellular and serum soluble VEGF-R2 (sVEGF-R2) has not been studied so far. *Aim.* Our study aims at evaluating the effect of Imatinib mesylate on angiogenic parameters viz; sVEGF-R2 and MVD after a minimum of three months of imatinib therapy and to correlate the clinico-hematological and molecular parameters in BCR-ABL positive CML patients prospectively. *Patients and Methods.* Thirty-two newly diagnosed CML patients, comprising of 21,8,3 patients in chronic phase (CP), accelerated phase (AP) and blastic phases (BP) respectively, were included in the study. BCR-ABL by RT-PCR, base line serum sVEGF-R2 and a bone marrow biopsy for MVD estimation were done and the patients were put on imatinib mesylate at a dosage of 400, 600, and 800 mg/d for CP, AP and BP respectively. These were repeated after a minimum of 3 months of imatinib therapy. MVD was determined by mean micro vessel counts per 10 high power fields on bone marrow biopsies subjected to immunohistochemistry with CD34 monoclonal antibody (Dako) by immunoperoxidase technique in all cases and in 16 chronic ITP cases which served as controls. Serum soluble VEGF-R2 was done by ELISA (Bender Med systems) in 32 patients and 16 normal healthy controls. *Results.* Fourteen of 21 (67%) patients in CML-CP achieved hematological remission (HR) only and 7/21(33%) achieved both HR and BCR-ABL negativity by RT-PCR. Only one patient in AP and none of the BP patients achieved HR and BCR-ABL negativity. Both angiogenic parameters before the therapy were higher in all phases of CML than the normal controls. They were highest in the BP followed by AP and CP. Seven of 21(33%) CP patients who had achieved both HR and BCR-ABL negativity were found to have a statistically significant (p<0.001) reduction in the levels of both angiogenic parameters after a minimum of 3 months of imatinib therapy. However, no significant reduction in these parameters was found in all AP, BP patients and in 14/21(67%) CP patients who achieved only HR. *Conclusions.* Imatinib mesylate therapy has a significant anti-angiogenic effect in CML- CP patients who achieved both HR and BCR-ABL negativity. No significant effect was seen in all AP, BP patients and CP patients who achieved only HR.

Reference

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0324

EXPRESSION LEVELS OF MIR-150 AND TRANSCRIPTION FACTORS PU.1, EGR2 INVERSELY CORRELATE WITH MYB AND MYCN IN CHRONIC MYELOID LEUKEMIA

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Background. Previously we found miR-150 downregulated in total leukocytes from peripheral blood of patients with chronic myeloid leukemia at diagnosis (n=5), hematological relapse (with no blasts in peripheral blood; n=5) and blast crisis (n=4). Normal levels were found in patients with major molecular response to imatinib (n=5) and imatinib suboptimal response/therapy failure (without relapse; n=5) (Machova Polakova *et al.*, ASH 2008, abstract 1082). Interestingly, miR-150 significant downregulation was found in native polycytemia vera reticulocytes (Bruchova *et al.* 2008, Haematologica, 93:1009). In contrast, upregulation was reported in chronic lymphocytic leukemia (Fulci *et al.* 2007, Blood, 109:4944). At the same time, the important role of miR-150 in hematopoiesis has been proved (Xiao *et al.* 2007, Cell, 131:146; Zhou *et al.* 2007, PNAS 104: 7080, Bruchova *et al.* 2007, Exp Hematol 35:1657). Aims.To further investigate the role of miR-150 in the pathogenesis of CML, we focused on the expression of its predicted targets PU.1 and Egr2, which are transcription factors regulating myelo-lymphoid lineage development. We also investigated expression of Myb as known target of miR-150, and miR-150 putative target MycN, both regulating cell proliferation. We aimed to correlate expression patterns of those genes with expression of miR-150. *Methods.* Real-time PCR was applied for quantification using the same samples from the previous study (see above). Relative fold changes of gene expression to normal control (expression average of 11 healthy donors) were assessed by Pfaffl *et al.* (2001, Nucleic Acid Res 29:e45). *Results.* Similarly as for miR-150, we found downregulation of PU.1 in blast crisis, diagnosis and most of hematological relapses. Downregulation of Egr2 was observed in blast crisis and hematological relapses. Significant PU.1 downregulation in CML at diagnosis (n=43) was described by Albajar *et al.* (2008, Cancer Lett, 270:328). Pospisil *et al.* (ASH 2008, abstract 473) reported downregulation of PU.1 and Egr2 in AML mononuclear cells. Authors described that PU.1 regulated transcription of the miR-17~92 cluster by inducing Egr2. Interestingly, we found upregulation of an oncogene miR-17~92 cluster in the CML blast crisis (Machova Polakova *et al.*, ASH 2008, abstract 1082) that inversely correlated to downregulated expression of PU.1 and Egr2. The expression of the known miR-150 target Myb was upregulated in the samples with downregulated expression of miR-150. Similarly, significant upregulation of MycN was found in blast crisis and diagnosis. *Conclusions.* At diagnosis and advanced phases of CML, expression of PU.1 and Egr2 genes encoding proteins that are necessary for differentiation during hemopoiesis is downregulated, while genes encoding proteins regulating cell proliferation Myb and MycN are overexpressed. Whether Myb and MycN overexpression may be the result of miR-150 downregulation in CML is for further investigation.

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PREDICTED COMBINATORIAL APPROACHES FOR OVERCOMING INTRINSIC IMATINIB RESISTANCE IN CHRONIC MYELOID LEUKEMIA IDENTIFY POTENTIAL TARGETS FOR INTERVENTION

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Background. Imatinib (Glivec, Novartis) has revolutionized therapy of chronic myeloid leukemia (CML). However, a subset of patients displays intrinsic imatinib resistance. Microarray gene profiling led to the identification of potentially useful genes in predicting cytogenetic responses of CML patients to imatinib (Frank *et al.*, 2006, Leukemia). Imatinib-treated patients were evaluated for hematologic, cytogenetic and molecular responses for a minimal observation time of 12 months. Patients who remain in a major cytogenetic remission (MCR; <=35% Philadelphia chromosome-positive [Ph^+] metaphases) after initial response were defined as responders (n=23), whereas those with a continuous minor cytogenetic remission (lack of MCR; >35% Ph^+ metaphases) were considered non-responders (n=11). Patients were included into the retrospective gene profiling study if they met the following criteria: (i) CML in chronic phase, (ii) failure of interferon-α treatment before imatinib therapy and (iii) definite clinical outcome based on cytogenetics measured within the first 12 months of treatment to discriminate responders from non-responders. Diagnostic groups were matched according to demographic and hematologic parameters. *Aims.*Since the identified gene expression signature (128 genes) points to involvement of BCR-ABL-independent resistance mechanisms, microarray data were further analyzed using an innovative in silico discovery approach capable of predicting rational combination therapies. *Methods.* The PIPE-line technology firstly enabled us to define those pharmaceutically tractable components contained within this resistance associated gene set (n=43). Further analysis of this subset using a text-mining approach allowed us to establish a *proteo-anatomical* model of clinically relevant proteins specifically associated with the resistance phenotype. *Results.* This model comprised a set of six chemically tractable proteins, which served as blueprint for the rational prioritization of commercial and pre-clinical compounds for combination testing with imatinib *in vivo*. Compounds targeting two of these components, a G protein-coupled receptor (GPCR), and farnesyl pyrophosphate synthetase (FPPS), were analyzed in more detail. Previously, the predicted efficacy of bisphosphonates for targeting FPPS in imatinib-resistant CML has been verified by others (Chuah *et al.*, 2005, Leukemia) demonstrating proof of principle of PIPE-line technology. For the GPCR, downregulation observed in imatinib non-responders as evidenced by microarray analysis might contribute to the oncogenicity of BCR-ABL since it is capable of attenuating the proliferative effects of this chimeric tyrosine kinase. Subsequently, Western Blot analysis detected differential expression of this GPCR in four human CML cell lines (K562, LAMA84, AR230, KCL22) of which imatinib-sensitive and -resistant clones were investigated. Treatment with two different putative GPCR-interacting agents alone or in combination with imatinib resulted in inhibition of leukemic cell proliferation as measured by MTS colorimetric assay, but not of normal fibroblast cells. Apoptosis was induced by a FDA-approved component as evidenced by various apoptosis assays, whereas an experimental agent did not modulate the apoptotic pathway. Currently, the underlying antileukemic mechanism is further investigated. *Conclusions.* Based on the predictive power of the PIPE-line technology, we suggest the existence of promising new target molecules for novel combinatorial treatments of CML.

0326

CCN3, A NOVEL PRO-APOPTOTIC FACTOR FOR CHRONIC MYELOID LEUKAEMIA

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Background. Chronic Myeloid Leukaemia (CML) is characterized by expression of the constitutively active BCR-ABL tyrosine kinase. Previously, we identified down-regulation of the negative growth regulator, CCN3, as a result of BCR-ABL kinase activity in both primary human CML cells and cell lines. We now show that CCN3 is a growth inhibitory factor and expression enhances imatinib induced growth inhibition. *Aim.* To investigate the role of CCN3 expression in CML cells. *Materials and Methods.* K562 cells were stably transfected with a construct containing CCN3 (pCMV82-23) and compared to cells transfected with vector alone (control) using Amaxa nucleofection; stable expression was selected using ampicillin resistance. Real-time PCR was performed for CCN3 expression in comparison with known standards for gene copy number. Colony formation assays were performed in methyl cellulose over a period of 5 days; cell growth was assessed using the CyQuant' assay (Invitrogen, UK). Western blot analysis was performed on cell lysates using antibodies for phosphorylated and total levels of levels of ERK, Akt, STAT5 (Cell Signalling Technology, USA) and for CCN3 using an anti-NH5 antibody. Flow cytometry was used to determine cell cycle status. *Results.* CCN3 expression was undetected by Real-time PCR in control cells whilst pCMV82-23 cells expressed 2.25×10^6 copies per 50ng of cDNA and this was reflected in CCN3 protein levels using Western blot analysis. pCMV82-23 cells showed reduced colony formation capacity (p=0.003) and reduced cell growth over a period of five days (p=0.005) compared to control. CCN3 expression resulted in significant downregulation of three major signaling pathways and demonstrated reduced phosphorylation of ERK (p=0.002), pAKT (p=0.017) and pSTAT5 (p=0.005) compared to control cells; protein levels for total ERK, AKT and STAT5 were unaffected by CCN3 expression. Sustained CCN3 expression resulted in an accumulation of cells within the subG0 stage

of the cell cycle (p=0.040). To determine if CCN3 expression could influence sensitivity to the BCR-ABL kinase inhibitor, imatinib, pCMV82-23 cells and control cells were treated with imatinib (5 uM) for 48h. Control cells treated with imatinib showed moderate growth inhibition (19.6%±2.5). pCMV82-23 cells showed a significant increase in the magnitude of imatinib induced growth inhibition (63.3%±10.5 (p=0.043)). This was associated with an increased accumulation of cells in the subG0 area of the cell cycle (pCMV82-23 cells 34.6%±5; control cells 21.7%±8; p=0.006). To determine if these effects could be reproduced using recombinant CCN3 (rCCN3), K562 cells were treated with imatinib (5 µM) alone or in combination with rCCN3 (10 nM) for 48h. K562 cells treated with rCCN3 and imatinib showed enhanced growth inhibition (71.8%±7.9) compared to cells treated with imatinib alone (81.1%±9.2 (p=0.008)). Loss of CCN3 expression is consistent with properties associated with the CML phenotype. Sustained expression of CCN3 in K562 cells restores growth control and re-establishes induction of apoptosis. Increased expression of CCN3 provides an additional benefit for imatinib induced growth inhibition thus providing a novel avenue for therapeutic intervention.

0327

3-DEAZANEPLANOCIN A (DZNEP) ACTIVITY AGAINST CD34+ LEUKAEMIA STEM CELLS

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Polycomb-repressive complex 2 (PRC2)-mediated histone methylation plays an important role in aberrant cancer gene silencing and is a potential target for cancer therapy. Recently it was shown that the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A (DZNep) effectively depleted cellular levels of PRC2 and induced efficient apoptotic cell death in cancer cells but not in normal cells. Chronic Myeloid leukaemia (CML) stem cells, which express high levels of BCR-ABL, cannot be completely inhibited by ABL tyrosine kinase inhibitors (TKI) such as imatinib (Glivec) or dasatinib (Sprycel) and up to 20% of patients develop drug resistance. Therefore it is important to search for other pathways to inhibit leukaemia stem cell growth, thus we investigated DZNep for activity against primary CML stem cells.We aimed firstly to test DZNep for an ability to kill CD34$^+$ stem cells harvested from leucapheresis from CML patients at diagnosis in chronic phase and from non-CML leucapheresis as controls. We investigated cell proliferation and apoptotic cell death in CD34$^+$ stem cells. Cell counts and viability were determined by trypan blue dye exclusion to establish IC50. Annexin-V binding and caspase-3 activation were measured by flow cytometry as proof of induction of apoptosis. Carboxyfluorescein succinimidyl ester (CFSE) staining was performed to investigate the effect of DZNep on cell division, the surviving CFSEmax (undivided) cells were enumerated. DZNep was toxic to BAF3Bcrabl but not BAF3 parental cells at concentrations up to 1 micromolar. The IC50 for DZNep in CML stem cells was 100 nanomolar at 72h. Despite the selectivity against Bcrabl$^+$ versus Bcrabl- BAF3 cell line, Philadelphia negative CD34$^+$ cells were equally inhibited by DZNep. Moreover, the anti-proliferative effect first seen with imatinib, was also observed after 72 hours of DZNep treatment whereby 0.11% of input CML CD34$^+$ cells were arrested undivided in untreated wells as compared to 23.9% with 100 nanomolar DZNep. Nevertheless, we observed 29.9% apoptosis (Annexin-V / Viaprobe positive) in CML CD34$^+$ cells versus 17.2% in non-CML stem cells at 100 nM at the same time point. Further, caspase-3 activation in CML cells at 72h was twice the incidence seen in non-CML. Having confirmed that DZNep kills CD34$^+$ cells, we now plan to investigate the effect of DZNep on the most primitive, quiescent CD34$^+$38$^-$ CML stem cell populations.

0328

A SET OF NEW GENES THAT MAY BE INVOLVED WITH THE PROCESS OF MODIFICATION OF GRANULOCYTIC CELLS IN CHRONIC MYELOID LEUKEMIA

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Background. Although the exact action mechanism of the protein BCR-ABL is unknown, studies *in vitro* and in animal models showed that the activity of protein tyrosine kinase (TK), by itself, is sufficient to cause the development of chronic myeloid leukemia (CML). This process occurs through the activation of several pathways of signal transduction, leading to: dysregulated cell proliferation, reduced adhesion of leukemic cells in bone marrow stromal and reduction of the apoptotic response to mutagenic stimuli. Thus, the progression of the disease to accelerated phase or blast crisis may be associated with genomic instability. The use of methods to detect the difference in gene expression between CML and normal granulocytes could bring new insights in the understanding of these complex mechanisms, highlighting new therapeutic targets for the CML treatment. *Aims.* Evaluation of the differential gene expression between CML and normal granulocytes using the Subtractive Suppression Hybridization (SSH) and investigation of the involvement of a set of differentially expressed genes in modulating the development of CML. *Methods and Patients*. The SSH libraries were constructed using a pool of RNA of granulocytes extracted from CML patients and from healthy blood donors. The study was approved by the Ethics Committee of the University of Campinas and prior to the study, in all cases, informed consent was obtained. *Results.* The comparison between granulocytes of CML patients and control granulocytes showed 39 genes exclusively expressed in CML and 169 genes in controls. These genes were related to different metabolic pathways like protein ligation (DCN1, KPNA6, SETDB1, FAM63A, KMT1E), transcription factors, (SETDB1, SIM1, IPOA7), apoptosis (PNUTL1, CDC42SE1, SRGN), lipid metabolism (TIMM23), ATP ligation (MTND4L), DNA repair (TOB1) and differentiation/cellular proliferation (PNUTL1, GP1B, SNAG1, ANXA9, MIER). Among these genes, some may play important roles in the CML development, as MIER, TOB1 and SRGN. The Mier protein is part of a complex responsible for the proliferation of T lymphocytes linked to the progression of CML; the TOB1 gene is a transcription factor related to cell proliferation and is involved in the control of cell cycle and hematopoiesis; and the gene SRGN, which is a regulator of apoptosis and acts in the neutralization of hydrolytic enzymes. Some of these genes were described as the first time in CML and represent new target for functional studies. *Conclusions.* Some important genes expressed exclusively in the library of CML may be related to the development and progression of this disease. The results showed in this study amplify the data published previously and may present new clues about gene regulation and the dynamic organization of genes and chromosomes in cells, improving the comprehension of the CML disease and the identification of new target genes for therapeutic purposes.

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HETEROGENEOUS MECHANISMS AT THE BASIS OF 5'BCR/3'ABL FUSION GENE GENERATION IN CHRONIC MYELOID LEUKEMIA

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Background. The t(9;22)(q34;q11), generating the Philadelphia chromosome, is found in more than 90% of patients with chronic myelocytic leukemia (CML). About 5%-10% of CML patients show variant translocations, involving one or more chromosomes in addition to 9 and 22. In a subset of CML patients the 5'BCR/3'ABL fusion gene is generated by cryptic rearrangements such as nonreciprocal insertions between chromosomes 9 and 22. Moreover, microdeletions on the der(9) chromosome next to the t(9;22) breakpoint or on the third derivative chromosome involved in variant t(9;22) have been recently described and appeared to be a valuable prognostic factor. *Aims.* To our knowledge, an accurate frequency assessment of different mechanisms at the basis of fusion gene generation or of concomitant chromosomal rearrangements other than the t(9;22) has never been performed. The aim of this study was to perform a detailed molecular cytogenetic characterization of a large series of CML patients to unveil the biological heterogeneity of CML. *Methods.* 404 CML patients in chronic phase were analyzed by conventional cytogenetic analysis and by FISH experiments with *home-brew* probes specific for ABL and BCR genes. Breakpoints characterization and deletions size definition were carried out with additional bacterial artificial chromosome (BAC) and Phage P1-derived artificial chromosome (PAC) probes selected according to the University of California Santa Cruz (UCSC http://genome.ucsc.edu/index.html; March 2006 release) database. *Results*. This molecular cytogenetic study identified 43 (10.6%) out of 404 CML cases showing heterogeneous chromosomal mechanisms accountable for generation of the 5'BCR/3'ABL fusion gene. These cases could be classified in three main groups: i) cases with variant chromosomal rearrangements other than the classic t(9;22)(q34;q11) (8.9%), showing a *masked der(9)* (7.4%) or a *masked Ph* chromosome (1.5%); ii) CML cases with cryptic insertions of ABL into BCR, or vice

versa (1.5%); iii) CML cases bearing additional concomitant chromosomal rearrangements, apart from the generation of the 5'BCR/3'ABL fusion gene (1.2%). *Conclusions.* This study provides an outline of the frequency and molecular features of the most relevant cytogenetic groups identified in a very large series of CML patients at diagnosis. A clear division has been made between cases with variant t(9;22) rearrangements, cases with cryptic insertions generating the 5'BCR/3'ABL fusion gene, and patients with additional concomitant chromosomal rearrangement other than the presence of 5'BCR/3'ABL. However, the biological significance and the prognostic impact of the cytogenetic molecular heterogeneity occurring in the generation of the 5'BCR/3'ABL fusion gene remain to be clarified.

0330

EVALUATION OF DSBS REPAIR IN IMATINIB RESISTANT CELL LINES

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Background. Chronic myeloid leukemia (CML) is characterized by having a fusion gene, BCR-ABL, with constitutive tyrosine kinase activity. Standard treatment is the tyrosine kinase inhibitor Imatinib (IM). Several studies report that BCR-ABL expression decreases cytotoxicity associated with DNA damage, while others suggest it enhances cytotoxicity and may induce chromosomal instability/mutation accumulation. BCR-ABL increases reactive oxygen species production and also DNA double-strand breaks (DSBs), upon treatment with DNA-damaging agents. It has also been reported that treatment of CML cells with IM can modify DSBs repair as well. *Aim.* Since altered DNA repair may affect resistance to therapeutic agents, the aim was to evaluate DSB repair ability and apoptosis in IM resistant cell lines after exposure to the oxidant H_2O_2. *Methods.* The K562 cell line (CML cell line expressing BCR-ABL) was incubated with increasing concentrations of IM in order to create resistant cell lines, and 3 cell lines were isolated showing resistance to 0.5 μM, 1.0 μM and 5.0 μM. Simultaneously, control cultures were maintained. Detection of DSBs was carried out using FITC-antibodies for phosphorylated histone H2AX (gamma-H2AX). We incubated cells (control cells and IM-resistant cells) with H_2O_2 (100, 250 and 500 μM) for 1 hour. For each sample group a negative control was also prepared. Images from immunofluorescence analysis were captured using a fluorescence microscope and analysis was performed with the freeware Cellprofiler. Apoptosis was evaluated by the TUNEL assay. Cells were incubated with 250 μM of H_2O_2, for 1 hour, and cultured for an additional 6 hours before evaluating apoptosis. For this assay we used the four cell lines referred previously, as well as the corresponding controls. BCR-ABL expression was evaluated by RT PCR.

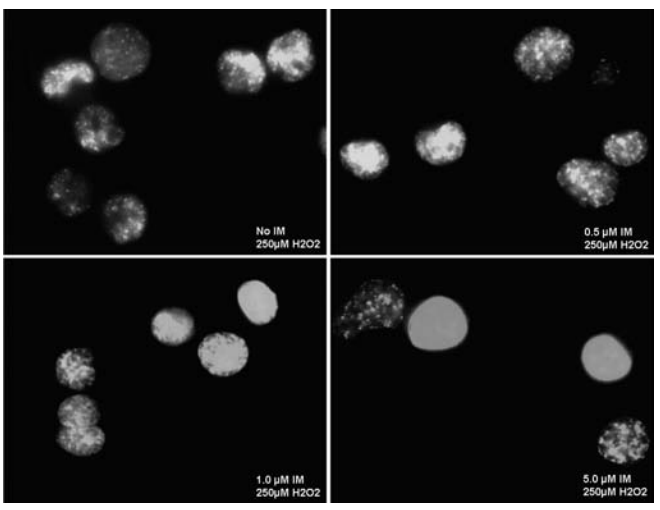

Figure 1

Results. Results suggest that IM-resistant cells are more sensitive to H_2O_2 damage. Comparing K562 negative controls vs. resistant negative controls we observed a slight increase in the basal level of gamma-H2AX foci with IM concentration. Additionally, the dose response curves showed a higher induction of gamma-H2AX foci in IM resistant cell lines compared to the K562 control cell line. Saturation of the gamma-foci was apparent at 250 μM H_2O_2. TUNEL assay did not show a significant variation on apoptosis in control and resistant cells incubated with H_2O_2. We did not find a significant variation in BCR-ABL expression in the different cell lines. *Summary and Conclusions.* IM-resistant cells showed higher levels of gamma-H2AX foci, indicating higher susceptibility to DNA damage upon exposure to H_2O_2 than non-resistant cells. In resistant cells, saturation of the gamma-H2AX signal was observed with lower concentrations of the oxidant, indicating either that these cells have a lower DNA repair capacity, or altered signaling in response to DNA damage. The increased number of DSBs with IM resistance does not seem to be associated with BCR-ABL expression, since its variation is not significant. We are evaluating other mechanisms of resistance in order to understand the increase of DSBs in IM-resistant cells.

0331

E T315I MUTATION CONFERS RESISTANCE AGAINST SMALL MOLECULES BY RESTORING THE ABL-KINASE ACTIVITY ACCOMPANIED BY TRANSPHOSPHORYLATION OF ENDOGENOUS BCR, EVEN IN LOSS-OF-FUNCTION MUTANTS OF BCR/ABL

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Philadelphia Chromosome (Ph) positive ALL and CML the fusion between BCR and ABL leads to the BCR/ABL fusion proteins, which induces the leukemic phenotype because of the constitutive activation of multiple signaling pathways down-stream to the aberrant BCR/ABL fusion tyrosine kinase. Targeted inhibition of BCR/ABL by ABL-kinase inhibitors induces apoptosis in BCR/ABL transformed cells and leads to complete remission in Ph-positive leukemia patients. However, a large portion of patients with advanced Ph^+ leukemia relapse and acquire resistance. Kinase domain (KD) mutations interfering with inhibitor binding represent the major mechanism of acquired resistance in patients with Ph^+ leukemia. The T315I mutations confers resistance against all actually approved ABL-kinase inhibitors. It seems not only to decrease affinity for kinase inhibitors but to confer additional features to the leukemogenic potential of BCR/ABL. To determine the role of T315I in resistance to the inhibition of oligomerization and in the leukemogenic potential of BCR/ABL, we investigated its influence on loss-of-function mutants with regard to the capacity to mediate factor-independence. Thus we studied the effects of T315I on BCR/ABL mutants lacking functional domains in the BCR portion indispensable for the oncogenic activity of BCR/ABL such as the N-terminal coiled coil (CC), the tyrosine phosphorylation site Y177 and the serine/threonine kinase domain (ST), as well as on the ABL-portion of BCR/ABL (#ABL-T315I) with or without the inhibitory SH3 (ΔSH3-ABL) domain. Here we report that i.) T315I restored the capacity to mediate factor independence of oligomerization-deficient p185BCR/ABL; ii.) resistance of p185-T315I against inhibition of the oligomerization depends on the phosphorylation at Y177; iii.) autophosphorylation at Y177 is not affected by the oligomerization inhibition, but phosphorylation at Y177 of endogenous BCR parallels the effects of T315I; iv.) the effects of T315I are associated with an intact ABL-kinase activity; v.) the presence of T315I is associated with an increased ABL-kinase activity also in mutants unable to induce Y177 phosphorylation of endogenous BCR; vi.) there is no direct relationship between the ABL-kinase activity and the capacity to mediate factor-independence induced by T315I as revealed by the #ABL-T315I mutant, which was unable to induce Y177 phosphorylation of BCR only in the presence of the SH3 domain. In summary our here presented data provide evidence that the T315I requires autophosphorylation at tyrosine 177 in the BCR-portion to mediate resistance against the inhibition of oligomerization and restores the capacity to mediate factor-independent growth of loss-of-function mutants due to an increase in or activation of ABL-kinase which is accompanied by phosphorylation of endogenous BCR, suggesting aberrant substrate activation by BCR/ABL harboring the T315I mutation. These data show that T315I confers additional leukemogenic activity to BCR/ABL, which might explain the clinical behavior of patients with BCR/ABL -T315I-positive blasts.

0332

SURVIVIN EXPRESSION IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS WITH IMATINIBE MESYLATE TREATMENT

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Background. Survivin protein is a member of the inhibitors of apoptosis protein (IAP) family that also regulates cell division. Present during fetal development and prominently expressed in cancer, survivin is undetectable in terminally differentiated adult tissues. Over 95% of patients with chronic myelogenous leukemia (CML) are Philadelphia chromosome positive [Ph^+] due to the translocation t(9;22)(q34;q11) resulting in the formation of BCR-ABL gene which codes for Bcr-Abl protein, a constitutively active tyrosine kinase. CML patients are treated with imatinib mesylate, an inhibitor of Bcr-Abl tyrosine kinase. Survivin was also found to be highly expressed in accelerated/blast phase (AP/BP) of CML but low in chronic phase (CP) and is regulated through Bcr-Abl/MAPK signaling at both transcriptional and posttranslational levels.[1] Aim of this study was to investigate SURVIVIN (SVV) and BCR-ABL expression in CML patients with imatinibe mesylate treatment in different phases of the disease. *Materials and Methods.* Twenty Ph^+ CML patients with fifty-seven blood samples (Twelve women and eight men, median diagnosis age 52, cytogenetic response: 63,2% complete, 6,2% partial, 2% minor, 12,3% minimal, 16,3% none) in different phases of the disease and twenty healthy control samples were investigated. Of these samples fifty were in CP and seven, in AP/BP. Total RNA was isolated from the blood cells. Then cDNA synthesis and quantitative real-time PCR with primers and hydrolysis probes for SVV, BCR-ABL and c-ABL (endogenous gene) sequences were performed. The quantification was relative with external standards and the SVV/c-ABL, BCR-ABL/c-ABL ratios were calculated. Results were statistically analyzed with T-test. *Results.* I. SVV expression is low-detected in healthy control samples (median ratio: 0,071) where BCR-ABL expression is absent. II. SVV expression is significantly higher (p<0,05) in CML patients in CP (median ratio: 0,17) in comparison with those in AP/BP (median ratio: 1,095) and follows BCR-ABL expression as it increases from CP (median ratio: 0) to AP/BP (median ratio: 0,66) (p<0,05). III. SVV expression is significantly higher (p<0,05) in CML patients in CP with complete cytogenetic response to treatment (median ratio: 0,178), where BCR-ABL expression (median ratio: 0) is undetected, in comparison with healthy controls. IV SVV and BCR-ABL expression are shown to be significantly higher (p<0,05) in CML patients in chronic phase with cytogenetic response less than complete to treatment (median ratios: 0,171 and 0,075) in comparison with healthy controls. But the comparison with the patients in complete cytogenetic response showed no significant difference. *Conclusions.* Survivin and Bcr-Abl expression follow the clinical expansion of CML with the first to be significantly higher in patients in CP with complete cytogenetic response to treatment.

Reference

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0333

AN APPROACH TO OVERCOME BCR-ABL INDEPENDENT STI571 RESISTANCE IN CML

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Background. Chronic- myelogenous leukemia (CML) is associated with high tyrosine kinase (TK) activity of the chimeric protein BCR-ABL. CML is treated with the TK inhibitor Imatinib (STI571), but in some cases STI571 resistance appears. BCR-ABL over expression, amplification or mutation in the kinase domain was shown. However, CML cells can evade the inhibitory effect of STI571 by different mechanisms, such as enhanced efflux by Multidrug Resistance P-glycoprotein (MRD-Pgp) or mutations in genes other than BCR-ABL. One possible approach to overcome STI571 resistance involves combination with Histone Deacetylase Inhibitors (HDACI). We have shown that Pivaloyloxymethyl butyrate (Pivanex), a HDACI, alone and combined with STI571, induced apoptosis, differentiation and reduced BCR-ABL protein levels in CML cell line (K562). *Aims.*Herein we study the mechanism of STI571 resistance in a CML STI571 resistant cell line and the effect of HDACI on these cells. *Methods.* We developed a STI571 K562 resistant cell line (K562-R) by gradually exposing the cells to elevated levels of STI571. The K562-R cells had an IC50 33 times greater than that of K562 wild type (K562-S) cells. The following assays were conducted: viability loss using Alamar blue test, cell cycle parameters by PI and Anexin V staining analyzed, respectively, on Flow-cytometer, erythroid differentiation by glycophorin-A and Tetramethyl Benzidine staining, BCR-ABL and signal transduction proteins by western blot analysis, bcr-abl and WT1 expression by RQ-PCR and mutation analysis, by chip-based matrix-assisted laser desorption-time-of-flight mass spectrometer (MALDI-TOF) for 45 previously reported different mutations that confer STI571 resistance. *Results.* K562-R cells had very low MDR-Pgp levels, high BCR-ABL protein and transcript levels same as K562-S. No mutations were found in the catalytic TK domain. However, K562-R cells demonstrated higher spontaneous apoptosis, G2M arrest and P21, a cell cycle regulatory protein, but lower S phase and WT1 levels. Glycophorin A expression was higher in K562-R cells although spontaneous hemoglobin synthesis was lower. A preliminary study of the signal transduction pathway showed elevated phosphorylated p38 in K562-R cells but no differences in p70S6, STAT3, Erk 1/2, CREB, Ikβ-α and JNK proteins levels (total and phosphorylated). Pivanex did not change BCR-ABL levels; p21 and differentiation were raised to a lesser extent. However, it induced viability loss, apoptosis and caspase activity enhancement in K562-R cells. *Summary*. We suggest that STI571 resistance mechanism in K562-R cells does not involve BCR-ABL but other regulatory mechanisms in cell cycle, differentiation and signal transduction pathways. The higher spontaneous G2M arrest, apoptosis and differentiation could be related to changes in p21 and p38 signaling pathways. Reduced WT1 levels in K562-R cells may also facilitate apoptosis induction in these cells and may explain both the relatively higher spontaneous apoptosis and the higher Pivanex induced apoptosis. K562-R with no change in BCR-ABL present a model for STI571 resistance in CML that does not involve BCR-ABL and a possible benefit of HDACI treatment in CML STI571 resistant patient with no mutation or changes in BCR-ABL protein.

0334

TARGETING CERAMIDE METABOLISM INCREASED SENSITIVITY OF PHILADELPHIA POSITIF CHRONIC MEGACARYOBLASTIC LEUKEMIA CELLS TO DASATINIB

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Background. Ceramide metabolizing genes have important functions in regulation of apoptosis, cell growth, proliferation, differentiation and senescent. While ceramide is a strong apoptotic molecule, conversion of apoptotic ceramide to antiapoptotic glucosylceramide by glucosyl ceramide synthase (GCS) results in resistance to anticancer agents. It was shown by different studies that GCS levels were increased in resistant cells and in patients resistant to different chemotherapeutic agents. Thus, inhibition of GCS or increasing intracellular concentrations of ceramides may sensitize cancer cells to anticancer agents. Dasatinib is a novel anticancer agent used for the treatment of Philadelphia chromosome positive chronic leukemias. *Aims.*In this study, we examined the possible outcomes of increased intracellular concentrations of ceramide through induction of *de novo* ceramide synthesis and inhibition of conversion of ceramide to glucosyl ceramide by a strong GCS inhibitor, PDMP, in human Meg-01 CML cells. *Methods.* The Ph^+ human Meg-01 cells were exposed to increasing concentrations of dasatinib, a ceramide analog (C8:ceramide) or PDMP. Then, we applied combinations of dasatinib and C8:ceramide or PDMP and cytotoxicity analyses were determined 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. *Results.* IC50 values of dasatinib and C8:ceramide or IC90 value for PDMP were found to be 4 nM and 70 μM or 50 μM, respectively. Combination of dasatinib with 70 μM C8:ceramide or 50 μM PDMP increased sensitivity of Meg-01 cells to dasatinib as compared to only dasatinib applied cells. There were 0-, 8-, 17-, 24-, and 46% decreases in cell proliferation in 1-, 5-, 10-, 20-, and 50 nM dasatinib applied Meg-01 cells, respectively, while the combination of these doses with 70 μM C8:ceramide resulted in 72-, 76-, 78-, 81-, and 82% decreases cell proliferation, respectively, as compared to untreated controls. In parallel experiments, we have shown that, application of the same doses of dasatinib with IC90 value of PDMP (50 μM) caused 67-, 76-, 77-, 78-, and 80% decreases in cell proliferation. There were 1,3- and 1,52 times increases in caspase-3 enzyme activity in 1- and 10 nM dasatinib treated Meg-01 cells while 70 μM C8:ceramide and 50 μM PDMP increased the loss of MMP 1.24 and 1.4 times, respectively. Combination of 1- and 10 nM dasatinib with 70 μM C8:ceramide or 50 μM PDMP increased caspase-3 enzyme activity 1.94-, and 3.58-, or 1.81-, and 4.38 times which is significant. Similar results were obtained for the changes in mitochondrial membrane potential. *Summary and Conclusions.* It was shown for the first time that manipulating ceramide metabolism to provide accumulation of intracellular ceramides increased cytotoxic effects of dasatinib on human Meg-01 chronic megacaryoblastic cells.

Chronic lymphocytic leukemia and related disorders - Biology I

0335

DELTA EX6, THE NOVEL TRANSACTIVATION-DEFECTIVE SPLICING VARIANT OF TP53, IS DIFFERENTIALLY EXPRESSED IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA AND CONFERS ACCENTED PROLIFERATIVE PHENOTYPE *IN VITRO*

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Background. The regulation of TP53 is substantially controlled at the transcriptional level. Nine different splicing variants of TP53 have been described so far, with distinct biological characteristics, resulting from combinations of an alternative splicing of intron 2, 9 and/or aberrant transcription, starting at so-far unrecognized cryptic promoter in intron 4. We have recently identified a novel splicing variant of TP53 gene, termed delta ex6, lacking the whole coding sequence of exon 6 and harboring an insertion at the exon9/10 boundary. The delta ex6 variant is differentially expressed in patients with chronic lymphocytic leukemia (CLL) as compared to healthy donors. Delta ex6 variant is devoid of transactivational activity as determined *in vitro* by FASAY (Functional Analysis of Separated Alleles in Yeast). *Aims.*The goal of the work was to test the biological properties of the novel TP53 transcript variant delta ex6 *in vitro*, using a TP53 double-knock-out model cell line H1299 with a stable integrated Tetracycline repressor (H1299TetR). *Methods.* We have cloned the whole delta ex6 coding sequence into a Tet-ON pcDNA4/TO vector and transfected the H1299TetR cell line to produce stable integrants. Four stable delta ex6 producing H1299TetR cell lines, as well as control cell lines in doublets (parental H1299TetR harboring the pcDNA4/TO cloning vector, and H1299TetR stably transfected with wild type TP53) were subjected to the Affymetrix GeneChip Human Exon 1.0 ST whole genome expression analysis. *Results.* Stable H1299TetR cell lines expressing the delta ex6 variant were distinguished by a remarkable loss of intercellular contacts and semi-suspension growth properties, in contrast to the strictly adherent growth of the parental cells and mock-transfected cells. The microarray data corroborated the accented and proliferative phenotype as observed *in vitro* in the tissue culture: overexpression of a number of cyclins (A1, G1, G2, F, I, B2, A2, T2), matrix metalloproteinases, hyaluronidases and caspase inhibitors; and downregulation of adhesion molecules and molecules of the intercellular matrix. *Summary*. Our data on the presence of the delta ex6 TP53 variant in CLL patients supports the recent evidence on dysregulation of TP53 splicing pattern in malignancies. Moreover, as assessed *in vitro*, overexpression of the delta ex6 variant leads to an accented and proliferative phenotype, a finding further supporting the biological role of the novel delta ex6 TP53 variant *in vivo*.

0336

THE TUMOR SUPPRESSOR MECHANISM IN 13Q14.3 INVOLVES MONOALLELIC EXPRESSION, NON-CODING RNA GENES AND EPIMUTATIONS AFFECTING THE MAJORITY OF CLL PATIENTS

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Background. Deletions in chromosomal band 13q14.3 occur in a variety of human neoplasms like chronic lymphocytic leukaemia (CLL), indicating a tumor suppressor mechanism (TSM) in this region. Intriguingly, several characteristics of the region of interest point to an epigenetic pathomechanism: a) candidate protein-coding genes and non-coding RNA genes including miR15a and miR16-1 lack point mutations in the majority of patients, yet b) these genes are significantly downregulated in almost all CLL patients, c) the presence of large non-coding RNA genes in 13q14.3 is reminiscent of imprinted regions where only one gene copy is active. We have recently shown that already in healthy tissue only one gene copy of 13q14.3 is active while one gene copy is randomly chosen for silencing. *Aims.*To identify an epimutation in the critical region in 13q14.3 in CLL patients. *Methods.* In order to elucidate the epigenetic regulatory mechanism, we analysed DNA- and histone-methylation of all CpG islands in the region in non-malignant B-cells and CLL cells. To this end, we used aPRIMES and ChIP-qPCR as screening

tools, BioCOBRA as a quantitative high-throughput method and bisulfite sequencing for validation. *Results.* We could identify two candidate regulatory elements with abnormal chromatin in CLL patients (n=80, median 57% DNA-methylation, range 0-100%) as compared to healthy probands (n=20, median 88% DNA-methylation, range 74-100%, $p<0.003$). Interestingly, this epimutation can be found in all cytogenetic subgroups of CLL patients and is independent of IgV(H) mutation status, making it a prime candidate for an underlying epigenetic defect in CLL. Pilot studies suggest that this epimutation regulates gene expression of the critical region via large non-coding RNA genes. *Conclusions.* We propose a model for the TSM in 13q14.3 where i) in healthy B-cells, only one gene copy is active while the second is epigenetically silenced, ii) 13q14.3 harbors an epimutation that is present in all cytogenetic subgroups of CLL patients and that iii) deregulates expression of long ncRNA genes directly and protein-coding and miRNA candidate tumor suppressor genes indirectly.

0337

INTEGRATIVE HIGH-RESOLUTION MICROARRAYS APPROACH IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA REVEALS THE PRESENCE OF MOLECULARLY DISTINCT SUBGROUPS IN PATIENTS WITH 13Q DELETION

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is the most recurrent leukemia in the western world, with a highly variable clinical course that reflects its heterogeneous genomic pattern. Aims. To define clusters of patients based on their genomic profiles and identify gene expression patterns specifically associated to them. *Methods.* We applied SNP array technology (Affymetrix GeneChip® Human Mapping 250K Nsp) to characterize allelic imbalances in a panel of 100 newly diagnosed, untreated B-CLL patients in early stage disease (Binet stage A). We performed an integrative approach between whole genome and gene expression profiling data (Affymetrix GeneChip® HG-U133A) for 60 patients for whom RNA material was available. Results. The non-negative matrix factorization (NMF) algorithm allowed the identification of four significant clusters (correlation coefficient = 0.95), mainly driven by the major chromosomal alterations. Groups I and II, containing respectively 27 and 13 cases, were characterized by the presence of 13q14 deletion, stratified into the two groups based on the deletion size and the presence of biallelic deletion; group III consisted of all the 21 patients with trisomy 12; the remaining 39 patients, among which patients showing 11q (8 pts) and 17p (4 pts) deletions without other well known aberrations, were placed in group IV. A SAM multi-class analysis between the four groups identified sixty well-characterized differentially expressed genes, with a prevalent deregulation of genes located at 13q14 (8%) and on chromosome 12 (60%) (group I, II and III, respectively), whereas no specifically modulated genes were recognized in group IV. As regard the putative functional features of the deregulated genes, we found a significant involvement in transcription regulation (9/60 = 15%); regulation of apoptosis (6/60 = 10%), among which 3 genes mapping at chromosome 12 (DYRK2, TEGT and BTG1) and 1 at 13q14 (TPT1); glycolysis (3/60 = 5%) and negative regulation of cell cycle (3/60 = 5%), all located on chromosome 13 (TRIM13, RB1 and DLEU1) and already described by others as involved in B-CLL pathogenesis. Conclusions. The natural grouping of genome profiles reveals that the complex scenario of copy number alterations affecting B-CLL is mainly driven by the presence of deletion at 13q14 (groups I and II) and trisomy of chromosome 12 (group III). The prevalent deregulation of genes located in these regions suggests that the modulation of gene expression observed in the comparison of the 4 groups could be mostly due to a gene dosage effect. These data further suggest that the deregulation of genes involved in fundamental biological functions, as a result of genomic alterations, may play an important role in the molecular pathogenesis of B-CLL.

0338

PROTEIN KINASE C INHIBITION WITH ENZASTAURIN PRIMES CHRONIC LYMPHOCYTIC LEUKEMIA CELLS FOR DEATH THROUGH DOWN-REGULATION OF MCL-1

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Background. Chronic Lymphocytic Leukemia (CLL) cells have many defects in the apoptotic machinery, among whom Mcl-1 plays a critical role, leading to drug resistance and, subsequently, poor clinical outcome. Aims We assessed the influence of Enzastaurin (an orally available PKC inhibitor) on CLL survival, based on emerging evidences that PKCβ does play a critical role in the establishment and maintenance of leukemic cells in transgenic mice models of CLL. *Methods.* Enzastaurin dose-effect (range 1-20 μM) was measured in 20 CLL samples, and correlated to Binet's stage, IgVH mutational status, caryotype/FISH and CD38 expression. Results Mean IC50 of 16/20 responding CLL patients was 6.9 μM (95% CI 3.6-10.4 μM), while sparing normal Peripheral Blood Lymphocytes. These data are reminiscent with published IC50 in Waldenstrom's macroglobulinemia cell lines, but lower than IC50 observed in CLL cell lines JVM3 and MEC2 (respectively 15 and 10 μM). Inhibition of phospho-serine 9-Glycogene Synthase Kinase 3β phosphorylation (see below as GSK3β activation), was also used as a surrogate marker for Enzastaurin activity. Enzastaurin induce significant GSK3 activation, except in the 20% resistant samples (including two 6q- and two 11q-deleted patients). A specific phosphatise assay demonstrated that Protein Phosphatase 2A (PP2A)-induced dephosphorylation of GSK3 was triggered by Enzastaurin in a dose-dependent manner, and its inhibition by 5 nM Okadaic Acid prevented totally GSK3 activation and partially cell death induced by Enzastaurin. As GSK3 regulates Mcl-1 expression through regulation of proteasome-mediated degradation, the influence of Enzastaurin on Mcl-1 expression was investigated. Pre-treatment with 5 μM Enzastaurin for 24 hours induced a decrease in Mcl-1 expression level in enzastaurin-sensitive samples. In these samples, Mcl-1 downregulation preceded apoptotic features, including cytochrome c release as well as caspase 3 and PARP cleavage. Finally, in 4/5 cases tested, we observed that Enzastaurin (E 5 μM) sensitized Rituximab (RTX 10 microgram/ml)-induced B-cell apoptosis (as measured at 24h by Annexin V binding : RTX alone 2-5% vs. RTX+E 15-55%, $p<0.05$) in peripheral mononuclear cells from gradient density-purified CLL blood samples, this effect being correlated with both GSK3 activation and, most importantly, Mcl-1 down-regulation. As Mcl-1 controls fludarabine resistance, combination of 5 μM Enzastaurin (E) and 1 μg/mL Fludarabine (F) for 4 days were assessed in 5 patients for synergistic activity. Yet, an additive but not synergistic effect was found, as evaluated by cell counting and PARP cleavage (living cells : F alone 74.4±20% vs. F+E 48.8±12.8%, two-sided student $p=0.016$). *Conclusion*. Our study shows for the first time that CLL cells are generally sensitive to enzastaurin, and that this drug should be tested alone or in combination with Rituximab. Effects of Enzastaurin are largely p53-independent, as 4/5 patients with 17p deletion were found to be good responders.

0339

ROLE OF CD38 IN HOMING OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Chronic lymphocytic leukemia (CLL) is clinically heterogeneous, with the presence of two distinct subsets of patients characterized by different survival, propensity to grow according to microenvironmental signals and overall response to treatment. A limited number of molecular markers have been identified so far to distinguish these two extremes. Aggressive CLL is defined by the absence of mutations in the IgVh genes, expression of the surface molecule CD38 and of the cytoplasmic kinase Zap70. Human CD38 is a glycoprotein, simultaneously acting as an ectoenzyme and a receptor. The products of the enzymatic activity are

involved in the regulation of cytoplasmic Ca^{2+} levels, while the receptorial functions lead to activation/proliferation and migratory signals in T, B, NK and dendritic cells. Further more, the interaction with CD31, the only non-substrate ligand for CD38, controls an active signaling pathway in circulating and residential lymphocytes. We reported that CD38 acts as a receptor on CLL cells mediating proliferation and survival and modulating the expression of a set of genes involved in migration and signaling pathways. Furthermore, the simultaneous expression of CD38 and Zap70 in neoplastic B cells identify a subset of cells functionally and genetically distinct from all other subsets and characterized by a higher migration and signalling ability in response to CXCL12, a critical chemokine in CLL homing from blood to lymphoid organs. The aim of this work is to explore the role of CD38 in CXCL12-mediated homing of CLL cells. The ultimate goal is to define the mechanisms regulating CLL re-circulation from blood to lymphoid organs, an essential step in the maintenance and progression of the disease. The results obtained analysing a large cohort of clinically and molecularly characterized patients indicate that i) the concurrent expression of CD38 and Zap70 defines a subgroup of patients endowed with the highest migratory potential in response to CXCL12; further, ii) the $CD38^+$/$Zap70^+$ clones are able to induce a significantly phosphorylation of Erk1/2, compared to the negative counterpart, after exposure to the chemokine, and iii) the interactions CD31/CD38 activate genetic pathways involved in proliferation and migration. Further confirmation of the functional link between CD38 and CXCR4 has been obtained evaluating the response of CLL cells to CXCL12 in presence of blocking monoclonal antibodies (mAbs). Pre-treatment with anti-CD38 mAbs resulted in a decreased response to the chemokine in terms of Erk1/2 phosphorylation and chemotaxis. Similar results have been obtained also in an *in vivo* model, using NOD/SCID mice: pre-treatment of CLL cells with anti-CD38 mAbs resulted in a reduced homing to spleen and bone marrow. Taken together, the results so far obtained indicate that CD38 play a crucial role in modulating CXCR4 signalling pathway, exerted also through a physical association on CLL cells, and represent a novel mechanism in the regulation of lymphocyte chemotaxis and homing. Furthermore, they represent preliminary data supporting the hypothesis that CD38 may be used in CLL as a target for human specific mAbs.

0340

THE FREQUENCY OF CIRCULATING CLL-LIKE B-CELL CLONES IN OTHERWISE HEALTHY INDIVIDUALS SIGNIFICANTLY INCREASES USING A HIGH-SENSITIVE MULTICOLOR FLOW CYTOMETRY APPROACH

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Background. Monoclonal B-cell lymphocytosis (MBL) is the international consensus term proposed to indicate the presence of low numbers of circulating monoclonal B cells (<5×10^9/L) in otherwise healthy individuals. Whether MBL represent an early stage of a chronic B-cell malignancy particularly chronic lymphoid leukemia (CLL), as in a large proportion of MBL cases, cells have a CLL-like phenotype is still unknown. Recently, it has been reported that circulating CLL-like B cells can be detected using 4- or 5-multicolor flow cytometry in 5%-7% of adults with normal lymphocyte counts. *Aim.* to investigate the frequency of circulating monoclonal B cells in a series of healthy adults who belong to a population living in Salamanca (Western Spain), by using a high-sensitive multicolor flow cytometry approach. *Methods.* A total of 608 healthy individuals (47% m / 53% f) older than 40 years (62±13 years) with normal lymphocyte counts ($2.2\pm0.7\times10^9$/L) were randomly recruited from the Primary Health Care system region of Salamanca. EDTA-anticoagulated peripheral blood (PB) samples were immunophenotyped using a high-sensitive flow cytometry approach, based on 8-color staining panels and the systematic screening for >5x106 total PB leucocytes. Analyses of genetic abnormalities and IgH gene rearrangements were performed on FACSorted clonal B cells (purity ≥98%) by conventional iFISH and PCR amplification/sequencing, respectively. *Results.* the frequency of PB monoclonal B cells was markedly higher than previously reported (12% for CLL-like B cells, found at frequencies of $0.17\pm0.13\times10^9$ cells/L); interestingly, the incidence progressively increased with age (5%, 5%, 14%, 19%, 23% and 50% of individuals aged 40-49, 50-59, 60-69, 70-79, 80-89 and >90 years, respectively), while no association ($p>0.05$) was found between age and the size of the clone, or between the presence of circulating clonal B-cells and gender (11% of females versus 13.8% of males). In 62% of cases the infiltration was below the maximum sensitivity of the techniques described by others (<0.01%), at median frequencies of 0.38% of all PB B cells (interquartile range: 0.14%-4.2%). Clonal origin of these B-cell expansions was confirmed (n=15) by the presence of either clonal IgH gene rearrangements or the presence of genetic abnormalities. *Summary and Conclusions.* We show that the frequency of PB monoclonal B cells in healthy adults individuals >40y with normal blood counts is markedly higher than that previously reported (12% for CLL-like B cells), due to the higher sensitivity of the flow cytometry approach here applied. As it has been suggested that the fundamental biologic issue is the presence of a clonal population, and no cutoff point ensures 100% efficient discrimination of individuals at risk of progression, the precise identification of cases having circulating clonal B cells becomes a key question.

0341

THE LOCAL MICROENVIRONMENT PLAYS A DIFFERENT ROLE IN THE SURVIVAL OF TUMOR CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH MUTATED AND UNMUTATED IMMUNOGLOBULIN (IG) VH GENES

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Background. Chronic lymphocytic leukemia (CLL) is a clinically heterogenous disease. The mutational status of the tumor immunoglobulin heavy chain variable region (IgVH) is a well established prognostic marker: patients with unmutated (UM) IgVH have a bad prognosis, whereas patients with mutated (M) IgVH have a good prognosis. It has already been reported that soluble factors (i.e. IL-4 and CD40L) and cellular components of the local microenvironment [i.e. bone marrow stromal cells (BMSC) and peripheral blood T cells (PBT)] are important survival factors for CLL B cells. It is currently unknown to what extent tumor cells from UM and M CLL patients depend on the local microenvironment for their survival. *Aims.* The aim of this work was to evaluate the different level of dependency of UM and M CLL cells from survival factors of the tumor microenvironment. *Methods.* M and UM CLL cells negatively selected by magnetic beads isolation were cultured in standard medium in the presence or in the absence of IL-4, CD40L, murine stromal cells (M2-10B4), CLL-derived BMSC and autologous T lymphocytes. Apoptotic and necrotic cells were quantified by annexin V and propidium iodide (PI) staining. The intracellular expression of Bcl2 and the nuclear translocation of NFkB were evaluated by flowcytometry and by EMSA, respectively. *Results.* Leukemic cells purified from the peripheral blood of UM CLL patients showed a significantly higher apoptotic rate than leukemic cells of M patients. Both M and UM CLL cells showed high level of expression of Bcl-2 and NF-kB soon after purification. *in vitro* spontaneous apoptosis of UM B-CLL cells was associated with a progressive time-dependent downregulation of the intracellular expression of Bcl2 and with a complete loss of the active nuclear form of NF-kB. On the contrary, the higher long term viability of M CLL cells was paralleled by the maintenance of Bcl2 and NF-kB expression. We next investigated whether the enhanced pro-apoptotic tendency of UM CLL cells could be reverted by extrinsic survival factors. We found that IL-4 and CD40L, used alone or in combination, as well as murine and human BMSC were capable of rescuing UM tumor cells from apoptosis. The pro-survival effect of these stimuli was exerted through the upregulation of Bcl-2 and was totally independent from the recovery of NF-kB expression. A pro-survival effect on UM CLL cells was also exerted by autologous T cells. Indeed, the cocolture of B and T cells at different ratios showed that the presence of sufficient numbers of viable T cells could prevent UM CLL cells apoptotic death by restoring the expression of the nuclear form of NF-kB and of Bcl2. *Conclusions.* These data indicate that the survival of tumor cells from UM CLL patients is dependent on the local microenvironment. On the contrary, tumor cells from M CLL patients are intrinsically more resistant to apoptosis and minimally influenced by the local microenviroment. This higher dependency of UM CLL cells from extrinsic signals might be exploited to develop new therapies targeting the tumor microenvironment to potentially improve patient outcome in more aggressive CLL.

0342

GENOME-WIDE DNA COPY NUMBER ANALYSIS BY SNP ARRAYS OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA REVEALS THE PRESENCE OF NOVEL MOLECULAR LESIONS

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is a genetically heterogeneous disease with a variable clinical course. Chromosomal changes have been identified by FISH in approximately 80% of patients, and the presence of specific lesions, such as trisomy 12 and 13q14, 11q23 and 17p deletions have been proven to be prognostic markers for disease progression and survival. Aims. To provide insights into the genomic complexity of B-CLL. *Methods.* A panel of highly purified neoplastic cells (>92%) from 100 untreated, newly diagnosed patients in Binet A was investigated. This series was characterized by fluorescence in situ hybridization (FISH) for the most recurrent genomic aberrations (trysomy 12, 13q14, 11q23 and 17p13 deletions) and for the major prognostic markers. Genome-wide profiling data were generated by means of Affymetrix GeneChip® Human Mapping 250K Nsp single nucleotide polymorphism (SNP) arrays. Copy number alterations (CNA) were calculated using the DNA copy Bioconductor package, which looks for optimal breakpoints using circular binary segmentation (CBS) (Olshen *et al.*, 2004). Results. FISH identified the presence of trisomy 12 in 21 cases; 13q14 deletion in 44 cases, 34 as the sole abnormality; 11q23, 17p13.1 and 6q23.3 in 15, 7 and 2 patients, respectively. In addition, ZAP-70 and CD38 expression resulted positive in 42 and 46 patients, whereas IgVH genes were mutated in 45 patients. The genome-wide analysis allowed the identification of CNAs in all cases. A total of 782 aberrations (from 1 to 31 per sample, mean and median values 7.82 and 7, respectively) were detected; losses (365/782=46.7% loss; 194/782=24.8% biallelic deletion) were found to be more frequent than gains (148/782=18.9% gain; 75/782= 9.6% amplification). The most recurrent alterations detected by FISH were all confirmed by SNP array analysis, strengthening further the good reliability of such high-resolution technology. We identified a total of 16 minimally altered regions (MARs) larger than 100 kb with a frequency higher than 5%. Among well known alterations, the largest was represented by chromosome 12 trisomy, followed by 6q, 17p and 11q23 deletions (32.87, 19.09 and 10.43 Mb, respectively) and 13q14 deletion (635 kb). Gain of 2p extended to almost the whole short arm of chromosome 2 in 6 cases, with smaller regions of gain encompassing cytobands 2p25.3-p25.2, 2p16.3-p16.2, 2p16.1-p15 and 2p12 in 5 additional patients. Among those alterations previously described in B-CLL, we found losses at 14q32.33 (12 pts) and 22q11.2 (5 pts) involving the IGH and IGLĬ loci, respectively. With regard to novel regions, we identified losses at 4q35.2 (5 pts), 8q24.23 (6 pts) and 11q25 (6 pts). In addition we found a high frequency of losses/gains at 14q11.2 (42 pts) and 15q11.2 (33 pts), two genomic regions reported to be affected by DNA copy number variations. Conclusions. Our data indicate that genetic abnormalities involving chromosomal gains and losses are very common in early-stage B-CLL and further support the application of high resolution SNP array platforms in the characterization of genetic changes in the disease. In addition we detected novel altered chromosomal regions that warrant future investigations to better define their pathogenetic role in B-CLL.

0343

THE SYK INHIBITOR R406 BLOCKS BCR SIGNALING, CELL ADHESION AND MIGRATION, AND INDUCES APOPTOSIS IN CLL CELLS: AN EXPLANATION FOR THE CLINICAL ACTIVITY OF R406 IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B cell receptor- (BCR-) signaling is increasingly recognized as factor promoting the expansion of Chronic Lymphocytic Leukemia (CLL) cells in tissue microenvironments. The spleen tyrosine kinase (Syk) is a key element of BCR signaling, and therefore represents a novel therapeutic target. R406, an ATP-competitive Syk inhibitor, displays clinical activity in CLL, characterized by transient increases in circulating CLL cells, and subsequent remissions in a significant number of patients (Friedberg J. *et al.* Ann Oncol. 2008 Jun; 19 Suppl 4). These findings suggest that R406 induces CLL cell mobilization form tissue compartments to the blood, and then induces CLL cell death. *Aims.* Because the effects of Syk on CLL cell migration, and BCR-dependent activation and survival are largely unknown, we examined the effects of R406 on CLL cell chemotaxis, expression of adhesion- and co-stimulatory-molecules, and survival. *Results.* Activation of BCR with polyclonal goat F(ab')2 fragments to human IgM significantly increased chemotaxis towards CXCL12 to 135.4±5.2% of controls and towards CXCL13 to 170.5± 31.5% of controls (Mean ± SEM, n=15). Engagement of BCR also significantly increased expression of CD40, CD44, CD54, and CD62L. Pretreatment with R406 abrogated the increased chemotaxis, and adhesion- and co-stimulatory molecule expression. BCR triggering also increased the viability of CLL cells in the majority of cases, preferably in ZAP-70$^+$ samples, and this pro-survival response was completely abrogated by R406. Co-culture with nurselike cells (NLC) represents an *in vitro* model for CLL cell interactions with their microenvironment, in which various pathways, including the BCR, become activated. We found that NLC-dependent CLL cell survival and anti-IgM- and NLC-dependent induction of the chemokines CCL3 and CCL4 in CLL cells was blocked by R406. Furthermore, R406 blocked BCR-induces phosphorylation of Syk, AKT and p44/42 MAP. *Conclusions.* Collectively, this study demonstrates that BCR engagement increases CLL cell migration, adhesion- and co-stimulatory-molecule expression, and survival. R406 effectively antagonizes these responses and induces CLL cells apoptosis in suspension and co-culture with NLC. The inhibitory effect of R406 on adhesion molecule expression and chemotaxis could be the reason for the transient mobilization of CLL cells into the blood of patients treated with R406, suggesting that our findings provide an explanation for the clinical activity of R406 in CLL patients.

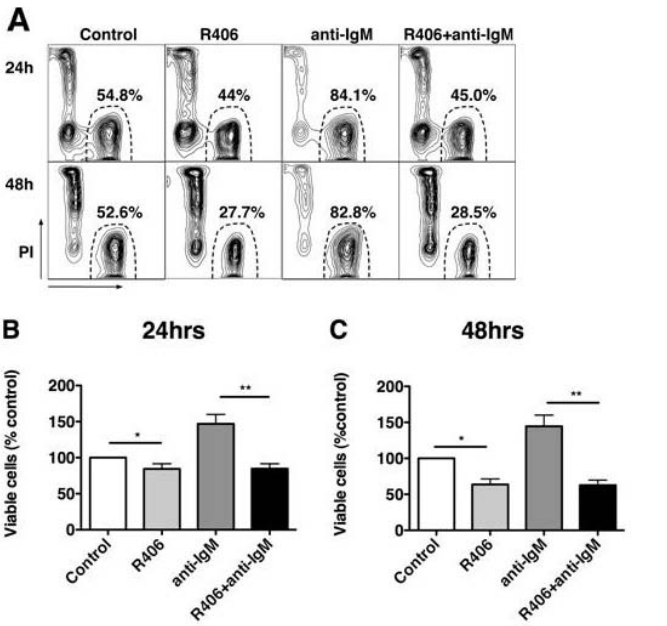

Figure 1. Effect of R406 on CLL cell viability.

0344

MICRORNAS EXPRESSION PATTERN IN AGGRESSIVE SUBTYPE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. MicroRNAs (miRNAs) are small RNA molecules involved in the regulation of cell differentiation, proliferation and apoptosis. miRNAs expression was shown to be associated with known prognostic markers in CLL (deletion of 13q14, expression of ZAP70, unmutated IgVH, mutated IgVH). *Aims.* Although, recent publications demonstrat-

ed the involvement of miRNAs in the pathogenesis of CLL, the role of miRNAs in prognostically unfavourable disease has not been clearly defined. Thus, we examined miRNA expression patterns in aggressive CLL subtype harboring a deletion and/or mutation of the p53 gene (TP53) compared to wild-type p53 samples (mutated and unmutated IgVH). *Methods.* Micro-arrays (LNA oligos-Exiqon, EMBL) and Real Time-PCR (TaqMan MiRNA Assays, ABI) were used to define the miR expression pattern in CLL cells. MicroArrays (470 miRNAs) were performed for 11 CLL samples (del./mut. TP53 n=6, wt TP53 n=5) and Real Time-PCR for 30 CLL samples (35 miRNAs; del./mut. TP53 n=12, wt TP53 n=18). MicroRNAs expression was also studied in 6 control $CD19^+$ B cell samples from tonsils (after tonsillectomy). All control tonsillar and peripheral blood samples from patients were separated by RosetteSep Human B Cell Enrichment (purity ≥95% of $CD5^+19^+$ CLL cells; ≥95% of $CD19^+$ control B cells) *Results*. Using micro-arrays we identified 14 microRNAs down-regulated in CLL cells harboring deletion and/or mutation of p53 gene. Interestingly, no miRNAs were up-regulated in these samples compared to wild-type p53 cells. Real-time PCR was performed for miRNAs identified by microArrays and miRNAs added based on literature data and target prediction. By RT-PCR we observed a statistically significant down-regulation of three microRNAs, miR-34a, miR-29c and miR-17-5p, in the TP53-abnormal samples ($p<0.05$). Interestingly, these miRNAs target genes which were previously proposed to be involved in CLL pathogenesis or cell cycle regulation. miR-34a has been reported to regulate Bcl2 and to be directly regulated by p53 protein in response to genotoxic stress. Furthermore, miR-29 regulates the expression of TCL1 and MCL1 (anti-apoptotic Bcl2 family member) in the CLL cells. On the basis of animal models, deregulation of the TCL1 and MCL1 oncogenes was suggested to be important in the CLL pathogenesis. The third differently expressed miRNA, miR-17-5p, is a part of the c-myc regulated cluster (miR-17-92) and targets E2F1, p21 and Cyclin D1. MiR-17-5p also acts at the G1/S-phase cell cycle checkpoint by targeting genes involved in this transition. Moreover, miR-17-92 is included in the c-myc-mediated repression of hypoxia-inducible factor-1a (HIF-1a). HIF-1a plays a role in cell proliferation during hypoxia, and its upregulation in CLL bone marrow biopsies was linked with microvessel proliferation. Interestingly, for other hypoxia-associated miRNA family miR-23 we observed a higher expression in the unmutated IgVH samples. *Conclusions.* We describe a complex miR expression pattern in patients with deleted and/or mutated p53 gene compared to other CLL subtypes. MicroRNAs deregulated in aggressive CLL subtype target genes, which were previously proposed to be involved in CLL pathogenesis or cell cycle regulation suggesting their possible role in disease progression/pathogenesis.

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0345

IDENTIFICATION OF NOVEL RECURRENT COPY NUMBER VARIATIONS BY HIGH RESOLUTION COMPARATIVE GENOME HYBRIDISATION

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Background. B-cell chronic lymphocytic leukaemia (B-CLL) is the most common form of adult leukaemia in the Western World. It is a heterogeneous disease and important biological and clinical differences have been identified. However, many questions remain about the molecular abnormalities underlying the genomic complexity of the disease. The technique of array based comparative genomic hybridization (aCGH) has revolutionized our ability to perform genome wide analyses of copy number variation (CNV) within cancer genomes. Most abnormalities noted previously have been very large, making it difficult to pinpoint specific candidate genes[3DOTS] By contrast, very small CNVs involving fewer genes are likely to have passed undetected due to insufficient array resolution. *Aim.* We therefore wanted to test a high resolution CGH array platform for its ability to detect small copy number variations (CNV). Method. To investigate this in more detail, we used a high resolution 244K aCGH platform to test and characterize enriched B-CLL peripheral blood samples (>80% $CD19^+$; $CD5^+$) from 40 clinically annotated patients collected at our institution. To distinguish CNVs seen commonly in the general population the results were compared with 'in house' control data sets and the Database of Genomic Variants (http://projects.tcag.ca/variation/. *Results.* Our results show that large abnormalities, already noted by FISH, were reliably identified and the boundaries of abnormalities at 11q22.3, 13q14.2 and 17p could be defined more precisely. In addition, novel and recurrent CNVs within the sample set were identified (1p33; 3p24.3; 3p14.2; 4q12; 4q13.3; 6q21; 6q27; 8p22; 10q24; 11p15.4; 11q12; 11q13.4; 11q14.1; 11q22.1; 11q23.3; 13q14.11; 14q21.1; 15q15.1; 15q25.3; 17p13.3; 17q22; 18p11.32; 18p23; 19p13.13; 19p13.12; 19p13.32; 22q11.21; 22q11.22). Interestingly, some of these abnormalities contain single gene alterations involving oncogenes, chemokine receptors, kinases and transcription factors important in B cell development and differentiation such as ETS-1. Assessment of smaller CNVs (less then 10 consecutive oligonucleotides), also revealed recurrent CNVs involving single genes, including gains of MAP2K5 (15q23) and NFKB activator (18q21.33) in the majority of samples. We are currently in the process of clustering single gene abnormalities into different groups according to gene function and pathways, ie oncogenes and tumour suppressors, receptors, signaling molecules, B-cell transcription factors, genes encoding for microRNAs and others. *Conclusions.* To date, the results suggest that genes belonging to pathways important for B cell development and differentiation are severely affected by the genomic instability observed in B-CLL samples. This work was supported by the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.

0346

VASCULAR ENDOTHELIAL GROWTH FACTOR ACTS VIA AUTO- AND PARACRINE MECHANISMS AS A CRITICAL MICROENVIRONMENTAL FACTOR FOR THE SURVIVAL OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Background. Over the last years studies giving attention to the vascular endothelial growth factor (VEGF) status in chronic lymphocytic leukemia (CLL) have been published frequently and clearly indicated a role of VEGF in CLL. Whereas most of those studies were solely descriptive by focusing on the correlation of the VEGF status to patients´ characteristics and clinical features, actual functional data are missing. *Aims*. To describe the effect and functional background of VEGF on CLL cells and to further identify a potential involvement of the bone marrow microenvironment. *Methods*. Primary cells from either CLL patients or healthy individuals were used. Gene transcription status was analysed by real time PCR and protein expression levels by immunoblotting. Secreted VEGF was measured by ELISA. Phosphorylation of the VEGFreceptor2 was analysed using intracellular phospho flow cytometry. Cell survival was assessed by annexin V/PI negativity. VEGF-specific siRNAs were used to knock down VEGF in HS5 cells. *Results*. CLL and healthy B-cells produce VEGF, but only CLL cells seem to secrete it. Moreover, we found the VEGFreceptor2 to be phosphorylated to a higher extent in CLL than in healthy B-cells. Stimulation of CLL cells with rhVEGF enhanced the VEGF expression in an autocrine fashion. VEGF stimulation went along with increased expression of the antiapoptotic proteins Mcl1 and XIAP. We used the bone marrow stromal cell line HS5 to partly mimic the *in vivo* microenvironment of the CLL cell. HS5 themselves produce and secrete VEGF to a high extent. HS5/CLL coculture lead to an increase of VEGF expression in CLL cells. Furthermore, coculturing enhanced CLL cell survival an average of 28% after 72h of *in vitro* culture over monoculture, clearly indicating a prosurvival effect. Neutralization of VEGF using an anti-VEGF antibody resulted in a reduction of the coculture-mediated survival advantage in CLL cells by about two/thirds. Additionally, a coculture of CLL cells with siRNA-mediated VEGF-depleted HS5 did not support survival, explicitly displaying the significance of VEGF in CLL cell survival promotion by the coculture system. Additionally, we found STAT3, which is constitutively Ser727-phosphorylated, to become phosphorylated on Tyr705 in CLL cells upon rhVEGF stimulation and under coculture with HS5. This lead to STAT3 activation as seen by induction of expression of the target genes antiapoptotic Bclxl and CyclinD1. Blockage of the VEGFreceptor2 using small molecules reduced Tyr705-phosphorylation, STAT3 target gene expression and induced apoptosis. *Conclusions.* CLL cells, but not healthy B-cells, exhibit positive auto- and paracrine VEGF feedback loops. Furthermore, a coculture with HS5 prevents CLL cell death *in vitro*. VEGF plays an essential role in this regard, since the depletion of VEGF in this coculture setting reduced or even completely abrogated CLL cell survival. A potential mechanism for VEGF mediated CLL cell survival support is the activation of the potent oncogene STAT3. Hence, VEGF at least participates in the prolonged survival of the CLL cell *in vivo*, which characterizes the major pathophysiological problem of the disease. Therefore,

VEGF and downstream effectors, such as STAT3 have to be considered promising targets for CLL therapy aiming on induction of CLL cell apoptosis and subsequent reduction of the malignant clone.

0347

LUMILIXIMAB INDUCES APOPTOSIS IN DRUG RESISTANT LYMPHOMA AND LEUKEMIA CELLS

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Background. Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in the western hemisphere. Although much progress has been made in CLL treatment, patients can develop resistance to currently available therapies, with CLL cells exhibiting an apoptosis-resistant phenotype via enhanced survival signaling due to the over-expression of anti-apoptotic proteins such as Bcl-2, Mcl-1 and XIAP, and activation of pro-survival kinases such as Akt. Lumiliximab, an anti-CD23 monoclonal antibody currently being evaluated in a registrational trial in CLL, differentially induces apoptosis in CLL cells via activation of capsases -9 and -3. In addition, lumiliximab downregulates anti-apoptotic proteins such as Bcl-2, Mcl-1, and XIAP, and reduces activation of pro-survival kinases such as Akt, enhancing the apoptotic effects of other therapeutic agents such as fludarabine and rituximab on CLL cells. *Aims.* To investigate the ability of lumiliximab to induce apoptosis in drug resistant cells *in vitro*. *Methods.* A preclinical, *in vitro* chemo-resistance model was generated by incubating SKW cells which over-express CD23 on the cell surface, with increasing concentrations of the chemotherapeutic drug adriamycin. *Results.* In the preclinical model, the drug-resistant cells (SKW-R) upregulated multi-drug resistance protein, MRP-2 and exhibited resistance to adriamycin and fludarabine as compared with parental cells (SKW-P). In addition, accompanying loss of CD20 expression rendered them insensitive to rituximab. Despite these changes, lumiliximab remained effective in inducing apoptosis in the drug-resistant cells. These results are consistent with findings in clinical trials of lumiliximab monotherapy and combination therapy: *in vitro* results from pre- and post-treatment patient samples obtained from a Phase 1 lumiliximab monotherapy trial in relapsed CLL (Study 152-20) showed activation activation of caspase-3, along with downregulation of Bcl-2 and Mcl-1, and a reduction in Akt activation. Lumiliximab induced apoptosis in samples from patients who did not respond well to prior fludarabine or rituximab treatment and apoptosis induction was independent of ZAP70 status. In addition, cytogenetic analysis of the available samples from this trial indicated that two of the three patients with a 17p deletion had decreases in CD5/CD19 counts. Furthermore all of the fludarabine-refractory subjects showed a reduction in ALC following treatment with lumiliximab and 50% had a reduction in lymph node bulk, as determined by SPD reduction. In a Phase 1/2 study of lumiliximab in combination with FCR, clinical response (including complete response) appeared to be unaffected by the presence at baseline of markers of poor prognosis (β2M levels greater than 2000 ng/mL; absence of IgVH mutation; $CD38^+$ CLL cells greater than 30% or $ZAP\text{-}70^+$ CLL cells greater than 30%) or by the presence of markers of rituximab resistance (CD55 or CD59). *Conclusions.* These data indicate that lumiliximab induces apoptosis in CLL cells and may be beneficial in the treatment of CLL, especially in those patients that have developed resistance to rituximab or fludarabine.

0348

HIGH-RESOLUTION MICROARRAY CHARACTERIZATION OF THE 13Q14 DELETION EXTENT IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA: CORRELATION WITH COPY NUMBER AND EXPRESSION LEVELS OF MIR-15A/16-1 CLUSTER

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Background. Hemizygous and/or homozygous loss at 13q14 has been identified as the most frequent genomic alteration in B-cell chronic lymphocytic leukemia (B-CLL). Two microRNAs genes, *mir-15a* and *mir-16-1*, are located at 13q14 within a 30-kb region of loss in B-CLL and have been found to be deleted and down-regulated in the majority of cases with respect to normal B-cells. Aims. To narrow down the extent of the 13q14 deletion, to validate the genomic status of *mir-15a/16-1* cluster and to correlate the copy number (CN) and expression levels of the two miRNAs. *Methods.* The whole genome profile was investigated by means of single nucleotide polymorphism (SNP) arrays (Affymetrix GeneChip® Human Mapping 250K Nsp) in a panel of 100 untreated B-CLL patients in early stage disease (Binet stage A). The *mir-15a/16-1* cluster DNA CN and transcription levels of mature *miR-15a* and *miR-16* have been detected by quantitative Real Time RT-PCR (Q-RT-PCR) using a custom TaqMan® assay and TaqMan® microRNAs assays (Applied Biosystems), respectively. *Results.* Del(13)(q14) was present in 44 of our patients, in 34 as a single aberration. Biallelic deletions encompassing the 13q14.2-q14.3 region were found in 14/44 cases (32%). SNP arrays showed that the deletions varied considerably in size, ranging from 291 kb to 56 Mb. The minimal monoallelic deletion was 635 kb long, extending from physical position 49,635,024 bp to 50,270,550 bp. Notably, the *mir-15a/16-1* cluster is located approximately 87 kb upstream to this region, and thus apparently not affected by the deletion. Overall, in our series we found that 4 cases retained one or two copies of the *mir-15a/16-1* cluster. We evaluated the miRNA genes DNA CN by means of Q-RT-PCR in the four patients and in a selected panel of 28 cases (10 biallelic deleted, 7 monoallelic deleted, 11 non-deleted patients); the estimated gene CN values showed a very good correlation with SNP array data (p=1.37×E-06, Kruskal-Wallis test). As regards the miRNAs expression, we found a significant difference between biallelic and monoallelic deleted B-CLLs (p=0.009 and 0.006 for *miR-15a* and *miR-16*, respectively), whereas expression levels associated with the retention of 1 or 2 alleles were not statistically different. Conclusions. Our data confirmed the previous evidence of a short common deleted region at 13q14, narrowing it down to 635 kb of extent. Furthermore we demonstrated that one or two copies of *mir-15a/16-1* cluster can be maintained at DNA level in patients with 13q14 deletion. As regards *miR-15a* and miR-16 expression levels, we found that they are significantly down-regulated only in patients with biallelic deletion.

Chronic lymphocytic leukemia and related disorders - Clinical I

0349

MICRORNA-29C AND 223 AND THEIR PROGNOSTIC VALUE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. MicroRNAs (or miR) are a novel class of small noncoding RNA involved in gene regulation. Aberrant microRNA expression has been recently associated with chronic lymphocytic leukemia (CLL) outcome. Currently, the heterogeneous evolution of this disease can be predicted by several prognostic factors. Nevertheless, a better individualization of the outcome in a given patient is still of utmost interest. *Aims and Methods.* In the current study, we investigated the expression of two microRNAs, miR-29c and miR-223, compared them to other biologic or clinical markers and proposed a real-time quantitative PCR (qPCR) score to better assess CLL outcome. *Results.* miR-29c and miR-223 expression decreased significantly with progression along Binet Stage A to C (p=0.0010 and p=0.0183, respectively), and were significantly lower in poor prognosis subgroup defined by cytogenetic abnormalities, IgVH mutational status, lymphocyte doubling time, solubleCD23, β2-microglobulin, ζ-associated protein 70 (ZAP70), lipoprotein lipase (LPL) and CD38 expression. Furthermore, miR-29c and miR-223 could predict TFS (n=110, p=0.0015 and p<0.0001, respectively) and OS (n=110, p=0.0234 and p=0.0008, respectively). Regarding all these results, we developed a qPCR score (from 0 to 4 poor prognostic markers) combining miR-29c, miR-223, ZAP70 and LPL in order to stratify treatment and death risk in a 110 patient cohort with a median follow-up of 72 months (range, 2-312). Patients with a score of 0/4, 1/4, 2/4, 3/4, and 4/4 had a median TFS of >312, 129, 80, 36 and 19 months, respectively (HR=17.00, p<0.0001). Patient with a score of 0-1/4, 2-3/4 and 4/4 had a median OS of >310, 183 and 106 months, respectively (HR=13.69, p=0.0001). Interestingly, during the first 50 months after diagnosis, only 10% of patients with a 0/4 score required a treatment, when compared to 100% of the 4/4. Furthermore, during the total follow-up (312 months), patients with a 4/4 score had a 27-fold higher risk to be treated and a 31-fold more risk to die comparing to patients with a 0/4 score. This score was validated by a 10-fold cross-validation (concordance of 82%) and a pre-validation study. Finally, in Binet stage A patients (n=77), this score remained relevant and significant for TFS and OS (HR=18.56, p<0.0001 and HR=12.5, p=0.0068, respectively). We also showed that miR-29c negative patients expressed higher level of Tcl1 oncogene (n=20, p=0.0068) making by this way a molecular connection of this our prognosis study. *Conclusions.* we showed that (i) miR-29c and miR-223 levels were decreased in poor prognosis patients regarding several well-known prognostic factors; (ii) a low level of these two microRNAs is thus associated to disease aggressiveness, high tumor burden and poor clinical evolution; (iii) we also showed that these two microRNAs could predict TFS and OS; (iv) we proposed a qPCR score to better individualized evolution of a particular CLL patient. This score will help to identify patients who will need early therapy and require thus a closer follow-up.

0350

PHARMACOKINETICS, SAFETY AND ANTI-TUMOR ACTIVITY OF ABT-263 IN PATIENTS WITH RELAPSED OR REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA/SMALL LYMPHOCYTIC LYMPHOMA

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Background. ABT-263 is an orally bioavailable BH3 mimetic that binds with high affinity (Ki ″1nM) and inhibits multiple antiapoptotic Bcl-2 family proteins. ABT-263 displays potent activity (EC50 ″ 1 μM) against human lymphoid and small cell lung cancer cell lines. Mechanism-based preclinical effects include apoptosis of circulating lymphocytes and apoptosis of circulating platelets mediated by inhibition of Bcl-2 and Bcl-XL, respectively. *Methods.* Two phase I monotherapy studies with continuous reassessment method (CRM) and 3+3 Fibonacci (3+3) designs are being conducted internationally to determine the pharmacokinetics, safety/tolerability and efficacy of ABT-263 in relapsed/refractory lymphoid malignancies (M06-814; 3+3) and CLL (M06-873; CRM). Patients (pts) were dosed on Days 1-14 of a 21-day dosing cycle with 10-440 mg (M06-814) or 10-250 mg (M06-873) ABT-263. Currently, continuous 21/21-day dosing (21d cycle) following a lead-in dose is being explored in the 275 mg (M06-814) and 300 mg (M06-873) cohorts from both studies. Tumor responses were evaluated by the NCI-WG criteria. *Results.* 72 pts have enrolled in these ongoing studies (n=53, 14/21-day dosing; n=19, 21/21-day dosing). Of these, 43 CLL/SLL pts were treated with ABT-263 (n=27, 14/21 day dosing; n=16, 21/21 day dosing). ABT-263 exposure increased proportional to dose from 10-440 mg with a terminal half-life of 18 h, supporting a QD schedule. Pts were heavily pretreated with a median of 4 prior treatments (range 1-12).The median progression-free survival has not yet been reached; the median time on study is 260 days [95% CI: 87, 364]. Among the 43 CLL/SLL pts, 4 had radiographically confirmed partial responses (PR) (99, 92, 72, and 64% reduction in lymphadenopathy) and 5 had unconfirmed regression in lymph node size at 72, 67, 55, 53 and 51%. 9 pts maintained a ≥50% decrease in circulating absolute lymphocyte count for ≥2 months (n=8, 14/21-day dosing; n=1, 21/21-day dosing). Stable disease (SD) was noted in 9 pts; 5 experienced minor radiographic responses (range from 15-49% reduction). Of these 43 patients, 10 were fludarabine and/or alemtuzumab refractory. Of the 6 fludarabine refractory pts, 2 had a 50% decrease in circulating lymphocytes, 1 a PR with a 92% reduction in lymphadenopathy, and 1 pt had a 49% reduction in lymphadenopathy. Of the 4 fludarabine and alemtuzumab refractory pts, 1 achieved a 46% reduction in lymphadenopathy and 1 had SD. Dose-dependent thrombocytopenia (TCP) resulting from on-target activity against Bcl-XL was observed. 27 pts on the 14/21-day schedule, had dose-limiting toxicities (DLT) occurring at 160mg (bronchitis), 315 mg (elevated ALT and Grade 4 TCP) and 440 mg (worsening pleural effusion in a pt with underlying afib) in M06-814, and at 110 mg (tumor lysis and Grade 4 TCP) and 250 mg (Grade 4 TCP) in M06-873. Among the 15 pts on the 21/21-day schedule, 3 experienced DLT; 2 Grade 4 TCP, 1 at 275 mg (M06-814) and 1 at 200 mg (M06-873), and 1Grade 2 nausea at 250 mg (M06-873). *Conclusions.* ABT-263 exhibited favorable pharmacokinetics and safety profiles with anti-tumor activity in relapsed/refractory CLL/SLL. Toxicities were predictable and manageable. Identification of optimal dose and schedule for phase II trials continues.

0351

RESULTS OF THE PHASE II NCRI CLL206 TRIAL OF ALEMTUZUMAB IN COMBINATION WITH HIGH-DOSE METHYLPREDNISOLONE FOR HIGH-RISK (17P-) CLL

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Background. Deletion of TP53 on chromosome 17p13 is the most ominous predictor of adverse outcome in CLL and is strongly associated with resistance to chemotherapy. Alemtuzumab and methylprednisolone are unusual in having established activity in 17p- CLL. However, these agents are of limited value when used alone. A combination regimen was therefore developed. Following a small pilot study that showed the *Cam-Pred* regimen to have promising activity in cases of CLL with TP53 defects [Leukemia 2006;20:1441-5], a multicentre phase II trial was developed to evaluate the regimen in more detail. *Aims.*To investigate the efficacy and toxicity of alemtuzumab in combination with high-dose methylprednisolone (HDMP) in high-risk (17p) CLL. *Methods.* Patients were eligible for the NCRI CLL206 trial if they had CLL requiring therapy and a 17p deletion in at least 20% of their malignant cells. The study treatment consisted of alemtuzumab 30 mg thrice week-

ly for up to 16 weeks (intravenous for the first 4 weeks and subcutaneous thereafter) plus methylprednisolone 1.0 g/m^2 for 5 consecutive days on weeks 1, 5, 9 and 13. Mandatory supportive care included antimicrobial prophylaxis with co-trimoxazole, aciclovir and itraconazole, G-CSF for neutropenia of <1.0×10^9/L and weekly CMV-PCR surveillance with pre-emptive (val)ganciclovir for CMV reactivation. Responses were assessed according to the criteria set out in the 2008 IWCLL guidelines. *Results.* 41 patients were recruited into the study between May 2006 and February 2008. Two patients died after registration but before receiving any treatment. Among the 39 remaining patients 56% were male and the median age was 61.5 (range 34-82). 46% were Binet stage C, 43% stage B, and 11% progressive stage A. 22 patients had received prior therapy whereas 17 were previously untreated. Data obtained up to October 2008 have been analysed. Response data were obtained for 37 patients and toxicity data for all 39 (Figure 1). 27 patients had CT scans performed, while reliable MRD data were available from 21 patients. The CR/CRi rate was 24% overall and 37% in previously untreated patients, with 3 patients achieving a confirmed MRD-negative CR/CRi. Grade 3-4 haematological toxicity and CMV reactivation rates were no greater than would be expected following alemtuzumab alone (67% and 23% respectively). However, there was a high rate of grade 3-4 non-CMV infection (41%) and glucocorticoid-related toxicity (38%). Rates of grade 3-4 infection were higher in previously treated patients (64% versus 41%). 12 of the 39 patients who received treatment died, 5 from disease progression, 2 from post-transplant complications, 1 from intracranial haemorrhage and 4 from infection including one case of suspected invasive aspergillosis and one of suspected viral encephalitis. Nine of the 12 deaths occurred among the 22 previously treated patients. Upated information on progression-free and overall survival will be presented. *Summary and Conclusions.* The Cam-Pred regimen appears to be the most active remission induction treatment for 17p- CLL hitherto reported, although it is also associated with a significant risk of infection and glucocorticoid-related toxicity.

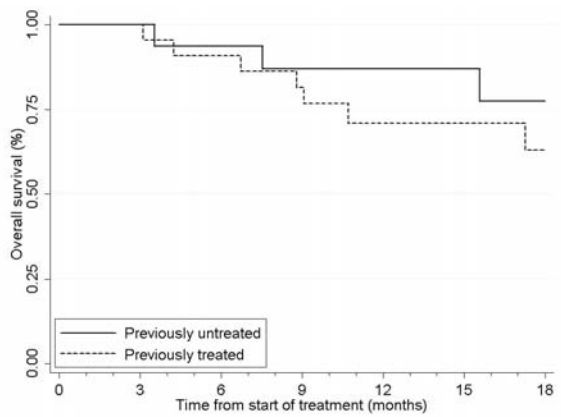

Figure 1. Summary of response and toxicity data.

0352

STEROTYPED B-CELL RECEPTOR AND IGHV4-39 USAGE REPRESENT INDEPENDENT RISK FACTORS OF CHRONIC LYMPHOCYTIC LEUKEMIA TRANSFORMATION TO RICHTER SYNDROME

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Background. Richter's syndrome (RS) represents the transformation of CLL to aggressive lymphoma. Despite the abundance of biological markers available for predicting CLL progression, only few have been shown to be useful for RS prediction. *Aim.* To test the role of stereotyped HCDR3 in RS transformation. *Methods.* The first step of the study consisted of a case-control analysis comparing IGHV gene usage and prevalence of stereotyped HCDR3 in RS (n=69; all DLBCL) versus a control group (n=715) of CLL not transformed to RS. The second step consisted of an actuarial assessment of the impact of IGHV gene usage and stereotyped HCDR3 at CLL diagnosis, on the risk of subsequent transformation to RS in a cohort of 754 CLL, of which 39 had transformed to RS. Cluster analysis was performed by aligning HCDR3 of RS and non-transformed CLL into a database of 2421 HCDR3 of CLL. Results. Case-control comparison of IGHV usage documented that IGHV4-39 was the sole gene preferentially utilized in RS compared to non-transformed CLL (p=0.002). Cluster analysis revealed a significantly higher prevalence of stereotyped HCDR3 in RS compared to non-transformed CLL when considering all cases (RS: 34/69, 49.3% vs non-transformed CLL: 152/714, 21.3%; p<0.001), unmutated cases only (RS: 28/48, 58.3% vs non-transformed CLL: 95/276, 34.4%; p=0.002), and mutated cases only (RS: 6/21, 28.6% vs non-transformed CLL: 57/438, 13.0%; p=0.043). Subset 8 utilizing unmutated IGHV4-39/IGHD6-13/IGHJ5 genes was the sole HCDR3 subset preferentially utilized by RS (5/34, 14.7%) compared to non-transformed CLL (2/152, 1.3%) (p=0.002). Actuarial univariate analysis revealed higher risk of RS: i) in CLL utilizing stereotyped HCDR3 (5-year risk: 14.2%) compared to CLL without stereotyped HCDR3 (5-year risk: 3.9%) (p<0.00001); and ii) in CLL utilizing IGHV4-39 (5-year risk: 35.4%) compared to CLL utilizing other IGHV genes (5-year risk: 5.6%) (p<0.000001). Bivariate analysis combining stereotyped HCDR3 and IGHV mutation status indicated that stereotyped HCDR3 was not a surrogate of IGHV homology for RS prediction. Multivariate analysis selected IGHV4-39 usage (HR:4.25; p=0.002) and stereotyped HCDR3 (HR:3.08; p=0.002) as independent predictors of RS transformation. The observation that all RS utilizing IGHV4-39 carried stereotyped HCDR3 prompted investigation of the interaction between IGHV4-39 usage and stereotyped HCDR3 in the model. Multivariate analysis selected the interaction between IGHV4-39 usage and stereotyped HCDR3 at CLL diagnosis as the strongest independent predictor of RS transformation (HR:5.13; p=0.001). The relevance of the interaction between IGHV4-39 and stereotyped HCDR3 was confirmed by bivariate analysis. Accordingly, CLL utilizing both IGHV4-39 and stereotyped HCDR3 were identified as the disease category with highest risk of transformation (5-year risk: 68.7%) (Figure 1). Neither IGHV4-39 usage nor stereotyped HCDR3 affected the risk of CLL progression occurring without transformation to RS. Conclusions. The implications of our results are fourfold: i) RS carry stereotype HCDR3 at a very high frequency; ii) RS display biased usage of IGHV4-39 with stereotyped HCDR3; iii) stereotyped HCDR3 at CLL diagnosis is an independent risk factor of RS transformation; iv) the combination of stereotyped HCDR3 and IGHV4-39 usage in the same patient identifies CLL with a very high risk of RS transformation.

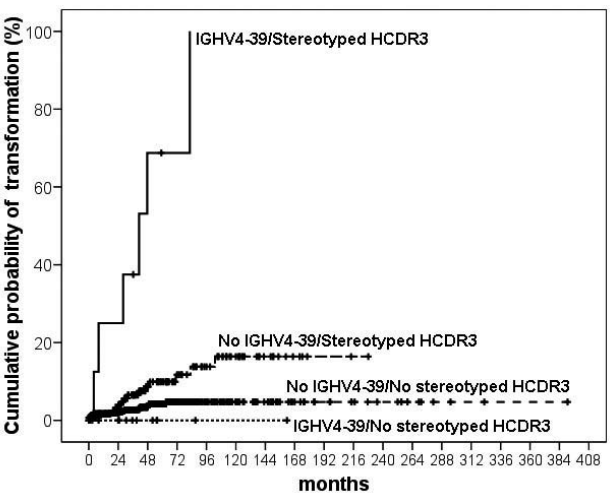

Figure 1. Cumulative risk of trasnformation to RS.

0353

FINAL RESULTS OF ORAL FLUDARABINE WITH CONCOMITANT SUBCUTANEOUS ALEMTUZUMAB IN RELAPSED/REFRACTORY B-CHRONIC LYMPHOCYTIC LEUKAEMIA (B-CLL): THE FLUSALEM STUDY

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Background. Relapse of B-CLL presents a crucial problem to the patient and clinician. Combination of alemtuzumab and fludarabine was effective in heavily pre-treated patients in a very small pilot trial (Kennedy, Blood, 2002). The German CLL study group presented data on 36 relapsed or refractory CLL patients using intravenous fludarabine combined with a greatly reduced dose of 3 times 30 mg of intravenous alemtuzumab per cycle (FluCam regimen; Elter, JCO 2005). They showed a high 83% ORR, but did not report risk factors or MRD analysis. *Aims.* The FLUSALEM protocol combines 4 cycles of oral fludarabine (40 mg/m^2 3d) with continuous alemtuzumab therapy (30 mg s.c. 3 x weekly for 16 weeks), thereby increasing the cumulative alemtuzumab dose, while allowing a continous outpatient mangement. The study followed a 2-step Gehan design evaluating efficacy and safety of the therapy, with a previously reported interim analysis (Egle, ASH, 2006). *Methods.* In addition to the primary response endpoint, progression-free survival, risk factor profiles and MRD measurements after therapy were analysed. *Results.* Sixteen patients were evaluable for response and PFS. Median age was 60 years, 50% of patients had stageRai III/IV and median follow up was 18 months. Overall response was 87.5% (CR rate 56%, as compared to 30% in the FluCam Study), median time to reaching clinical CR was 59 days. Considering that 50% of included patients had Rai III/IV, bone marrow clearance by this regimen occurred very rapidly. Median PFS was 486 days or 16 months (as compared to 12 months in the dose-reduced FluCam) and 5 of 16 patients are in continued remission after 500-1300 days of follow-up. Therefore, 20% of our patients have a longer PFS than the longest reported from the FluCam study. Grade III infection was observed in 4 patients (25%, 3 CMV reactivations and 1 pneumocystis pneumonia in a trimethoprim-intolerant patient on pentamidine inhalation prophylaxis). No fatal infections were observed. One spontaneously resolving pulmonary grade 3 toxicity was observed after the end of therapy. CD4 depletion was profound (median 69/µL after 3 cycles) and prolonged (3/4 of patients did not reach CD4 counts >200/µL withinin 6 months). CD38 risk groups as well as mutation status are available for 14 and 11 patients with PFS follow up, respectively. Neither was predictive of PFS or response quality. MRD analysis by 4 colour flow cytometry was performed according to Rawstron (Blood 2001). MRD data at 2 months after treatment are available for 10 patients with 6 patients achieving an MRD negative state. MRD negativity at 2 months after end of therapy predicted a trend in PFS (p=0.09) and identified all but one patient with a PFS above the median. The exceptional case displayed a close to negative MRD measurement. *Summary and Conclusions.* Oral fludarabine combined with 16 weeks of subcutaneous alemtuzumab was feasible in an out-patient setting. Infectious complications were surprisingly low, and all were manageable. Response rates are high for previously treated patients and show high remission qualities, irrespective of risk factors. Long term progression-free survival is observed in MRD negative patients.

0354

LARGE BUT NOT SMALL COPY-NUMBER ALTERATIONS CORRELATE TO HIGH-RISK GENOMIC ABERRATIONS AND SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA: A HIGH-RESOLUTION GENOMIC SCREENING IN NEWLY DIAGNOSED PATIENTS

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Background. Recurrent genomic aberrations (i.e. deletions of 11q, 13q, 17p and trisomy 12) are of great prognostic value in chronic lymphocytic leukemia (CLL). Identification of these alterations is routinely performed by fluorescence in-situ hybridization (FISH). However, less is known about the overall genetic complexity such as small copy number alterations (CNAs) and loss of heterozygosity (LOH) in CLL, and their contribution to prognosis. Moreover, the fact that no common genetic aberration has been described in the pathogenesis of CLL suggests that application of genome-wide techniques with high resolution would be valuable for identification of yet unidentified genetic lesions. *Aims.* To investigate genomic CNAs and CNN-LOH in newly diagnosed chronic lymphocytic leukemia (CLL) patients by applying high resolution SNP-arrays. *Methods.* 203 CLL patients were selected for inclusion in the present study from the Swedish part of a population-based Scandinavian cohort Array experiments were performed according to the standard protocols for Affymetrix GeneChip® Mapping 250K arrays. Copy-number analysis was performed by applying the BioDiscovery Nexus Copy Number 3.0 software. The evaluation of CNN-LOH was performed by applying a newly developed method, detecting tumor-specific CNN-LOH. *Results.* CNAs were identified in 90% of samples where deletions were more commonly detected than gains (70% vs. 30%, respectively). The known recurrent alterations were found in 75% of patients; del(13q) 52%, del(11q) 13%, trisomy 12 11% and del(17p) 4.4%. Interestingly, 5 samples (2.5%), which all carried del(11q), displayed a gain of chromosome 2p. Furthermore, 8 samples with homozygous del(13q) showed CNN-LOH of large parts of 13q, suggesting that a chromosomal duplication occurred after mono-allelic deletion. The prognostic impact of known recurrent aberrations was verified, although 11q-deleted and trisomy 12 patients displayed a similar poor-risk profile and patients with homozygous del(13q) had superior survival. Large aberrations (>5Mb) were strongly associated with poor-prognostic markers, i.e. 11q-/17p- and unmutated IGHV genes, and predicted poor survival. In contrast, small aberrations (<1Mb) were commonly detected in all samples, but were most often non-overlapping, and did not predict outcome. *Summary*. Whole-genome screening with SNP-arrays revealed a high frequency of known recurrent alterations in newly diagnosed CLL patients. Moreover, the SNP-technique allowed detection of additional large and small CNAs, which gave the opportunity to evaluate the overall complexity in CLL patients. As a result, we identified genomic complexity as a poor prognostic marker, but noted that this feature was strongly linked to established poor-risk molecular markers. Finally, this technique allowed identification of CNN-LOH in CLL, which were recurrently detected on chromosome 13. In conclusion, high resolution genomic arrays are applicable for the evaluation of recurrent aberrations and genomic complexity in newly diagnosed CLL samples.

0355

BENDAMUSTINE VERSUS CHLORAMBUCIL IN TREATMENT- NAIVE PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: UPDATED RESULTS OF AN INTERNATIONAL PHASE III STUDY

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Background. Bendamustine (BEN) is a purine analogue / alkylator hybrid agent that provides effective treatment for a number of haematological and non-haematological malignancies. In Germany, it is used since more than three decades in first line therapy and pretreated CLL as well as for other types of non-Hodgkin's lymphomas. *Aims.* To compare the efficacy and safety of BEN versus chlorambucil (CLB) in treatment-naïve patients (pts) with CLL Binet stage B/C. This study was designed as pivotal trial for the approval of BEN in a number of European countries. *Patients and Methods.* Within this prospectively randomised phase III trial, pts received either BEN (100 mg/m^2 on days 1+2) or CLB (0.8 mg/kg on days 1+15) for up to 6 cycles. Primary endpoints were overall remission rate (ORR) and progression-free survival (PFS). Secondary endpoints were duration of remission, overall survival (OS), and safety, Also, type of remission (CR, nodular PR, PR) was analysed. Follow-up was for ≥12 months after completion of treatment of the last patient, or until progression for patients with CR, nPR or PR and stable disease, or until death or lost to follow-up. A 5-stage, adaptive-group, sequential procedure was used with planned interim analyses to adjust the number of patients. In addition to external data monitoring, efficacy was evaluated according to the NCI working group criteria by an independent committee in a blinded fashion. *Results.* 45 centres recruited 319 pts who were randomised to receive BEN (n=162) or CLB (n=157). All these pts were subject to the efficacy analysis (intention-to-treat population). As 7 pts did not receive any study medication, 312 pts were included in the safety analysis. Median age was 64 years; 71% had Binet stage B and 29% Binet stage C disease; median number of treatment cycles/patient was 6. ORR was significantly higher with BEN than with CLB (68% vs 31%; p<0.0001), with CR of 31% vs. 2%, respectively. Median PFS was 21.6 months with BEN and 8.3 months with CLB (p<0.0001), and median duration of remission (Kaplan-Meier estimate) was 21.8 months with BEN and 8.0 months with CLB (p<0.0001). No significant difference in OS is seen so far. Infection rates (CTC grades III+IV) were low in both treatment groups (7% BEN; 4% CLB). *Conclusions.* BEN was significantly more effective than CLB in treatment-naïve pts with CLL Binet stage B/C with respect to remission induction, PFS and duration of remission. Furthermore, safety data indicate that BEN toxicities are manageable and the drug is well tolerated. On the basis of these results, BEN should be considered as first-line chemotherapy for patients with CLL Binet stage B or C.

0356

EXPRESSION OF MUTATED IGHV3-23 GENES HAS INDEPENDENT PROGNOSTIC RELEVANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA AND IDENTIFIES A DISEASE SUBSET WITH PECULIAR MOLECULAR AND BIOLOGICAL FEATURES

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Background. In the last years, the B cell receptor (BCR) has become a key molecule in chronic lymphocytic leukemia (CLL), given the correlation between mutational status of immunoglobulin heavy chain variable (IGHV) genes and disease prognosis. Recently, a fraction of CLL has been shown to preferentially express specific IGHV genes, often in a non-random combination with homologous heavy chain complementarity-determining region-3 (HCDR3) and peculiar light chains. Some of these stereotyped BCR mark CLL subsets with peculiar clinical behaviours regardless of IGHV mutations. These data suggest a role for BCR in defining the clinical and biological features of CLL, also beyond the mutational status of IGHV genes. *Aim.* To discover novel associations between specific IGHV gene usage and prognosis in CLL. *Methods.* A HCDR3-driven clustering of 1,426 IG sequences (1,398 patients) was performed using ClustalX(1.83). Time to treatment intervals (TTT), Rai staging, IGHV mutational status, CD38, ZAP-70, and karyotype abnormalities evaluated by FISH were available for 644 patients. Gene expression profiling (GEP) was performed on purified CLL cells, using a dual labelling strategy. Validation experiments were performed utilizing real-time quantitative PCR (QRT-PCR). *Results.* Taking into account the distribution of IGHV genes in 71 identified stereotyped BCR clusters, IGHV3-23 was totally absent in clusters despite being the second most frequently used IGHV gene (134/1,426). Although 109/134 IGHV3-23 CLL were mutated (M), alignment of IGHV sequences revealed a high degree of conservation in the context of the 13 AA positions involved in superantigen binding by IGHV3 subgroup genes, suggesting that the majority of M IGHV3-23 cases maintained the capacity to mediate superantigen recognition and binding. Median TTT (73 months) of M IGHV3-23 CLL (46 cases) was shorter than median TTT (153 months, p=0.0201) of the whole series of M CLL (350 cases), as well as of a series (342 cases) of M CLL in which 8 cases belonging to the bad prognosis IGHV3-21/IGLV3-21 cluster were excluded (median TTT=253 months, p=0.0115). The independent prognostic value of IGHV3-23 expression was tested in 396 M CLL. All the prognosticators and IGHV3-23 usage correlated with an increased risk of progressive disease by univariate analysis. Multivariate Cox proportional hazard analyses selected IGHV3-23 usage (p<0.05), Rai stage (p<0.0001) and FISH group (p<0.0001) as independent markers of disease progression for the whole cohort of M CLL, and in a cohort in which 8 M CLL from the IGHV3-21/IGLV3-21 cluster were excluded. By comparing 5 M IGHV3-23 and 22 M non-IGHV3-23 CLL for their differential GEP, 212 genes were selected, 108 up-regulated and 104 down-regulated in M IGHV3-23 CLL. Using the "Gene-Ontology Tree Machine" platform, a set of growth/tumor suppressor genes (PDCD4, DIDO1, TIA1, RASSF5), all down-regulated in M IGHV3-23 CLL, were significantly enriched in several gene-ontology categories related to apoptosis. The down-regulated expression of these genes was confirmed by QRT-PCR experiments. *Conclusions.* Expression of IGHV3-23 marks a subset of M CLL with a worse prognosis; such a peculiar clinical behaviour may be related to superantigen stimulation combined with down-regulation of specific growth/tumor suppressor genes.

0357

5-YEAR FOLLOW-UP OF HEAVILY PRETREATED PATIENTS WITH RELAPSED/REFRACTORY CLL IN PHASE 3 STUDY: SIGNIFICANT SURVIVAL BENEFIT WITH OBLIMERSEN PLUS FLUDARABINE/CYCLOPHOSPHAMIDE IN RESPONDING PATIENTS

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Background. Oblimersen (OBL) decreases the antiapototic protein, Bcl-2, and enhances cytotoxic activity of agents used to treat CLL. A randomized Phase 3 study of fludarabine/cyclophosphamide (FC) with/without OBL was conducted in patients who had relapsed/refractory CLL and at least 2 prior cycles of fludarabine. Informed consent was obtained; patients were stratified according to prior response to fludarabine (relapsed or refractory). The distribution of patients between groups was generally well balanced with respect to demographic and disease-related characteristics at baseline. In both groups, more than 80% of patients had disease-related symptoms, the median number of prior regimens was 3, and the median number of prior fludarabine cycles was 6. The primary endpoint -the relative proportion of patients who achieved a complete response (CR; defined as complete or nodular partial response)- was met: the CR rate was significantly greater among patients treated with OBL-FC than among those treated with FC (17% versus 7%; p=0.025; O'Brien *et al.*, J Clin Oncol 2007). With at least 2 years of follow-up, duration of CR also significantly favored OBL-FC (median not

reached [36+ months] versus 22 months; p=0.03; Moore *et al.*, ASH 2006). *Aim.* We evaluated whether the OBL-FC regimen would provide a long-term survival benefit. *Methods.* Patients received up to six 28-day cycles of FC (25 mg/m^2/d and 250 mg/m^2/d x 3d, respectively) with or without OBL (3 mg/kg/d x 7d by continuous IV infusion beginning 4 days before FC). Response and progression were determined according to NCI-WG criteria by a clinical expert blinded to treatment. An independent hematopathologist blinded to treatment and clinical information assessed bone marrow specimens. CT was required to confirm CR in patients with baseline abnormalities. Patients were followed for up to 5 years after randomization. *Results.* Five-year follow-up data were obtained for 97% of the 241 patients randomized (OBL-FC, 120 patients; FC, 121). Post-study CLL treatment was balanced between groups. In the intent-to-treat population, the 5-year survival rate was improved (25% versus 15%) with OBL-FC, but not significantly different. Among responding patients (ie, those with complete or partial response), survival was significantly increased in the OBL-FC group (hazard ratio=0.60; p=0.038). Patients with fludarabine-sensitive disease (as defined at baseline) had the greatest benefit from the addition of OBL: a four-fold increase in the CR rate (p=0.016) and a 50% reduction in the risk of death were observed (Figure). No between-treatment survival difference was apparent in nonresponding patients (hazard ratio=1.0; p=0.9). *Summary and Conclusions.* In heavily pretreated patients with relapsed/refractory CLL, adding OBL to FC significantly increases the rate of CR and durable CR and provides a significant survival benefit in patients who achieve a complete or partial remission. Greatest benefit was noted in the prespecified group of patients who retained sensitivity to chemotherapy.

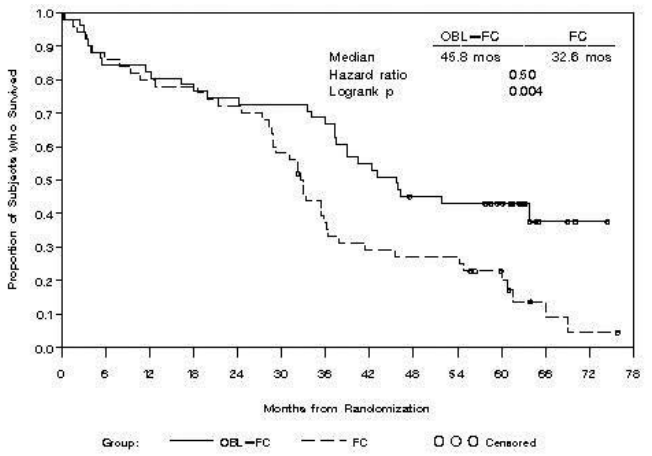

Figure. 5-year survival in fludarabine-sensitive subjects

0358

CD49D EXPRESSION AND SHORT TELOMERE LENGTH INTERACT IN CHRONIC LYMPHOCYTIC LEUKEMIA AND IDENTIFY PATIENTS AT RISK OF PROGRESSION AND SHORT SURVIVAL

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Background. The rationale of the study stems from two considerations: i) CD49d expression and short telomere length (TL) represent novel prognostic markers for chronic lymphocytic leukemia (CLL); ii) both CD49d expression and short TL associate with clinico-biological features of highly proliferating CLL. *Aim.* To test the impact of the interaction between CD49d expression and short TL on CLL prognostication. *Methods.* The study was based on a consecutive series of 180 previously untreated CLL. CD49d expression was analyzed by flow cytometry. Peak TL was determined by Southern blot. Best CD49d and TL cut off points for CLL prognostication have been previously identified (Gattei *et al.*, Blood 2008; Rossi *et al.*, Leukemia 2009). *Results.* Figure 1A represents the relationship between CD49d expression and TL in a consecutive series of 180 CLL. When treated as categorical variables, CD49d>30% and TL<5000 bp were not associated (p=0.097). Clinico-biological variables at diagnosis and outcome were stratified according to CD49d and TL status. For this purpose, CLL were grouped into 3 categories: i) CD49d-low/TL-long (CD49d<30%/TL>5000bp); ii) CD49d-high/TL-short (CD49d>30%/TL<5000bp); iii) discordant cases (CD49d<30%/TL<5000bp and CD49d>30%/TL>5000bp). The prevalence of unfavorable variables at diagnosis progressively increased from CD49d-low/TL-long CLL to discordant cases and to CD49d-high/TL-short CLL (Rai stage III-IV: 3.8% vs. 15.5% vs. 24.1%, respectively, p=0.006; IGHV homology >98%: 19.0% vs. 41.4% vs 69.4%, respectively, p<0.001; CD38 >30%: 8.8% vs. 39.4% vs. 62.1%, respectively, p<0.001; unfavorable karyotype: 21.3% vs. 46.5% vs. 65.5%, respectively, p<0.001).

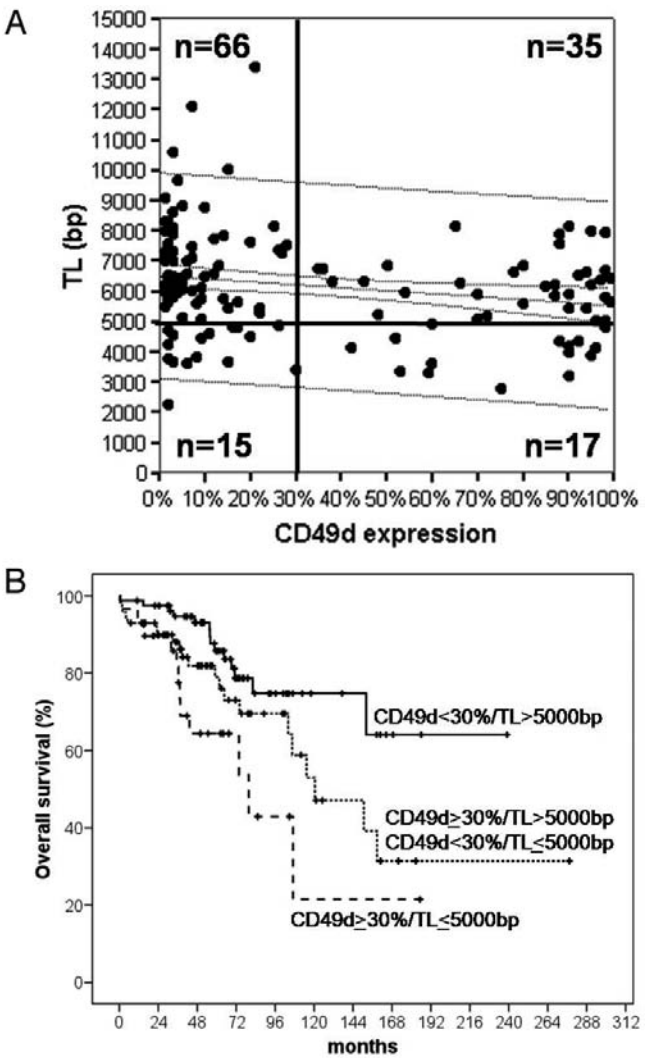

Figure 1. A: CD49d and TL correlation. B: survival.

Occurrence of markers of rapid cell turnover also progressively increased from CD49d-low/TL-long CLL to discordant cases, and to CD49d-high/TL-short CLL (median β-2-microglobulin: 2.1 mg/L vs 2.7 mg/L vs. 3.3 mg/L, respectively, p<0.001; median LDH: 323 U/L vs 370 U/L vs. 399 U/L, respectively, p<0.001). Time to lymphocyte doubling progressively decreased from CD49d-low/TL-long CLL to discordant cases, and to CD49d-high/TL-short CLL (54.2 months vs 28.6 months vs. 18.3 months, respectively, p<0.001). Time to progression to a more advanced stage progressively decreased from CD49d-low/TL-long CLL to discordant cases, and to CD49d-high/TL-short CLL (68.9 months vs 34.6 months vs. 23.0 months, respectively, p<0.001). Treatment free survival progressively decreased from CD49d-low/TL-long CLL to discordant cases, and to CD49d-high/TL-short CLL (130.8 months vs. 37.2 months vs. 21.5 months, respectively, p<0.001). Survival progressively decreased from CD49d-low/TL-long CLL to discordant cases, and to CD49d-high/TL-short CLL (not reached vs. 120.5 months vs. 79.8 months, respectively, p=0.006) (Figure 1B). Other variables associated with short survival were IGHV homology >98%, presence of del17p13 or 11q22-q23, Rai stage III-IV, and β-2-microglobulin >2.5 mg/L (p<0.05). Multivariate analysis selected the interaction between CD49d expression and TL as independent predictor of survival (HR:2.17; p=0.023)

after adjusting for IGHV homology, unfavorable FISH karyotype, Rai stage and β-2-microglobulin. Conclusions. The implications of our results are twofold: i) CD49d expression and short TL interaction identifies CLL with rapid tumor kinetics, high risk of progression, and short survival; ii) the interaction between CD49d expression and short TL is an independent predictor of short survival in CLL.

0359

UPDATE OF THE FRENCH LGL PROLIFERATION REGISTRY: REPORT OF 229 CASES

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Background. Large granular lymphocytes (LGL) leukemia is a rare lymphoproliferative disorder associated with autoimmune diseases and impaired hematopoiesis. Only few series have reported on a large number of patients. Clinico-biological presentation and therapeutic options need to be clarified. *Aims.* This study describes the largest series of clinical and biological characteristics of 229 patients with LGL leukemia. *Methods.* The French national LGL proliferations registry has included clinico-biological data from 250 patients suspected of LGL leukemia and coming from 35 medical centres. Using the updated criteria, the diagnosis was based on a LGL expansion (>0.5×10^9/L) lasting more than 6 months (excluding transient or reactive LGL proliferations). Monoclonal TCR γ gene rearrangement was detected in all T LGL leukemia. The term chronic NK lymphocytosis was used for patients with pauci or asymptomatic disease while patients with tissue LGL infiltration of spleen, liver or bone marrow with aggressive clinical behaviour were considered as having NK LGL leukaemia. Response to therapy was determined by monthly clinical and blood count assessments. The primary response criterion was defined based on results of blood count at 3 months.

Table 1.

T or NK subtype	T	NK	T or NK subtype	T	NK
Nb of patients	201	28	hyperlymphocytosis	51%	58%
Median age	59	58	LGL > 4 x 10.9/L	14%	21 %
Sex ratio (M/F)	90/111	14/14	LGL<1.10.9/L	55%	59 %
Non symptomatic	18%	25%	Neutropenia Severe neutropenia	61% 26%	48% 16%
Splenomegaly Hepatomegaly Adenopathy	24% 10% 6 %	25% 14% 7%	Anemia Anemia Hb < 8 g/dl Thrombocytopenia	24% 7% 19%	28% 4% 8%
B Symptoms	7%	10%	LGL Marrow infiltration	71%	61%
Infections	23 %	18%	Polyclonal gammapathy	35%	56%
RA	17%	11%	Monoclonal gammapathy	10%	6%
Autoimmune cytopenia	7%	11%	RF AAN	40% 48%	43% 63%
LGL related deaths	14/201	1/28	Need for treatment	44%	39%

Results. The diagnosis of T-LGL leukemia was confirmed in 201 cases, chronic NK lymphocytosis in 27 cases and NK LGL leukemia in one case. The main clinico-biological characteristics are summarized in Table 1 as follows. We did not observe any significant difference between T and chronic NK lymphocytosis. The classical phenotype of T-LGL leukemias was TCRαβ/CD3$^+$/CD4$^-$/CD8$^+$/CD16$^+$/CD57$^+$. γδ+ T cell LGL leukemia was uncommon (18%). Their common phenotype was similar (CD3$^+$/CD4$^-$/CD8$^+$/CD57$^+$) but 35% of those patients had a double negatif CD4$^-$/CD8$^-$ phenotype. NK LGL leukemia mainly expressed a CD3$^-$/CD16$^+$/CD56$^+$ phenotype. Vβ repertoire was analysed in 43 T LGL cases and showed a monoclonal V β expression in 39 cases (ie most frequently observed; Vβ14: n= 7, Vβ 8: n= 5, Vβ 7.1: n= 4), a potential defective repertoire in 2 cases and 3 patients showed two monoclonal clones. We did not observe any specific restricted Vβ subset. Associated auto-immune diseases or other neoplasms were associated in 70 and 34 cases, respectively. With a median follow-up of 58 months, 100 patients (44%) required a treatment, mainly for infections due to severe neutropenia (n=48), symptomatic auto-immune disease (n =28), transfusion-dependant anemia (n=15), and other causes (n=9). The onset of therapy was at diagnosis (n=43), within (n=31) or after (n=26) the 12 months following diagnosis. Patients were treated using steroids (n= 33), methotrexate (n=62), cytoxan (n=32), cyclosporine (n=24) either at first, second, third or fourth line therapy. The complete response rate (CR) and overall response rate (ORR) were as follows: steroids (12% and 3%), methotrexate (55% and 21%), cytoxan (66% and 45%), cyclosporine (21% and 5%), respectively. Four out of 13 patients responded to splenectomy. The mean number of treatment was 3.4 (1-7). There were 15 LGL leukemia related deaths. Estimated overall survival at 5 years was 75%. *Conclusions*. T LGL leukemia and chronic NK lymphocytosis dysplay similar clinico-biological features and response to treatment. Cytoxan induced a better response rate than methotrexate does.

0360

MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) IN FIRST-DEGREE RELATIVES OF PATIENTS WITH SPORADIC (NON-FAMILIAL) CHRONIC LYMPHOCYTIC LEUKEMIA

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Backgrounds. Although biological similarities have been described among MBL and CLL, the relationships between these two conditions are not fully understood, and new epidemiological studies in different populations and different countries continue to be reported. *Aims.* The goal of the study was to determine the prevalence, biological characteristics and evolution of MBL in first-degree relatives of families with just one case of CLL (sporadic CLL). *Methods.* We investigated 167 individuals from 42 families by 4-color flow cytometry assay as recommended by Rawstron *et al.* (Blood 2001;98:29-35). All subjects gave their written informed consent prior to entering the study. Purified B-lymphocytes CD19$^+$ of peripheral blood were selected by magnetic sorting and used for PCR (IgH/T-cell receptor) and FISH experiments (del 17p, del 13q, del 11q and trissomy of 12) in MBL cases. *Results.* MBL was found in seven individuals, from five families, of a total of 167 subjects (4,1%). The prevalence according to the age was 0 (0/54) in individuals with less than 40 years, 2,5% (2/81) between 40 and 60 years and 15,6% (5/32) in individuals over than 60 years. The Table shows the characteristics of MBL cases. With a median follow-up of 23 months, none individual has progressed to CLL.

Table. Age, sex and laboratorial characteristics of MBL cases.

Name	Family	Age/Sex	Ly#	MBL type	κ:λ ratio	PCR IgH
LPA	MPA	46/F	1,8	CD5$^+$/CD20$^{+bright}$/CD79b^{+dim}	0,08:1	Monoclonal
DRK	JRK	75/M	1,7	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	0,25:1	Negative
AAJ	TAO	72/M	1,3	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	1,28:1	Monoclonal*
BFR	LARB	53/M	1,2	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	1,12:1	Monoclonal
ARP	LARB	62/M	1,8	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	1,35:1	Monoclonal*
JGZ	MJZC	75/M	1,1	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	4,35:1	NP
PCZ	MJZC	61/F	4,0	CD5$^+$/CD20^{+dim}/CD79b^{+dim}	4,94:1	Negative

#Lymphocytes (x 10^6/mL)
*Also monoclonal for T-cell receptor.
NP: Not Performed.

From the 7 individuals that have been detected with MBL, 6 were analyzed by PCR and FISH. Clonal gene rearrangements of IgH were detected in 4 subjects. In two, we also found a clonal rearrangement of the gene TCRG of T-cell receptor in the MBL population. FISH experiments did not show any abnormality. *Summary and Conclusions.* This is the first study of MBL in first-degree relatives of patients with sporadic CLL. Monoclonality was detected in all cases either by an abnormal κ:λ ratio, or by PCR, or by both. Although chromosomal lesions associated with poor prognosis are rare in MBL, 13q deletion and trissomy 12 have been found in similar proportion in MBL to that seen in CLL. Our not significant FISH results are probably related to the small number of MBL individuals studied. The overall prevalence of 4.1% found in our population is similar to that of MBL in individuals of general population,

which varies between 3.5% and 5.5%. However, with regard to the prevalence in age groups, we have found a value of 15.6% (5/32) in individuals over 60 years, which is higher than values found for the same age group in general population, but very similar to the prevalence of 16.7% (3/18) found by de Tute *et al.* (Leukemia 2006;20:728-729) in familial CLL subjects over 60 years. Provided that the higher risk of emergence of clinical CLL in relatives of CLL families is probably related to the high prevalence of MBL in these subjects, our data strongly suggests that in old first-degree relatives of patients with sporadic CLL, the risk of MBL detection is as high as in old first-degree relatives from CLL families, which could render these individuals belonging to *sporadic CLL families* as susceptible as individuals belonging to *familial CLL* to the development of clinical CLL.

0361

NCRN CLL207 STUDY OF ALEMTUZUMAB CONSOLIDATION IN CHRONIC LYMPHOCYTIC LEUKAEMIA: REPORT OF THE PLANNED INTERIM ANALYSIS

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Backgrounds. The duration of remission in patients with chronic lymphocytic leukaemia (CLL) is highly dependent on the level of minimal residual disease (MRD) that remains at the end of therapy, regardless of the therapy used to achieve remission. The conversion of remissions from MRD positive to negative should prolong remissions and survival. Several small studies have used alemtuzumab as consolidation therapy following conventional chemotherapy with good efficacy but concerns over toxicity; primarily due to immune suppression and infections. The dose and timing (i.e. interval between prior chemotherapy and alemtuzumab) seem to be critical. *Aims.* The NCRN CLL207 Trial is designed to assess alemtuzumab consolidation post-chemotherapy in a phase II setting. Herein are the results from a planned interim analysis on the primary endpoints: eradication of MRD and assessment of safety. 25 patients have completed treatment, with a maximum of 54 patients planned to be recruited. *Methods.* The alemtuzumab dose of 30 mg was administered subcutaneously 3 times a week for 6 weeks, at which time patients had a bone marrow to assess response. MRD negative patients and non-responders stopped therapy; MRD positive patients with a significant response compared to pre-alemtuzumab continued therapy for a further 6 weeks. MRD was assessed in peripheral blood and marrow using 6-colour flow cytometry with a 0.01% detection limit. All patients received prophylaxis with co-trimoxazole (or equivalent) and aciclovir as well as weekly CMV monitoring by PCR. *Results.* Of the 25 patients who have completed alemtuzumab therapy, 23 had received fludarabine combinations as the latest therapy and 4 had received rituximab combined with fludarabine-based treatment. Alemtuzumab was stopped in 2 patients before week 6 due to toxicity, 19 patients received 6-8 weeks of treatment and 4 patients received a 12-week course. 8 (32%) patients required G-CSF during alemtuzumab. There were 12 reported SAE's in 10 (40%) patients, most of which were infections. 3 patients have died: one as a direct result of a treatment-related SAE (parainfluenza); one due to treatment-related MDS presumed to be related to prior therapy; and one due to progressive CLL. Positive CMV PCRs were detected in 12 (48%) patients, all of whom were successfully treated with pre-emptive antiviral therapy. After alemtuzumab, of 24 patients with known results, 18 (75%) became MRD negative, 4 (16.7%) remained MRD positive and 2 (8.3%) were not evaluable. 8 out of 9 (89%) patients who were MRD positive CR's at initiation of alemtuzumab became MRD negative. 10 out of 15 (67%) patients who were MRD positive PR's at initiation of alemtuzumab became MRD negative. *Conclusions.* 18 of 24 patients (75%) treated with alemtuzumab as consolidation therapy achieved MRD negative responses in their marrows. Such an approach is associated with mainly infective toxicities, which are largely manageable with recommended prophylaxis and close monitoring. These results support the continued investigation of alemtuzumab consolidation in CLL but primarily within a clinical trial setting and with appropriate monitoring of patients.

0362

NEW PROGNOSTIC MARKERS OF CHRONIC LYMPHOCYTIC LEUKEMIA IN THE EVERYDAY HEMATOLOGICAL PRACTICE. A MULTICENTER ANALYSIS

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Background. The opinions about the pathogenesis and prognostic factors of chronic lymphocytic leukaemia (CLL) have undergone significant changes over the past decade and a variety of new prognostic factors have been discovered. On the other hand, however, indication for the start of therapy continues to be based on the conventional hematological and clinical findings, while a range of new and effective therapeutic procedures is now used in the CLL therapy. Additionally, the methods used to detect new prognostic factors are not standardized, and it is also not clearly defined when these factors should be examined in CLL patients. The exact current role of the new prognostic markers of CLL therefore remains undefined. *Aims and methods.* The goal of the study was an analysis of the influence of conventional as well as new prognostic factors on overall survival (OS) of CLL patients. We retrospectively analyzed data of all patients entered into the databases of four big and independent hematological centers. The analysis of individual influence of each factor on OS, as well as multiparametric analysis of prognostic factors were performed. *Results.* The total of 1146 patients (median age 61 years, 32-90; sex: 707 male, 439 female) underwent the analysis. The median follow-up was 53 months (1-362). The median OS of all patients was 180 months; after excluding the patients who died from a cause different from CLL, the median OS was 233 months. In 353 patients (31%), the treatment of CLL was indicated. Through the use of uniparametric analysis, it was determined that advanced stage of CLL (Rai II-IV) ($p<0.001$), unmutated IgVH status ($p<0.001$), del/mut p53 ($p<0.001$), whether the cut-off is 5% or 20%, and increased expression of CD38 ($p=0.01$) have significant negative influence on OS. By contrast, uniparametric analysis did not prove either an unfavourable or a favourable influence on OS of these markers: +12, del ATM, del RB1 and cZAP70. The patients with unmutated IgVH, del/mut p53, del ATM, +12, cZAP70$^+$ or CD38$^+$ were treated significantly more frequently than patients without these changes. Multiparametric analysis revealed that OS is significantly influenced by sex (men with CLL have approximately three times higher risk of death than women; $p=0.02$), age ($p=0.04$), IgVH status ($p=0.03$) and del/mut p53 ($p=0.007$). When the patients who died from other causes than CLL were excluded from the analysis, a statistically significant influence on OS could only be found in one single factor: del/mut p53 ($p=0.004$). *Conclusion.* According to our analysis, the condition of p53 is the only prognostic factor that really and under all circumstances significantly influences the OS. Our analysis showed that this marker has the strongest predictive value even in an unselected large group of patients from a *real hematological practice*. For a non-selected patient group, the role of other factors is much less significant, and is likely diminished as well by the influence of new and more effective curative procedures.

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0363

LENALIDOMIDE INDUCED UPREGULATION OF CD80 ON LEUKEMIC CELLS CORRELATES WITH T-CELL ACTIVATION, THE RAPID ONSET OF A CYTOKINE RELEASE SYNDROME AND CLEARANCE OF TUMOR CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The immunomodulatory drug lenalidomide demonstrated encouraging activity in several lymphoid malignancies including chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). The mechanism of action has been hypothesized to include clearance of tumor cells through immune effector mechanisms. Despite the

absence of a pro-apoptotic effect on CLL cells *in vitro* patients typically have a rapid decrease in leukemic cell count with the start of treatment. Unique to CLL, lenalidomide causes a tumor flare syndrome that appears to be immune mediated and that has resulted in fatal complications. Whether immune activation correlates with anti leukemic activity has been controversial. Also, the basis for the particular side effect profile of lenalidomide in CLL is unclear. *Aims.*1) to describe and quantify the immune mediated effects in CLL patients treated with lenalidomide; 2) to test for correlations between immune activation and treatment response; 3) to derive predictors of immune activation, side effects, and treatment response. *Methods.* we conducted correlative analyses on clinical samples from patients with relapsed CLL enrolled on an investigator initiated, IRB approved, phase II study (registered under clinicaltrials.gov NCT00465127) of lenalidomide 20 mg (n=10) or 10 mg (n=8) daily for 3 weeks on 6 week cycles. We determined expression of costimulatory molecules on tumor cells and the activation marker CD69 on T-cells in PBMCs exposed to lenalidomide *in vitro*. We quantified cytokine levels in patient's serum, changes in lymphocyte subsets (CD4, CD8, NK, B) and effects on the lymph node environment during treatment (pretreatment vs day 8 biopsies). For comparison we included samples from patients with leukemic MCL in the *in vitro* analyses. *Results.* lenalidomide upregulated the costimulatory molecule CD80 on CLL and MCL cells but not on normal peripheral blood B cells *in vitro*. T-cell activation was apparent in CLL, weak in MCL, and absent in normal PBMCs and correlated with the upregulation of CD80 but not CD86 on B-cells. Strong CD80 upregulation and T-cell activation in the *in vitro* assay predicted more severe side effects (r=0.71, p=0.001), manifesting in 83% of patients as a cytokine release syndrome within 8-72 hours after the first dose. Within days, lenalidomide increased the inflammation marker CRP and caused a spike in inflammatory cytokines including TNFa, and IL-6. In most patients, lenalidomide rapidly reduced the number of circulating tumor cells but equally also the counts of T- and NK cells. In the matched lymph node biopsies we found no increase in T- or NK cells, even in patients with prominent lymph node swelling. CD80 upregulation on tumor cells correlated with clearance of leukemic cells from the peripheral blood (r=-0.79, p=0.006). In contrast, neither the clinical severity of the cytokine release syndrome, nor the degree of T-cell activation correlated with clinical response. *Conclusions.* upregulation of CD80 on tumor cells and T-cell activation correlates with unique toxicities of lenalidomide in CLL. T-cell activation appears to be dispensable for anti-tumor effects. This provides a rationale for combinations of lenalidomide with fludarabine or alemtuzumab.

0364

STANDARD AND REDUCED INTENSITY ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATIONS FROM RELATED AND UNRELATED DONORS FOR CHRONIC LYMPHOCYTIC LEUKEMIA. A LONG-TERM FOLLOW-UP (10 YEARS) STUDY FROM THE EBMT REGISTRY

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This retrospective analysis concerned 374 patients (pts) who underwent an allo-HSCT for chronic lymphocytic leukaemia (CLL) reported to the EBMT registry. There were 282 males (75%) and 92 females, median age of 53 years (24-69). The median interval diagnosis-transplantation was 53 months (3-308). Forty-five pts (12%) have received a previous hematopoietic stem cell transplantation (HSCT). At transplant, 302 among 323 evaluated patients had a good performance status (PS) (93%), 51 pts were in CR (14%), 163 in PR (45.5%), 39 in SD (11.5%) and 105 in PD (29%) among 353 evaluated patients. Two hundred and ninety-two pts received a standard (Std) and 82 a RIC; 314 pts received PBSC, 55 BM and 5 cord blood cells from 202 HLA siblings (Sib), 2 mismatched related donors and 170 unrelated donors (UD). There were 136 (36%) sex-mismatched (90 F/M and 46 M/F), 150 pairs (40%) had an ABO incompatibility (61 minor, 99 major) and for CMV: 80 pairs were +, 148 - and 112 mixed. 359 pts engrafted, 201 pts developed an AGVHD (gr I: 76, gr II : 79 , gr III: 30 and gr IV:16) and 153 presented a cGVHD (75 limited and 78 extensive). At day 100 after transplant, the cumulative incidence of AGVHD for the total population was 17% (13-22) for gr I, 31% (26-36) for gr ≥II. [Sib: 19% (13-25) gr I and 27% (21-34) gr ≥II; UD: 16% (9-22) gr I and 38% (29-47) gr ≥II] (Table 1). At 1 year after transplant for the total population, the cumulative incidence of limited and extensive cGVHD were 15% (6-24) and 29.5% (18-41) for Std; 18.6 (13-24) and 18% (13-23) for RIC respectively. With a median follow up of 38 months, the probability of 3-year and 5-year OS and DFS for the total group were 56% (51-62) and 47.4% (42-53); 49% (43-56) and 42% (36-48.5) respectively. We observed a significant difference concerning 5-year OS according to the pretransplant disease status [CR: 73% (60-89), PR: 57% (48-68) and PD: 35% (26-46)] (p<0.00001).There was no significant difference between standard and RIC HSCT in term of OS with 52.4% (42-66) and 47% (40-55.5) respectively (p=0.44) (Figure 1) [Std and RIC Sib: 51% (37-70) and 56% (47-67); Std and RIC UD: 60% (44-83) and 40% (29-55) respectively]. The multivariate analysis showed a significant impact of 3 factors on OS: age: HR=1.061 (1.02-1.10) p<0.0001, gender: HR=2.29 (1.02-5.11) p=0.04 and PS: HR=3.15 (1.40-7.10) p=0.005.The cumulative incidence of non-relapse and relapse mortality (NRM and RM) at 3 months and 1 year after transplant were: 10% (7-13), 5% (3-7) and 24% (19-28), 15% (12-19) respectively [Std:23% (11-35.6), 6% (0-14) 1 year; RIC: 22.5% (17.5-27), 18% (13-22) at 1 year]. This large retrospective analysis showed a high percentage of long-term OS after HSCT for CLL either after Std or RIC conditioning without any difference between the 2 groups except for the AGVHD (gr I, III and IV) where we had an higher incidence in the Std group, and for the RM with a higher level in the RIC group. Moreover, we demonstrated the important impact of disease status pretransplant (univariate); age, PS and sex-matching (multivariate) on the global OS.

Table 1. Cumulative incidence of AGVHD, NRM and RM according to HLA typing and kind of conditioning.

		RIC	Std
HLA siblings			
AGVHD (D100)	gr I	15% [9 - 20,5]	34% [19 - 48]
	gr II	15% [9 - 21,4]	13,5% [7 - 48]
	gr III -IV	11% [6 - 16]	12% [2 - 22]
NRM at 1 year		18,4% [12 - 25]	23% [11-35,6]
RM at 1 year		13% [8 - 18]	6% [0-14]
Unrelated Donors			
AGVHD (D100)	gr I	13% [6 - 20]	22% [4 - 39]
	gr II	22% [13 - 30]	26% [7,5 - 45]
	gr III -IV	14% [7 - 21]	22% [4 - 39]
NRM at 1 year		24% [16 - 32]	38% [17-65]
RM at 1 year		16% [9 - 23]	4% [0-12]

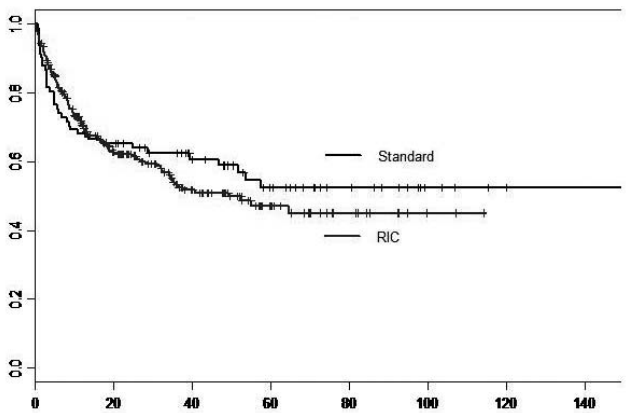

Figure 1. Probability of OS for standard conditioning group and RIC group.

Myeloma and other monoclonal gammopathies - Biology I

0365

INCREASED EXPRESSION OF MACROPHAGE INFLAMMATORY PROTEIN-1 ALPHA ON TREPHINE BIOPSIES CORRELATES WITH ADVANCED MYELOMA, EXTENSIVE BONE DISEASE AND ELEVATED MICROVESSEL DENSITY IN NEWLY DIAGNOSED PATIENTS WITH MULTIPLE MYELOMA

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*Background/Aims.*Macrophage inflammatory protein-1 alpha (MIP-1α) is a potent osteoclast stimulator and a chemoattractant for monocytes. Recent studies showed that monocytes may give rise to vascular endothelium in the myeloma microenvironment. The aim of our study was to evaluate, for the first time, the expression of MIP-1α in marrow biopsies of MM patients and explore possible correlations with clinical data, including angiogenesis. *Methods.* We evaluated MIP-1α and microvessel density (MVD) in formalin fixed paraffin-embedded bone marrow sections of 130 newly-diagnosed MM patients (66M/64F, median age 68 years) before any kind of therapy and in 10 MGUS patients. Immunohistochemical staining was performed using an anti-MIP-1α monoclonal antibody (Santa Cruz, CA, USA), while identification of microvascular endothelial cells was performed using an anti-CD34 monoclonal antibody (DAKO, Glostrup, Denmark). MVD was measured according to standard methodology. The MIP-1α immunoreactivity was examined on the basis of positive plasma cells (PCs) with the following cut-off values: <20% positive PCs (negative expression), 20-50% positive PCs (intermediate expression) and >50% positive PCs (high expression). Serum MIP-1α was measured in MM patients and 20 controls using ELISA (R&D, Minneapolis, MN, USA). *Results.* Fifteen patients (11.5%) had asymptomatic myeloma and 45 (35%) ISS3 disease. Plain radiography of the skeleton showed that 43 (33%) patients had no lytic lesions or osteoporosis only (grade A bone disease), while 24 (18%) had 1-3 lytic lesions (grade B), and 63 (48%) >3 lytic lesions and/or a pathological fracture (grade C). Thirty-seven (28%) patients had negative MIP-1α expression, 17 (13%) intermediate expression and 79 (59%) high expression of MIP-1α in the trephine biopsies, while low- (MVD 1-2), intermediate- (MVD 3-6) and high-grade (MVD >6) angiogenesis was observed in 42%, 28% and 30% of the patients, respectively. All MGUS patients had negative MIP-1α expression and low-grade angiogenesis. There was a significant correlation between MIP-1α expression on marrow PCs and MVD measurement (ANOVA p=0.015). MM patients had elevated median values of serum MIP-1α compared with controls [22 pg/mL (2.1-135 pg/mL) vs. 14 pg/mL (0.02-54 pg/mL); p<0.0001]. There was a strong correlation between serum MIP-1α ≥and MIP-1α expression on PCs (r=0.32, p<0.001). MIP-1α expression significantly correlated with the extent of bone disease. The median values (range) of MIP-1α positive PCs in biopsies was 10% (5-35%), 40% (18-60%) and 90% (70-100%), for patients with grade A, B and C bone disease, respectively (ANOVA p<0.0001). Serum MIP-1α also correlated with the extent of osteolytic disease (ANOVA p<0.0001). ISS3 patients had increased MIP-1α (+) PCs (median 60%, range: 20%-100%) compared with ISS1 (35%, 5%-100%; p=0.03) and ISS2 (45%, 5%-100%; p=0.04). Similar results were observed with serum MIP-1α (p<0.04). Increased immunoexpression of MIP-1α correlated with low platelet count, hypercalcemia, elevated serum creatinine, and PC infiltration. *Summary and Conclusions.* Our findings underline the high incidence of MIP-1α positive PCs in the biopsies of MM patients. The correlation between high MIP-1α expression with advanced myeloma, extensive lytic bone disease and increased angiogenesis confirms the role of MIP-1α in the biology of MM and reveals MIP-1α as a rational therapeutic target for the development of novel anti-myeloma agents.

0366

SECRETOME ANALYSES OF PRIMARY BONE MARROW FIBROBLASTS ISOLATED FROM MGUS AND MULTIPLE MYELOMA PATIENTS SUPPORT THE ROLE OF THE MICROENVIRONMENT FOR MYELOMA PATHOGENESIS

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Background. The microenvironment of tumor cells in the bone marrow may be actively involved in the progression of malignant diseases. In multiple myeloma (MM), interactions of bone marrow stromal cells with the malignant plasma cells have gained significant importance as targets for novel therapeutic agents. *Aims.*Based upon these observations, we aimed at analyzing in detail the secretory capacity of bone marrow (BM) fibroblasts obtained from patients with MGUS and MM in order to better understand their contribution to disease progression. *Methods.* We analyzed the secretome of primary bone marrow fibroblasts of patients with MGUS and MM by proteome profiling based on highly sensitive mass spectrometry. Results were compared to those obtained with normal BM fibroblasts. *Results.* The secretome profile of normal BM fibroblasts included various extracellular matrix (ECM) proteins including fibronectin, collagens and laminins, in addition to some chemokines and cytokines including CXCL12, follistatin-like 1, insulin-like growth factor binding proteins 4, 5 and 7; and SPARC. In contrast, the secretion profile of BM-derived fibroblasts from MGUS patients was altered: This included secretion of CSF-1, chitinase-3-like protein 1, insulin-like growth factor II (IGF-II), and insulin-like growth factor binding protein 6. All proteins specific for the MGUS background were also found in BM fibroblasts from MM patients. In addition to those we found specific secretion of stem cell growth factor, stanniocalcin-1, hamartin, and matrix metalloproteinase 28 as well as increased amounts of α-fetoprotein. Co-culture of primary MM cells with these fibroblasts further stimulated the secretion of ECM proteins. *Conclusions.* BM fibroblasts from MGUS and MM background display a specifically altered secretion profile, and BM fibroblasts from MGUS background already show tumor-promoting activities as indicated by the expression of IGF-II. Proteome profiling of secreted proteins may thus help to identify relevant tumor-associated proteins, to increase our understanding of cell cooperativity and thereby increase our understanding of progression events in monoclonal gammopathies.

0367

MICRORNAS AS KEY REGULATORS OF MULTIPLE MYELOMA CELL GROWTH

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Background. Detailed genomic studies have shown that cytogenetic abnormalities contribute to multiple myeloma (MM) pathogenesis and disease progression. Nevertheless, little is known about the characteristics of MM at the epigenetic level, and specifically how microRNAs (miRNAs) regulate MM progression in the context of the bone marrow milieu. Therefore, multi-level genetic characterization of MM is required to improve our understanding of the underlying molecular changes that lead to the initiation and progression of this disease; as well as to develop novel therapeutic agents that specifically target epigenetic abnormalities of clonal plasma cells. *Aims.* 1) To identify miRNA signature in MM. 2) To identify and validate the functional role of deregulated miRNAs in MM pathogeneis. 3) To provide the basis for the development of miRNA-based targeted therapies in this disease. *Methods.* We performed miRNA-expression-profiling of bone marrow-derived $CD138^+$ cells isolated from patients with MM, compared to their normal cellular counterparts and validated data by qRT-PCR. MM cell lines (MM.1S/RPMI8226/U266) were also studied. *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 on signaling cascades have been evaluated by western blot and immunofluorescence. NF-kB activity has been investigated using a DNA-binding-enzyme-linked-immunosorbent-assay-based-assay. *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.* We identified a MM-specific miRNA signature characterized by decreased expression of miRNA-15a, -16 and increased expression of miRNA-222/-221/-382/-181a/-181b ($p<0.01$). MM cell lines showed similar miRNA expression pattern to primary MM tumor cells. qRT-PCR was performed on matched samples and showed expression patterns similar to those observed in miRNA analysis. Using algorithms commonly used to predict human miRNA gene targets (miRanda/TargetScan/PicTar), predicted targets of the decreased miRNAs in MM patients included pro-angiogenic cytokines, oncogenes, cell cycle regulators, NFκB activators. Conversely, predicted target genes for the increased miRNAs in MM included cell cycle inhibitors, suppressors of cytokine signaling, and pro-apoptotic factors. Functional studies revealed that miRNA-15a and -16 regulate proliferation and growth of MM cells. Indeed, transfected cells showed decreased DNA synthesis; decreased cyclinD1/cyclinD3/cdc25a/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-NFκB 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 were validated both *in vitro* and *in vivo*. *Summary.* These data indicate that miRNAs play a pivotal role in the biology of MM; providing the basis for the development of new miRNA-based targeted therapies in this disease.

0368

LENALIDOMIDE INDUCED P27 KIP1-ASSOCIATED GROWTH ARREST IN MYELOMA CELLS THROUGH DOWN-REGULATION OF BOTH C-MYC AND SCFSKP2 UBIQUITIN LIGASE

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Background. Multiple Myeloma (MM) is a plasma-cell malignancy characterized by the accumulation of malignant plasma cells within the bone marrow. Lenalidomide is a new effective treatment of relapsed and/or refractory MM. Both the immuno-modulatory and anti-angiogenic effects of Lenalidomide emerge as potentially important mechanisms of its action. Furthermore, Lenanidomide counteracts the proliferation and survival pathways of MM cells but its mechanisms of action are still poorly understood. *Aim.* In the present study, we have investigated the molecular mechanisms responsible of the anti-proliferative effects of Lenalidomide on a large panel of human MM cell lines representative of MM molecular heterogeneity (n=14). *Methods.* Expression and regulation of key cell cycle and signal transduction pathway proteins were assessed by immunoblot analysis and quantitative PCR. *Results.* Lenalidomide significantly inhibits the proliferation of 10 of 14 MM cell lines (with IC50 values ranging from 0.2 μM to 8 μM). The sensitivity to Lenalidomide is independent of the genetic background of MM (types of 14q32 translocations). Four cell lines (JIM-3, Karpas 620, XG-6 and XG-7) of different molecular subtypes are resistant to Lenalidomide. Lenalidomide suppress the Erk/Mapkinase signaling pathway, either by inhibiting Erk1/2 or Erk5 whereas it has no effect on STAT-3 and PI3-kinase signaling pathway. Analysis of cell cycle distribution indicates that Lenalidomide provokes a marked decrease of the S phase and an accumulation of MM cells into the G1 phase. Cell cycle arrest was accompanied by a decrease of both PCNA and survivin expression and an up-regulation of p27. Kinetics study demonstrates that p27 expression increases during the first 72 hrs of stimulation, reaching a maximum at 72hrs. It is known that p27 expression is controlled both at the transcriptional level by c-myc and by multiple post - translational mechanisms. Indeed, p27 was thought to be regulated through the ubiquitin-proteasome pathway. In fact, p27 is recruited by SCFSkp-2 to be poly - ubiquitinated and the level of Skp-2 was found to be the rate-limiting regulator for the degradation of p27. For these reasons, we examined the level of c-myc and Skp-2 into MM cells exposed to Lenalidomide. We demonstrate that, parallel to p27 upregulation, both c-myc and Skp-2 protein expression were down-regulated in Lenalidomide sensitive cell lines. By performing kinetics study, we show an early down-regulation of c-myc which coincides with the increase of p27 followed by a decrease of Skp-2 and a stronger p27 up-regulation. Finally, we demonstrate by quantitative RT-PCR that p27 up-regulation is due in part to an increase of its transcription probably through c-myc down-regulation.

0369

THE MTOR PATHWAY ACTIVATION PREDICTS A SHORT TIME TO PROGRESSION AND OVERALL SURVIVAL IN RELAPSED MULTIPLE MYELOMA PATIENTS

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Background. The mammalian target of rapamycin (mTOR) is a serine/threonine-specific protein kinase, downstream of the phoshatidylinositol 3-kinase (P13-K/AKT) pathway. Recent findings highlight on the involvement of the mTOR signaling pathway in cancer cell proliferation, survival and in multiple drug resistance mechanisms. Previous studies demonstrated that rapamycin has preclinical potential as therapy for multiple myeloma (MM), especially when associated with other drugs. *Aims.* To evaluate the phoshporylation status of the mTor pathway (AKT, mTOR, P70S6K and 4E-BP1) and to correlate mTor positive status with clinical parameters, cytogenetic data and clinical outcome separately in newly diagnosed and relapsed MM patients. *Methods.* Immunohistochemical analysis with p-AKT (Ser 473), p-mTOR (Ser2448), p-P70S6K (Thr389) and p-4E-BP1 (Thr37,Trh46) was performed on bone marrow sections of 92 symptomatic MM patients (64 newly diagnosed and 28 relapsed). For each case, a value designed HSCORE was obtained multiplying the semi-quantitative intensity (0, no staining ; 1,weak staining; 2, moderate staining; and 3, strong staining) with the corresponding percentage of positive cells [HSCORE =Σ(1XPC), where 1 and PC represent intensity and percentage of cells, respectively]. The median value for each antibody was calculated and considered as the cut-off value. Fisher exact or chi square tests were used to correlate p-mTOR positivity to clinical data (age, isotype, β2-microglobulin, albumin, hemoglobin, creatinine and LDH serum levels) and molecular cytogenetic performed by FISH analysis. Curves for time to progression (TTP) and overall survival (OS) were plotted according to the method of Kaplan and Meier. *Results.* 27 out 64 (42.1%) newly diagnosed (group A) and 14 of 28 (50%) relapsed (group B) MM patients stained positive p-mTOR, respectively. In group A, 30 (46.8%), 30 (46.8%) and 28 (43.7%) cases also stained positive for p-AKT , p-P706SK and p-4E-BP1, respectively and a similar pattern was found in group B with 14 (50%), 13 (46.4%) and 15 (53.5%) p-AKT, p-P706SK and p-4E-BP1 positive, respectively. Overall, p-mTOR expression significantly correlated with p-AKT (r=0.4), p-P70S6K (r=0.5), and p-4E-BP1 (r=0.55) positive staining consistent with the hypothesis that the mTOR pathway is activated in a subset of myeloma cases. No difference was found between m-Tor positive and m-Tor negative patients in both group A and group B with respect to presenting clinical and cytogenetic characteristic. In group B, relapsed patients who expressed mTOR positivity (14 out 28) had a significantly shorter TTP (median time 6 vs 14 months $p=0.002$) and OS (median time 9 vs 16.5 months $p=0.03$) when compared with patients who did not. Multivariate Cox regression analysis adjusted for β2-microglobulin, del 13, del 17 and t(4;14) confirmed that mTOR positivity independently predicted short TTP in group B both as dicotomic (median value) and continuous variable. *Conclusions* In relapsed myeloma patients , mTOR positivity is closely associated with a short TTP that may be related to chemo-resistance. The mTOR pathway activation may be considered as a novel therapeutic molecular targets in relapsed myeloma patients to overcame chemo-resistance and in newly diagnosed myeloma patients to enhance chemo efficacy.

0370

ANTIBODY RESPONSES TO CANCER-TESTIS ANTIGENS IN MULTIPLE MYELOMA PATIENTS

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Background. Cancer-testis (CT) antigens show an expression restricted to malignancies and the human germline among healthy tissues and, therefore, represent attractive targets for immunotherapies. We and others have recently shown that CT antigens, such as MAGE-C1/CT7 and MAGE-A3, are frequently and specifically expressed in malignant plasma cells of patients with multiple myeloma. Furthermore, expression of CT antigens in myeloma represents an independent prognostic factor associated with higher rates of recurrence and reduced survival. Importantly, CT antigens are known to be capable of inducing spontaneous antibody and T cell-mediated immune responses. However, little is

known regarding spontaneous and therapy-induced immune responses against CT antigens in multiple myeloma. *Aim.* We set out to perform the first longitudinal analysis of antibody responses against a variety of CT antigens in multiple myeloma patients. This study was conducted to answer the question if and under which conditions humoral responses against CT antigens would occur in myeloma patients and if the existence of such immune responses had an influence on the patient's clinical outcome. *Methods.* Antibodies directed against CT-antigens (MAGE-C2/CT10, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A8, MAGE-A11, SSX-2, SSX-4, NY-ESO-1, PRAME) were analyzed by an ELISA using full-length recombinant proteins. The specificity of those antibodies was confirmed by Western blot analysis. *Results.* We screened 1100 sera of 194 patients with multiple myeloma at different time points for antibody responses against the 10 CT-antigens. The sera of 100 healthy volunteers were used as controls. Importantly, we identified a number of patients with significant titers against CT antigens, some of them consistently showing high-tittered responses. 51% of all myeloma patients were at least at one point of time antibody positive for MAGE C2 and MAGE A11, 33% for MAGE A8, 26,8% for MAGE A1, 26,8% for PRAME, 16,5% for SSX-2, 15,5% for MAGE A3, 15,5% for SSX-4 and 8.2% for NYESO-1. The control samples were all negative for antibodies directed against those antigens. Remarkably, in many cases titers were only induced by allogenic stem cell transplantation. Interestingly, patients with this transplantation-induced immune response seemed to have a longer overall survival compared to antibody-negative myeloma patients. *Conclusions.* We show in this first comprehensive and longitudinal analyses of humoral responses against CT antigens in myeloma that spontaneous occur and often persist over the course of the disease. Furthermore, allogenic stem cell transplantation can induce high titer humoral responses targeting CT antigens in multiple myeloma patients. This antigen-specific immune effect seems to improve the prognosis of these patients. We plan to further investigate these responses on the T cell level and we will analyse if the stimulation of the adaptive immune system is able to reverse the negative effect of CT antigen expression on the patients' prognosis.

0371

RARA2 PLAYS A CRUCIAL ROLE IN RETINOID-BASED THERAPY OF MYELOMA STEM CELLS

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Despite major advances in treatment by combining autotransplantation with the newer drugs such as thalidomide, bortezomib and lenalidomide, multiple myeloma (MM) remains largely incurable because of the existence of a drug-resistant population, which may be the myeloma stem cell compartment. Previous investigation showed that the $CD138^-$ myeloma cell fraction contains the MM stem cells. However, little is known about the molecular characteristics of MM stem cells, which makes it difficult to specifically target such cells. In this study, comparing gene expression profiling (GEP) analysis of $CD138^+$ and $CD138^-$ cells isolated from MM cell lines, we discovered that RARα is the top one up-regulated gene in CD138- MM stem cells. RARα has two major isoforms, RARα1 and RARα2. Real-time PCR detected significantly higher expression of RARα2 but not RARα1 in $CD138^-$ MM stem cells than $CD138^+$ cells. Using RT-PCR, we also found that RARα1 was ubiquitously expressed in myeloma cells, but RARα2 was expressed in myeloma cells from about 30% of newly diagnosed patients and MM cell lines. Furthermore, patients with RARα2 expression had shorter overall-survival. These results strongly suggest that RARα2 may be critical for *stemness* properties and drug-resistance of MM stem cells. Both RARα1 and RARα2 are specific receptors for ATRA; interestingly, we found that ATRA selectively killed RARα2-overexpressing CD138- MM stem cells while spare the $CD138^+$ MM tumor cells and over-expressing RARα2 in RARα2-deficient myeloma cells restored the sensitivity of MM cells to ATRA-induced myeloma cell death and growth inhibition, demonstrating a crucial role of RARα2 in ATRA-induced cytotoxicity in MM stem cells. Wnt signaling pathway is activated in $CD138^-$ MM stem cells and ATRA down-regulates Wnt signaling in RARα2-overexpressing MM cells. Interestingly, previous investigation showed that down-regulation of COX-2 is required for ATRA-mediated inhibition of Wnt signaling pathway. In line with this investigation we found that a combination treatment of ATRA and a COX2 inhibitor induced synergistic effects on the inhibition of Wnt signaling pathway, as assessed by β-catenin levels, cell death and growth inhibition of RARα2-overexpressing MM cells. Altogether, our study suggests a crucial role of RARα2 in maintaining the *stemness* of myeloma stem cells and provides new approaches for targeting MM stem cells in MM therapy.

0372

A NOVEL FISH-BASED NORMALIZATION PROCEDURE FOR WHOLE GENOME MICROARRAYS DETECTING ACCURATE LOCAL COPY NUMBERS: APPLICATION TO MULTIPLE MYELOMA

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Background. The introduction of Mapping arrays have significantly contributed to understanding of genomic disorders, providing high-resolution profiles of DNA copy number (CN) aberrations. However, CN inference using available conventional analysis procedures shows limitations in identifying the correct ploidy whenever the median CN value of the analyzed profile differs from normal ploidy; this is the case of marked aneuploidy characterizing most of human myeloma cell lines (HMCLs) and a significant fraction of primary myeloma tumors (MM). Aims. To correlate the local ploidy information given by FISH and Mapping arrays data, in order to robustly normalize genomic profiles and correctly infer local CNs. *Methods.* We developed a FISH-based normalization (FBN) algorithm, based on the evidence that Mapping arrays values of a genome profile tend to cluster as they represent different CNs. For each sample, at least one FISH probe information derived from highly purified populations was collected showing the same number of signals in >90% of cells. Using a k-means based algorithm, we determined all the clusters appearing on the frequency distribution of the array values, and linked them to exact inferred local CNs based on ploidy assessed by FISH. This allowed to estimate the scaling factor used to normalize the raw CNs of the entire array to their corresponding nominal multiplicity values. *Results.* We generated the FBN-corrected profiles of a subset of 25 HMCLs (profiled on GeneChip® Human Mapping 250K NspI arrays); we collected FISH data of 12 chromosomal loci critically involved in MM, and found complete and unambiguous information in the whole dataset for 4 probes (mapping to 1p31.3, 4p16.3, 13q14.3 and 16q23.1-23.2). We obtained a set of clusters of CN values for each sample, and correlated the ploidy as assessed by FISH with the cluster containing those values corresponding to the associated FISH probe, in order to infer the exact local CN values and subsequently the entire normalized CN profiles. Finally, we determined thresholds corresponding to different CN values. To validate the obtained profiles, the entire normalization procedure was repeated four times using each of the mentioned probes. After each of the four normalization processes, the most part of the available FISH data were correctly recognized using FBN procedure (93.3% accuracy of the estimated profiles). The FBN procedure was further applied and validated on a dataset of 45 MM samples profiled on GeneChip® Human Mapping 50K XbaI arrays. After normalization and generation of accurate thresholds, the obtained CNs were validated with additional FISH data, showing a concordance of 93.2% and thus indicating the correctness of FBN procedure. Moreover, the normalization allowed to identify a large part of MM samples (15%) showing marked aneuploidy, which conventional normalization procedures failed to identify. Conclusion. We propose a novel normalization procedure to detect the real multiplicity of local CN alterations combining FISH and mapping arrays; the method was robustly validated on MM and HMCLs datasets, providing an important contribution to define the genetic of myeloma tumors and, overall, to mapping arrays analysis procedures in the presence of complex aneuploid karyotypes.

0373

CARFILZOMIB: A SELECTIVE INHIBITOR OF THE CHYMOTRYPSIN-LIKE ACTIVITY OF THE CONSTITUTIVE PROTESOME AND IMMUNOPROTEASOME HAS ANTI-TUMOR ACTIVITY ON MULTIPLE MYELOMA, LYMPHOMA AND LEUKEMIA CELLS WITH MINIMAL EFFECTS ON NORMAL CELLS

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Background. Carfilzomib, the first in a new class of specific and selective proteasome inhibitors, induces apoptosis in multiple myeloma cells

with minimal off-target effects. Carfilzomib primarily targets the chymotrypsin-like (CT-L) active site in both of the broadly expressed constitutive proteasome (subunit β5) and the hematopoietically-restricted immunoproteasome (subunit LMP7) with less pronounced effects on non-CT-L proteasome active sites (β1 and β2 of the constitutive proteasome and LMP2 and MECL1 of the immunoproteasome). We and others have proposed that inhibition of the CT-L activity of the constitutive proteasome and the immunoproteasome is sufficient for anti-tumor efficacy while inhibition on non-CT-L activities may reduce the therapeutic window (Cancer Res 67:6383-91; J Biol Chem 281:8582-90). *Aims.* To quantitate constitutive proteasome and immunoproteasome levels in normal and transformed hematopoietic cells and evaluate the relative contribution of CT-L and non-CT-L activities to proteasome substrate accumulation, apoptotic signaling and cell viability. *Methods.* Immunoproteasome and constitutive proteasome levels in non-transformed peripheral blood mononuclear cells (PBMCs) and hematopoietically-derived malignant cells were determined using an ELISA that accurately quantitates proteasome active site subunits. The relative impact of CT-L and non-CT-L activities on proteasome substrate accumulation (transfected GFPu), apoptotic induction (caspase-3 activation) and cell viability was evaluated in myeloma (MM1.S, RPMI-8226), lymphoma (Arh77, HS-Sultan) and leukemia (Molt4) tumor cell lines or PBMCs with the following reagents: carfilzomib, novel proteasome inhibitors that selectively target β5 or LMP7 and a shRNA targeting β5. *Results.* Immunoproteasome expression was high in both primary patient samples and hematopoietic tumor cell lines. Based on LMP7 expression levels, the immunoproteasome constitutes >97% and 85-88% of the total proteasome in PBMC and CD138+ cells from healthy volunteers, respectively, and 61-80% of the total proteasome in CD138$^+$ cells from multiple myeloma patients. For hematopoietic tumor cell lines, the immunoproteasome expression accounts for 39-45%, 48-68%, and 49% of the total proteasome levels in myeloma, lymphoma and leukemia cells, respectively. Inhibition of a single CT-L subunit activity using LMP7- or β5-selective inhibitors, was insufficient to induce protein accumulation in HS-Sultan and had no impact on the viability of either hematopoietic tumor cell lines or PBMCs. However, inhibition of both β5 and LMP7, using a combination of a LMP7- and a β5-selective inhibitors or a β5/LMP7-selective concentration of carfilzomib, initiates a cascade of cellular events that starts with proteasome substrate accumulation followed by caspase-3 activation and finally cell death in tumor cells without causing cell death in PBMCs. Knockdown of β5 expression in HS-Sultan cells with an shRNA resulted in enhanced anti-tumor activity with the LMP7-selective inhibitor. Lastly, we noted that inhibition of all proteasome catalytic activities resulted in maximal induction of proteasome substrate accumulation, caspase-3 activation and cell death in both tumor cell lines and PBMCs. *Conclusions.* The immunproteasome is a major form of the proteasome expressed in hematopoietically-derived cells, including primary CD138+ myeloma cells. Inhibition of proteasome CT-L active sites, either by a combination of β5- and LMP7- selective inhibitors or carfilzomib is sufficient to produce an anti-tumor effect with minimal toxicity in non-transformed cells.

0374

CORRELATIONS BETWEEN ANGIOGENESIS, MIP-1 ALPHA EXPRESSION BY MALIGNANT CELLS AND MACROPHAGE COUNTS IN WALDENSTROM'S MACROGLOBULINEMIA: IMPLICATIONS INTO THE BIOLOGY OF THE DISEASE

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Background. Angiogenesis represents an essential step of disease progression in several hematological malignancies. A Mayo Clinic study reported that microvessel density (MVD) was increased (intermediate- or high- grade angiogenesis) in 30% of patients with Waldenstrom's Macroglobulinemia (WM), showed only weak correlation with marrow infiltration and had no impact on patients' survival [Rajkumar *et al.*, Semin Oncol 2003]. Macrophage inflammatory protein-1 alpha (MIP-1α) is a potent chemoattractant for macrophages and we have shown that serum levels of MIP-1α are elevated in WM [Terpos *et al.*, BJH 2006]. *Aim.* The aim of the present study was to evaluate the role of MIP-1α and macrophages in angiogenesis of WM. *Methods.* We investigated the association between MVD, MIP-1α expression by WM cells and the macrophage counts in trephine biopsies of 34 untreated patients with newly-diagnosed WM (3 with asymptomatic disease), and 11 with IgM-MGUS. Identification of microvascular endothelial cells was performed using an anti-CD34 monoclonal antibody (DAKO, Glostrup, Denmark) and MVD was measured according to standard methodology. When the microvessel count was 1-2, angiogenesis was characterized of low grade, while intermediate grade angiogenesis was defined by the presence of a microvessel count of 3-6 and high grade angiogenesis by the presence of microvessel count of ≥7. Bone marrow biopsies were also studied using double immunohistochemical staining for CD34 (endothelial cells) and CD68 (macrophages/mast cells) using respective antibodies from DAKO & Santa Cruz (CA, USA). We have also used double immunohistochemical staining for CD20/MIP-1α and for CD138/MIP-1α using an anti-MIP-1α human monoclonal antibody (Santa Cruz, CA, USA) to evaluate the MIP-1α expression by both CD20$^+$ and CD138$^+$ WM cells. *Results.* Thirteen patients out of 31 with symptomatic WM (41%) showed intermediate-grade and 4 (12%) high-grade angiogenesis. All patients with IgM-MGUS and asymptomatic WM had low microvessel count (median: 1, range: 1-3), while the median microvessel count for symptomatic WM was 4 (range: 1-8, p<0.01). There was a strong correlation between MVD and the number of macrophages into the *hot-spots* (r=0.823, p<0.0001). Furthermore, significant correlations were observed between the percentage of lymphoplasmacytoid cell's marrow infiltration with MVD (r=0.554, p=0.002) and macrophage counts into the *hot spots* (r=0.457, p=0.011). WM patients with intermediate or high grade angiogenesis had increased IgM levels (p=0.007), lower hemoglobin levels (p=0.024), and reduced platelet counts (p=0.043), while patients with high-grade angiogenesis had a tendency to higher incidence of lymphadenopathy compared with all others (3/4, 75% vs. 9/30, 30%; p=0.054). There was no correlation between angiogenesis and survival. We also observed that both CD20$^+$ and CD138$^+$ WM cells of all patients produced MIP-1α. Patients with increased counts of CD68$^+$ macrophages had also increased numbers of MIP-1α positive WM cells in their trephine biopsies (r=0.732, p<0.001). *Summary and Conclusions.* The results of this ongoing study suggest that intermediate to high-grade angiogenesis is present in a substantial number of WM patients. WM cells produce MIP-1α which possibly contributes to the chemoattraction of macrophages in the WM marrow microenvironment. These macrophages correlate with MVD and are possibly implicated into the biology of WM.

0375

ABNORMAL SERUM FREE-LIGHT CHAIN RATIO IN PATIENTS WITH MULTIPLE MYELOMA IN LONG-LASTING COMPLETE REMISSION: STRONG ASSOCIATION WITH OLIGOCLONAL BANDS

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Background. Complete remission (CR) in multiple myeloma (MM) is defined by negative immunofixation. The serum free light chain (FLC) assay can potentially allow the detection of free kappa and lambda levels below the threshold of immunofixation. The International Myeloma Working Group (IMWC) has recently proposed the so-called Uniform Response Criteria for Multiple Myeloma, incorporating the FLC kappa/lambda assay in serum to define stringent complete response (sCR). A normal FLC ratio (0.26-1.65) in addition to negative immunofixation in serum and urine plus less than 5% bone marrow plasma cells is required to fulfill the sCR criteria. However, there is limited data on the prognostic significance of sCR. *Aims.* To investigate the incidence of abnormal serum FLC kappa/lambda ratio in patients with MM in long-lasting complete response (CR) seen at a single institution. *Methods.* 29 patients (12M/17F, median age 51 years) with MM who reached a CR were studied. All patients had a negative serum and urine immunofixation for the original monoclonal myeloma protein. The serum FLC measurement (FREELITE' assay, The Binding Site Ltd., Birmingham, U.K.) was performed by immunonephelometry. *Results.* CR was achieved after autologous (20) or allogenic (6) stem cell transplantation or after chemotherapy alone (3). The median follow up in CR was 7.4 years (range 1.5-23 years). Sixteen of the 23 patients (55.2%) had an oligoclonal band in serum and/or urine. Nine of the 29 patients (31%) had an abnormal serum FLC ratio. All of them had a ratio above the upper normal limit, indicating a kappa excess production. Seven of the patients with abnormal FLC ratio (77.8%) had an oligoclonal immunoglobulin. In contrast, among the 20 patients (69%) with normal serum FLC ratio, only 6 (30%) had an oligoclonal spike (p=0.04, Fisher's exact test). More-

over, the median value of FLC ratio was 2.09 in patients with an oligoclonal band versus 0.83 for those with no oligoclonal immunoglobulines (p=0.003, T-test for independent samples). No statistical significance was found between abnormal FLC ratio and time from CR achievement and FLC assay. The 4 relapsed patients who have relapsed all had a normal FLC ratio. Updated results will be presented at the meeting. *Summary and Conclusions.* 1) 55% of patients with MM in prolonged CR had oligoclonal bands, 2) 31% of patients with MM in CR had an abnormal FLC ratio and almost 80% of these had an oligoclonal band, 3) Since the presence of oligoclonal bands is associated with good outcome, our results question the value of sCR.

0376

DASATINIB ENHANCED APOPTOSIS OF RAPAMYCIN IN MULTIPLE MYELOMA CELLS THROUGH DOWN-REGULATION OF THE MTOR PATHWAY BY ABROGATING SIGNALING VIA AKT AND ERK

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Background. Multiple myeloma (MM) is a malignant plasma cells disorder and as far as now remains an incurable disease. Previous studies have shown the *in vitro* and *in vivo* activity of the mTor inhibitor rapamycin and of the tyrosine kinase inhibitor dasatinib in MM. *Aims.* We evaluated the anti-myeloma activity of dasatinib in combination with rapamycin in MM cell lines and in primary human MM cells and investigated the critical role of the Src activity in the deregulation of mTor signalling pathway. *Methods.* MM cell lines KMS18 and RPMI8226 were used. Bone marrow samples of 10 MM patients were subjected to CD138 immunomagnetic purification. Rapamycin was used at the dose of 100 nM in combination with increasing doses of dasatinib (50, 250 and 500 nM) and fixed dose of 250 nM dasatinib was combined with increasing doses of rapamycin (10, 100 and 500 nM). Apoptosis was assayed at 24 and 48h after treatment by flow cytometry evaluating annexin V marker. Inhibition of cell proliferation was evaluated with tripan blu exclusion assay. Src expression was reduced in cell lines by stable expression of a plasmid encoding small interfering RNA (siRNA) to Src. Pharmacological down-regulation of Src expression was obtained with the selective Src inhibitor PP2 (10 μM). Western blot analysis was performed to evaluate the anti-myeloma activity of the compounds and to assess the effects of the disruption of Src kinase activity on the phosphorylation status of AKT, mTOR, P706SK, Src and Erk. *Results.* Single agents rapamycin and dasatinib resulted in 20% and 32% (p<0.05) annexin V staining in KMS18 cells at 24 and 48h, respectively. The combination of rapamycin 100 nM and dasatinib 250 nM resulted in the highest level of apoptosis (47% at 48h, p<0.05). The RPMI8226 cells were less sensitive, values ranging between 15-25% for annexin V staining to the drug combination treatment. Rapamycin/dasatinib combination also enhanced apoptosis at 48h in MM cells of four out five mTor positive patients (38-75% annexin staining, p<0.05) while were ineffective in all MM cells of the five mTor negative patients. In KMS18 cells rapamycin determined a significant down-regulation at 24 and 48h of p-mTor (60%) and p-P706SK (55%) and a slight decrease of p-Erk (30%) while was ineffective on p-Akt. The rapamycin/dasatinib combination resulted in a more robust dephosphorylation of AKT (90%), mTor (88%), P706SK (85%) and Erk (70%). Pharmacological (PP2) and molecular (siRNA) down-regulation of Src and Dasatinib treatment strongly dephosphorylate mTor, P706SK, Src, AKT and Erk (24 and 48h). These data consistent with the hypothesis that dasatinib enhanced rapamycin activity and down-regulated the mTor pathway by dephoshporylating Src, Akt and Erk. A similar, but less evident pattern was expressed by the RPMI8226 cells studied in the same conditions. *Conclusions.* The dasatinib/rapamycin combination appears to induce significant cell death in MM cell lines and in a subset of MM patients with complete down-regulation of p-AKT. The synergism of the combination resulted in the down-regulation of the mTor signalling mediated by Src by abrogating signalling via Akt and Erk.

0377

PTEN IS DOWN REGULATED IN MULTIPLE MYELOMA

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Background. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumor suppressor gene that degrades the product of phosphatidylinositol 3-kinase (PI3K). The loss of function of PTEN causes accumulation of critical messenger lipids, which in turn increase AKT phosphorylation and activity, leading to decreased apoptosis and/or increased mitogen signaling. PTEN has essential roles in restricting the activation of the hematopoietic stem cells, in lineage fate determination, and in the prevention of leukemogenesis. It is frequently deficient in human cancer such as breast, brain and prostate cancer, due to mutations or through an epigenetic gene silencing mechanism. PTEN has a pivotal role in affecting myeloma cell apoptosis and growth thus linking the PI3-kinase-AKT pathway to myeloma leukemogenesis, where mutation and deletion have been observed as the major mechanism of PTEN inactivation. The present study aimed to investigate the expression of PTEN in primary bone marrow samples from multiple myeloma (MM) patients. Methylation analysis of PTEN gene promoter was performed in a subset of samples. *Patients and Methods.* Thirthy MM patients (male 15; female 15; 68.8 median age. ISS stage: 47% stage I, 8% stage II and 45% stage III.), and 11 MGUS patients were retrospectively examined for PTEN expression. Diagnosis was established on the bases of standard clinical and laboratory parameters and the disease stage was graded according to the International Prognostic Staging System (ISS). Relative quantification in real-time RT-PCR of PTEN gene transcript in comparison to ABL as reference gene was done on an ABI Prism 7500 (PE Applied Biosystems, Foster City, CA). Relative quantitative determination of target gene levels was done by comparing Ct and presented as *fold change* of matched tumor samples vs normal cells.Ten patients with monoclonal gammopathy of undetermined significance (MGUS) and 4 peripheral blood from controls were also evaluated. The methylation pattern of PTEN were determined using the methylation-specific polymerase chain reaction after bisulphite modification of genomic DNA from bone marrows. For statistical analysis Student t and Fisher exact tests were used. *Results.* PTEN was found to be differentially expressed in MM, MGUS and controls. The mRNA levels of PTEN were significantly decreased in MM compared with MGUS with a 5,1-fold difference, (p=0.0013). PTEN expression resulted down-regulated (> 5 fold reduction) in the 54% of MM examined. Three out of 30 (10%) MM showed a down regulated PTEN expression with a differences ranging from 7- to 48-fold. Clinical and laboratory parameters showed that MM showing the lowest expression of PTEN evolved from MGUS after 7-11 years, had IgG K isotype and methylation of the p16 gene. When PTEN promoter hypermethylation was examined, aberrant methylation pattern was found in 5/30 (16%) MM but none of MGUS or control samples were methylated. Of these, 5 patients had IgG ∫ isotype, 4 had abnormal cariotype with 13q deletion, one hyperdiploidia, one IgH rearrangements. Two patients were simultaneously hypermethylated in p16 gene promoter. Aberrant PTEN methylation correlates with the lowest levels of mRNA suggesting gene promoter methylation as mechanism of PTEN inactivation in a subset of MM patients. No statistically significant correlation between loss of PTEN expression and clinical parameters: gender, age, isotype of M component, type of light chain, haemoglobin, serum albumin level, calcium, β2 microglobulin, serum creatinine level, LDH, lytic bone lesions were found. *Conclusions.* Our results, eventhough on small number, demonstrate PTEN down regulation as a result of promoter hypermethylation in a subset of MM patients. Since talidomide seems to have a role in the PTEN/PI3K/AKT pathway, the PTEN function is worthy to be further investigated in multiple myeloma patients.

0378

DIFFUSE MRI PATTERN CORRELATES WITH INCREASED ANGIOGENESIS, EXTENSIVE BONE DISEASE AND POOR PROGNOSIS IN NEWLY-DIAGNOSED, PRIOR-UNTREATED, PATIENTS WITH MULTIPLE MYELOMA

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Background and Aims. Magnetic resonance imaging (MRI) has been a useful technique for the assessment of patients with multiple myeloma (MM). Bone lytic disease is a major feature of MM, while increased angiogenesis is implicated in the myeloma cell growth and survival. The aim of this study was to evaluate the MRI pattern of marrow infiltration in newly-diagnosed myeloma and explore possible correlations with clinical and laboratory data, including myeloma bone disease, angiogenesis and survival. *Methods.* Eighty-two patients with newly diagnosed MM before the administration of any kind of treatment were studied. MRI of the thoracic and lumbar spine was performed and MR images were analyzed for patterns of myelomatous involvement. Four MRI patterns were identified: the normal, the focal, the diffuse and the variegated. Microvessel density (MVD) was evaluated in formalin fixed paraffin-embedded bone marrow sections of all patients using an anti-CD34 monoclonal antibody for identification of microvascular endothelial cells, while immunohistochemical staining for VEGF expression was also evaluated in all biopsies. Evidence of bone involvement at the time of diagnosis was documented using plain radiography. The following biochemical indices were measured in the serum of the patients on the day of MRI performance using ELISA methodology: i) osteoclast regulators (sRANKL, osteoprotegerin, osteopontin and MIP-1α), ii) osteoblast inhibitor dickkopf-1 (Dkk-1), iii) bone resorption markers (CTX, NTX, and TRACP-5b), iv) bone formation markers [bone alkaline phosphatase (bALP), and osteocalcin], and v) angiogenic cytokines [(VEGF, VEGF-A, angiogenin, angiopoietin-1, angiopoietin-2, and bFGF). *Results.* In MRI, 34 (41.5%) patients had focal pattern of marrow involvement, 26 (31.7%) diffuse, 18 (22%) normal, and 4 (4.9%) had a variegated pattern. In terms of MVD, 39% had low- (MVD 1-2), 41% intermediate- (MVD 3-6) and 20% high-grade (MVD >6) angiogenesis. Myeloma patients had increased values of sRANKL, osteoprotegerin, sRANKL/osteoprotegerin ratio, MIP-1alpa, NTX, CTX, TRACP-5b, Dkk-1, VEGF, VEGF-A, angiogenin, angiopoietin-2 and bFGF and reduced values of angiopoietin-1/angiopoietin-2 ratio compared to 36 healthy controls ($p<0.001$ for all comparisons). Patients with normal MRI pattern had normal values of Dkk-1, bone formation markers, angiogenin, and bFGF and only 39% of them had osteolysis in skeletal survey in comparison with 84% of patients with other MRI patterns who had osteolytic lesions ($p<0.001$). Patients with diffuse pattern of marrow involvement had reduced values of bone formation markers (OC and bALP, $p<0.01$), while patients with focal pattern had only a borderline reduction ($p=0.09$), although both diffuse and focal MRI patterns had increased Dkk-1 levels. MVD and VEGF expression in trephine biopsies was increased in patients with diffuse pattern compared to patients with focal ($p=0.037$) or normal pattern ($p=0.01$). Diffuse and variegated patterns of infiltration correlated with advanced disease stage ($p=0.018$), extensive bone disease ($p=0.033$) and poorer survival ($p=0.009$). *Summary and Conclusions.* These results suggest that patients with diffuse MRI pattern have suppressed bone formation compared to patients with focal or normal MRI patterns and subsequent extensive bone lytic disease. Diffuse MRI pattern correlates also with increased angiogenesis, advanced stage and poor survival suggesting a possible role of more aggressive therapies for these patients.

0379

CORRELATION BETWEEN LOSSES OF THE IGH OR ITS SEGMENTS AND DELETIONS OF 13Q14 IN MULTIPLE MYELOMA WITH TRANSLOCATION T(11;14) (Q13;Q32) DETECTED BY COMBINED ANALYSIS OF MORPHOLOGY AND FISH

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Background. Multiple myeloma (MM) is a malignancy of the plasma cells (PCs) characterized by a wide variety of genetic and chromosomal abnormalities. In recent years, major attention was drawn to the significance of chromosomal aberrations as a prognostic indicator in MM. The prognostic contribution of translocation t(11;14)(q13;q32) is ambiguous. Initially this translocation was reported by several groups as associated with a poor prognosis. Recently larger studies have demonstrated the improved outcome of MM patients with t(11;14) (q13;q32). Knowledge of the association between the translocation t(11;14) (11q13;q32) and established prognostic factors, such as Δ 13 and ploidy level, is important for evaluating the prognostic significance of this translocation and can give insight into the biology of MM. However, no correlation was found yet between deletions of the long arm of chromosome 13 [Δ13] and t(11;14)(q13;q32). The aim of this study was to analyze the correlation of the translocation t(11;14)(11q13;q32) with Δ13 on the level of single plasma cell (PCs). *Methods.* In this study we applied a combined cell morphology and FISH method for the analysis of coexistence of Δ13 and t (11; 14) (q13; q32) in PCs of 51 MM patients using several probes for the 13q14, 11q13 and IgH regions. *Results.* We found 15 different variants of the translocation t (11; 14) (q13; q32). These variants are the result of deletions of Variable or Constant IgH segments and also duplications and losses of the IgH gene on the normal non-translocated chromosome 14 as well as into IGH/ Cyclin D1 (CCND1) fusion on der 14 and CCND1/IGH fusion on der (11). A strong association between Δ13 and specific variants of t(11;14)(q13;q32) was found: variants with deletion of the IgH gene or its segments were found only in MM cases with deleted chromosome 13, while the common translocation t(11;14)(q13;q32) was found only in the MM cases with normal chromosome 13q. *Conclusions.* We were able to demonstrate a high heterogeneity of translocation t(11;14) in BM samples from MM patients using a combination of morphology and FISH analyses , that was overlooked in previous investigations. Moreover, we have shown the strong association between two chromosome alterations, Δ13 and deletions of the IgH gene or its segments in the group of MM patients with translocation t(11;14)(11q13;q32). Therefore we suggest that the prognostic impact of the deletions of the IgH gene or its segments should be studied in this group.

Myeloma and other monoclonal gammopathies - Clinical I

0380

BUSULPHAN PLUS MELPHALAN (BUMEL) VERSUS MELPHALAN-200 MG (MEL) AS CONDITIONING REGIMENS FOR MULTIPLE MYELOMA (MM): A SEQUENTIAL PROSPECTIVE COMPARATIVE STUDY OF EFFICACY AND TOXICITY

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Introduction. Autologous stem cell transplant (ASCT) has become the standard of care in young newly diagnosed MM patients. Few studies have been prospectively conducted in order to evaluate different conditioning regimens, and in fact Melphalan 200 mg/m^2 is universally used as a standard.

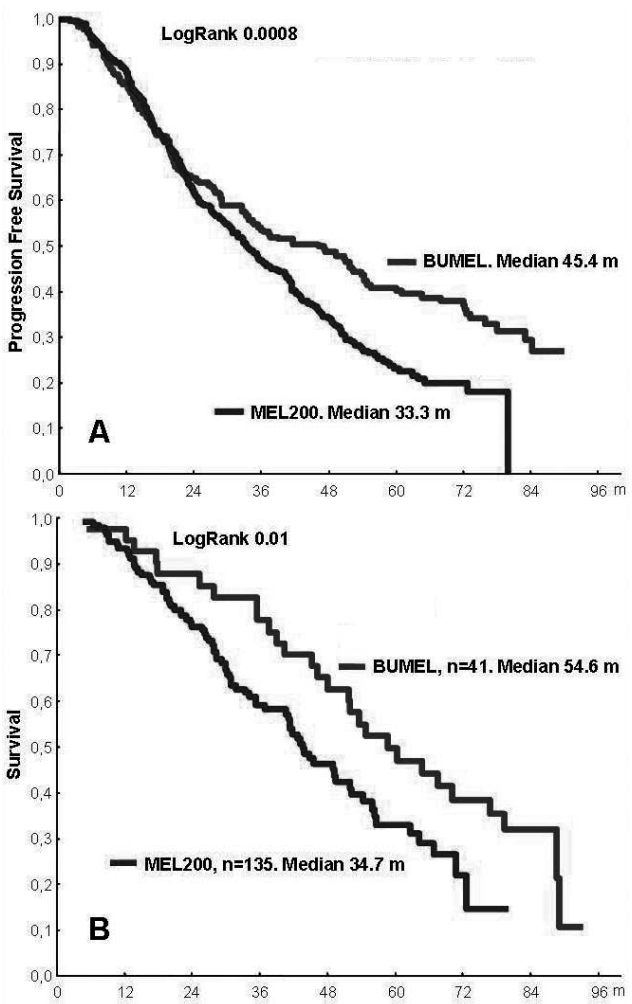

Figure.

Material and Methods. We have evaluated the efficacy and toxicity of two conditioning regimens in newly diagnosed MM patients included in the Spanish Pethema/GEM 2000 trial. 248 patients were conditioned with oral Busulphan at dose of 12 mg/Kg in combination with MEL 140 mg/m^2 and 554 with MEL 200 mg/m^2. All patients had previously received six cycles of alternating vincristine, BCNU, melphalan, cyclophosphamide, prednisone/vincristine, BCNU, Adriamycin, dexamethasone (VBMCP/VBAD). *Results.* Baseline characteristics were similar in both cohorts of patients, as well as the response rate to induction VBMCP/VBAD chemotherapy. Time to engraftment after transplant was also similar in both groups. Overall non-hematologic toxicities were higher in the BuMel arm (64% vs. 52%; *p*:0.002), as well as BuMel-related mortality (7,4% vs. 3,2%; *p*=0.009); hepatic toxicity, reported as sinusoidal obstruction syndrome (SOS) was the most relevant non-hematologic toxicity (observed in 8% of patients in the BuMel arm as compared to 0,4% in MEL arm; *p*:0.0001). Mortality directly associated to SOS was 2% and 0,2% in BuMel and MEL arms, respectively (*p*:0.026). There were not differences in the response rate (≥ partial response) at 100 days after ASCT: 92% in the MEL arm (36% CR with negative Immunofixation) vs. 91% for BuMel (38% CR IF-). With a median follow-up of 51 m, the median progression free survival (PFS) was significantly longer for BuMel (46,4 vs. 33.4m; *p*=0.002); however, not differences were observed in median overall survival (OS) between both arms (BuMel 79.3 vs MEL 70m; *p*=0.4). Nevertheless, the rescue therapy administered at the moment of first relapse clearly influenced the final outcome: patients who received bortezomib-based combinations had a significantly longer OS as compared with thalidomide-based combinations (41,3 vs. 34.3m; *p*=0.0008) vs. chemotherapy or steroids (25,8 m; *p*<10-5). Nevertheless, patients receiving BuMel were treated at an earlier time period (1999-2002) and the availability of IMiD's and bortezomib-based combinations as rescue therapies was more limited for these patients as compared to those subsequently treated with MEL. If we select the group of patients who received novel agents at the moment of relapse, median OS from diagnosis was significantly better for patients receiving BuMel as compared with pts treated with MEL and rescued with novel agents (54.6 vs. 34.7m; *p*=0.01). *Conclusions.* Conditioning with BuMel is associated with longer PFS but equivalent OS as compared with MEL. The absence of differences on OS could be due to the limited availability to novel rescue therapies in patients conditioned with BuMEL. Since we have used oral Busulphan without monitorization of serum levels and appropriate dose adjustment, it could be argued that the use of intravenous Busulphan would reduce the toxicity and eventually lead to a higher efficacy in MM patients.

0381

TANESPIMYCIN AND BORTEZOMIB IN PATIENTS WITH RELAPSED AND RELAPSED AND REFRACTORY MULTIPLE MYELOMA: FINAL RESULTS OF A PHASE 1/2 STUDY

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Background. Tanespimycin disrupts HSP90, a key molecular chaperone for signal transduction proteins critical to myeloma (MM) growth, survival, and drug resistance. Preclinical data show antitumor synergy between tanespimycin and bortezomib and suggest tanespimycin may be neuroprotective, including reversibility of bortezomib-induced peripheral neuropathy. A phase 1 study of single agent tanespimycin in advanced MM showed favorable tolerability and modest clinical activity. *Aim.* To assess the efficacy and safety of the combination of tanespimycin and bortezomib in patients with relapsed/refractory MM. *Methods.* Seventy-two (72) patients with relapsed/refractory MM received 0.7-1.3mg/m^2 bortezomib IV bolus followed by a 1-hr infusion of 100-340 mg/m^2 tanespimycin on days 1, 4, 8, and 11 in each 21-day cycle, with 42 patients receiving the highest dose of both drugs as part of a phase 2 expansion. Toxicities were assessed by CTCAE v3 and response rates were measured using modified EBMT criteria. *Results.* Of 72 patients, 72% had IgG subtype with a median age of 60 years. Median time since MM diagnosis was 50 months with median of 5 (range 1-15) prior regimens. Prior treatments included stem cell transplant (69%), thalidomide (74%), bortezomib (69%), lenalidomide (28%) and Hsp90

inhibitors (13%). Fifty-eight (58) patients with measurable disease were treated at 1 or 1.3 mg/m^2 bortezomib. Response rates (≥ MR) were 41%, 20%, and 14% in the bortezomib-naive, pretreated, and refractory patients, respectively. In the subgroup of patients with 1 to 3 prior therapies and who were bortezomib-naive, response rate was 56%. Median duration of response was 10.7 months (n=14 patients), including 3 bortezomib-refractory patients each with durable partial responses (PRs) through months 12, 22, and 28, respectively. Three additional patients remain in response through 24 months. The most frequent adverse events (AEs) were diarrhea (60%), nausea (49%), fatigue (49%), thrombocytopenia (40%), and AST elevation (28%), which proved manageable with dose reduction and supportive care. Most frequent Grade ≥3 AEs included: thrombocytopenia (25%); diarrhea, anemia and fatigue (7% each); back pain and AST elevation (4% each). Only 21% of patients had Grade 1 or 2 peripheral neuropathy; no Grade ≥3 peripheral neuropathy was observed. Only 4.2% of patients had any Grade neutropenia and 2.8% of patients had Grade ≥3 neutropenia. *Summary and Conclusions.* The combination of tanespimycin and bortezomib is active and well tolerated in relapsed/refractory MM, with durable responses in bortezomib-naive, pretreated, and refractory patients. Median duration of response for the combination compares favorably with historical bortezomib monotherapy. Final PFS data will be presented. No severe peripheral neuropathy has been observed, consistent with tanespimycin's neuroprotective effect in preclinical models. The incidence and severity of neutropenia compares favorably with historical bortezomib monotherapy. A phase 3 study of the combination of tanespimycin and bortezomib versus bortezomib monotherapy is ongoing.

0382

COMBINATION OF THALIDOMIDE, PEGYLATED LIPOSOMAL DOXORUBICIN, DEXAMETHASONE AND BORTEZOMIB (THADD-V) IN RELAPSED/REFRACTORY MULTIPLE MYELOMA: RESULTS OF A PHASE II STUDY

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Background. ThaDD combination has been found to be an effective treatment for patients both with newly diagnosed and relapsed/refractory multiple myeloma (MM) (Offidani *et al.*, Blood 2006, Haematologica 2007). *Aim.* In an attempt to enhance the efficacy of ThaDD regimen we added bortezomib in a phase II study. *Methods.* ThaDD-V regimen consisted of thalidomide 100 mg/d continously, dexamethasone 20 mg days 1-2, 4-5, 8-9, 11-12, pegylated liposomal doxorubicin 30 mg/m^2 on day 4 and bortezomib 1.3 mg/sm days 1, 4, 8, 11. Due to a high incidence of > grade 2 peripheral neuropathy in the first 20 patients, the regimen was subsequently amended (thalidomide 50 mg/day, bortezomib 1.3 mg/sm days 1, 4, 11). Each course was repeated every 28 days for a total of 6 courses followed by 3 cycles consolidation with bortezomib 1 mg/sm days 1, 8 and dexamethasone 20 mg days 1-2, 8-9 alternated with 3 courses including thalidomide 50 mg/day for 28 days and dexamethasone 20 mg on days 1-4 and by maintenance with Thalidomide 50 mg/day until relapsed or toxicity. Patients received anti-bacterial (ciprofloxacin 250 mg bid), anti-viral (valaciclovir) and anti-thrombotic (ASA 100 mg/d or enoxaparin 40 mg/d) prophylaxis. Responses were assessed as per the International Response Criteria. *Results*. Forthy patients, median age 62 years (range 31-83) with relapsed or refractory MM were enrolled. ISS at least II in 73% and 14% of patients presented impaired renal function. Twenty-eight patients received ThaDD-V as second line of therapy, 12 as third or subsequent lines. Twenty-one (52%) had undergone previous ASCT whereas 21 (53%) and 6 patients (15%) had received prior thalidomide or bortezomib, respectively. Among 28 patients with FISH cytogenetics available, 12 (43%) showed unfavourable abnormalities. After at least 4 courses of therapy, 85% of patients achieved at least a PR, 67.5% at least a VGPR and 35% at least a CR with a 15% of patients obtaining a sCR. After a median follow-up of 25 months, median TTP, PFS and OS were 28 months, 23 months and not reached, respectively. Response to ThaDD-V was the only significant factor affected TTP and PFS whereas ISS, β2m, cytogenetics and previous thalidomide did not. Patients achieving at least a VGPR had a significantly higher TTP if compared with those obtaining a lower response (median 30 vs 8 months; p<0.001). Moreover, all 6 patients achieving sCR are still in remission and alive. Grade 3-4 thrombocytopenia and neutropenia occurred in 17% and 5% of patients. Main non-hematologic toxicities included grade 3 peripheral neuropathy (12%), fatigue (7%), diarrhoea (5%) and skin rash (2%). Four patients developed grade 3-4 infections (one fatal) and 2 DVT. Only one patient was withdrawn from the protocol because of toxicity, but 10 patients interrupted bortezomib because of neuropathy (5 patients) and thrombocytopenia (5 patients). Four patients discontinued thalidomide because of neuropathy (2) and thrombotic events (2). *Conclusions.* ThaDD-V is a very active and well tolerated regimen in patients with relapsed/refractory MM; it seems to induce durable responses besides adverse prognostic factors provided at least VGPR is achieved.

0383

A MULTICENTER PHASE II CLINICAL TRIAL OF LENALIDOMIDE, MELPHALAN, PREDNISONE AND THALIDOMIDE (RMPT) IN PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA

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Background. Lenalidomide has shown significant antimyeloma activity in different clinical studies. In newly diagnosed multiple myeloma (MM) patients the addition of new drugs, lenalidomide or thalidomide or bortezomib, to the standard oral melphalan and prednisone (MP) combination significantly increased response rate and event-free survival. In advanced MM, the 4 drug combination VMPT further improves response rate. *Aims.*We conducted a multicenter open label phase I/II trial combining lenalidomide, melphalan, prednisone and thalidomide (RMPT) to determine safety and efficacy of this novel regimen in patients with relapsed/refractory multiple myeloma. *Methods.* Oral lenalidomide was administered at 10 mg/day on days 1-21, oral melphalan at 0.18 mg/kg on days 1-4 and oral prednisone at 2 mg/kg on days 1-4. Thalidomide was given at 50 mg/day (Arm A) or 100 mg/day (Arm B) on days 1-28. Each course was repeated every 28 days for a total of 6 courses. Maintenance therapy included lenalidomide alone at 10 mg/day on days 1-21, until progression. Aspirin 100 mg/day was given as a prophylaxis for thrombosis. *Results.* Forthy-four patients with relapsed or refractory MM were enrolled. Median age was 69 years (range 47-80). All patients had been already treated with a median of 2 previous lines of treatment: 26 patients received RMPT as second line of therapy and 18 as third line. Twenty patients received prior autologous transplant, 3 allogeneic stem cell transplant, 8 patients received conventional chemotherapy, 10 patients received thalidomide-based regimen and 9 bortezomib-based regimen. After a median of 5 courses, 75% of patients achieved at least a partial response (PR), including 20% very good partial response (VGPR) and 14% of patients achieving near or complete response (CR or nCR). Among 26 pts who received RMPT as second line therapy the PR rate was 73%, including VGPR 23% and CR/nCR 19%. Among patients who received thalidomide 100 mg, the PR rate was 82% (including VGPR 23% and CR/nCR 23%) compared to 68% of thalidomide 50 mg. The 1-year-progression-free survival was 51,5% and the 1-year survival from study entry was 72%. Grade 3-4 hematologic adverse events included: neutropenia (63%), thrombocytopenia (33,8%) and anemia (34,1%). Grade 3-4 non hematologic adverse events included: infections (22,3%), neurological toxicity (6,6%) and fatigue (6,8%). No thromboembolic events grade 3-4 were reported. *Conclusions.* RMPT is an effective salvage therapy with a high proportion of responses. Side effects were predictable and manageable. No thromboembolic complications were reported. An update of these data will be presented at the meeting.

0384

THALIDOMIDE-INTERFERON VS. INTERFERON MAINTENANCE THERAPY AFTER INDUCTION WITH THAL-DEX OR MP IN ELDERLY PATIENTS WITH MULTIPLE MYELOMA

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Background and Aims. Thalidomide maintenance therapy has been studied in a limited number of trials mainly after high dose therapy.

Here, we evaluated the efficacy of Thalidomide in combination with Interferon-α2b (Thal-IFN) in comparison to Interferon-α2b (IFN) maintenance treatment after first line treatment with either Thalidomide-Dexamethasone (Thal-Dex) or Melphalan-Prednisolone (MP). *Methods.* 289 patients (median age 72 years) had been randomized to either Thal-Dex or MP induction therapy. 249 of those received ≥2 cycles and 135 achieved stable disease or better. 128 of the 135 patients with ≥ stable disease after either Thal-Dex or MP induction therapy had finally been randomized to either Thal-IFN or IFN maintenance treatment. The starting dose for Thal maintenance was 100mg/day, and that for IFN maintenance 3 Mega U, TIW. Progression-free and overall survival have been estimated by the product limit method. *Results*. The outcome of the induction therapy has already been presented (BLOOD 2009). In short, Thal-Dex achieved a higher response rate compared to MP (69% vs. 50%, $p<0.0023$), but overall survival with Thal-Dex was shorter (41.5 vs. 49.4 months, $p=0.024$) mainly due to increased toxicity in elderly (age >75 years) patients with poor PS. The median follow up of after randomization to maintenance therapy was 32.3 months. The median duration of maintenance therapy was longer with Thal-IFN compared to maintenance with IFN only (10.2 months vs. 7.7 months, $p<0.02$). The cumulative dose of Thal was 27.200 mg (median) and the average daily dose was 75 mg. Maintenance with Thal-IFN resulted in significantly longer progression-free survival compared to IFN (24 vs. 12.6 months, $p<0.024$). Overall survival from start of maintenance treatment, however, did not differ between the Thal-IFN (52.6 months) and the IFN group (52.2 months, $p=0.68$) and was remarkably long. Likewise, no difference was observed when survival after start of maintenance was analyzed by first line therapy (Thal-Dex or MP). Progression free survival tended to be shorter in patients with unfavourable cytogenetics (t(4;14), t(14;16), del 17p, gain of 1q21) compared with standard risk patients (15.4 vs. 29.8 months, $p=0.088$). Median survival was 51.4 months in the former group while the median of survival has not yet been reached in the standard risk patients ($p=0.42$). Thal-Dex maintenance was associated with more grade 1-3 neuropathy (51% vs. 38%, $p=0027$), grade 1-2 constipation (44% vs. 18%, $p<0.0007$), and grade 1-2 skin toxicity (29% vs. 115, $p<0.009$). Maintenance IFN alone was initially relatively well tolerated but was discontinued earlier. *Summary and Conclusions.* Maintenance therapy with Thal-IFN resulted in significantly longer progression-free survival but overall survival did not differ between both groups

0385

REVERSAL OF ACUTE RENAL IMPAIRMENT BY BORTEZOMIB-DOXORUBICIN-DEXAMETHASONE IN MULTIPLE MYELOMA. RESULTS FROM AN PHASE II STUDY

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Background. Acute light chain induced renal failure (ARF) is a severe complication of progressive multiple myeloma (MM), often leading to permanent renal dysfunction and dependence on chronic hemodialysis in a substantial proportion of patients. Reversal of kidney failure can only be achieved by fast and substantial suppression of pathogenic light-chains with effective anti-MM therapy. *Aims.* To evaluate prospectively the efficacy of the bortezomib-doxorubicin-dexamethasone (BDD) regimen in restoring renal function and in achieving tumour control in patients with light chain induced acute renal failure. *Patients and Methods.* 72 patients have been enrolled (age: median 66 years, range 40.7-82 years, DS stage I: 4%, II: 10%, III: 76%. 81% of patients presented with *de novo* MM, and 19% with progressive disease. ARF was defined in newly diagnosed patients as reduction of GFR to <50 mL/min and in previously treated patients as an acute reduction of GFR by >25% to <60 miL/min in patients who had a GFR of >60ml/min within 4 weeks before deterioration of renal function and documented progressive disease. Light-chain induced nephropathy had to be established as cause of ARF either by clinical findings or by renal biopsy. Treatment regimen: Bortezomib (1.3 mg/m^2, d 1, 4, 8, 11 until the first safety analysis and thereafter of 1.0 mg/m^2 d 1, 4, 8, 11), doxorubicin (9 mg/m^2, d 1, 4, 8, 11 until first safety analysis and thereafter of 9 mg/m^2, d 1, and 4) and dexamethasone 40 mg (d 1, 4, 8, 11). Mandatory antibiotic and antibacterial prophylaxis was instituted after the safety analysis. Cycles were repeated every 21 days. *Results*. 58 patients have completed at least 2 cycles and are evaluable for response as yet. Twenty seven patients (46.5%) achieved CR/nCR, 7 VGPR, 5 PR and 5 MR (CR-PR: 67%, CR-MR: 75.8%): Median GFR at baseline was 16.8 ml/min (range: 4-48 mL/min) and improved to 58 ml/min (range: 6.7 - 134 ml/min). Improvement of GFR correlated weakly with tumour response. GFR increased to a median of 64.6 ml/min (20.2-134 mL/min) in 34 patients with CR/nCR or VGPR, to 30.7 mL/min (14.7-55.3 mL/min) in 10 patients with PR or MR, and remained rather stable at 19.6 mL/min (6.7-57.9 mL/min) in 10 patients without anti-myeloma response (SD or PD). 62% of patients achieved a renal response and 31% a complete renal response (GFR>60 mL/min). Median time to best renal response was 4.4 months. Patients with severe renal impairment (anuria/oliguria and/or very low GFR (<15 mL/min) were less likely to achieve reversal of their renal failure even if they responded to anti-myeloma therapy. Overall Survival @ 2 years was 64% (CI: 50-82) in the ITT and 73% (CI: 59-92) in the evaluable patient population. A tendency towards better OS was seen in patients with renal response. Toxicity data are available in 49 patients as yet: The following G3-4 toxicities were observed: infections (16%), neutropenia (16%), cardiovascular (10%), weakness (10%), and thrombopenia and acute hearing loss in 5%. Grade 1-2 toxicities: infections 21%, neutropenia 16% and weakness, diarrhoea, GI bleeding in 5%. Of note, 4 of the 7 infectious complications were due to herpes virus infections/reactivations. *Summary and Conclusions.* The BDD regimen resulted in an overall renal response rate (improvement in stage of renal impairment by 2 stages-or better) of 62% and in a complete renal response rate (GFR>60 mL/min) of 31%. Significant anti-myeloma activity (CR/nCR/VGPR) was seen in 58% and CR-PR in 67% of patients. The increase in GFR correlated weakly with major anti-myeloma activity. The BDD regimen was well tolerated after dose adjustment. Updated results will be presented at the meeting.

0386

THE PRESENCE OF RESIDUAL NORMAL PLASMA CELLS BY MULTIPARAMETER FLOW CYTOMETRY IN THE BONE MARROW OF SYMPTOMATIC MULTIPLE MYELOMA PATIENTS AT DIAGNOSIS IDENTIFIES A GOOD PROGNOSTIC SUBGROUP

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Background. Multiparameter flow cytometry (MFC) immunophenotyping allows the discrimination between normal (N-PC) and myelomatous plasma cells (PC) within the bone marrow plasma cell compartment (BMPC). While in most MGUS and smoldering MM the BMPC contains N- PC, in symptomatic MM patients usually only clonal PC are identified. *Aims.*The aim of the present study is to investigate the frequency, characteristics and outcome of MM patients showing at diagnosis >5% N-PC among the BMPC. *Methods.* A total of 811 untreated symptomatic MM patients were included in the present study, all of them uniformly treated following the Spanish GEM2000 protocol. For the correlation with cytogenetics, we selected patients at diagnosis (N=501) in which cytogenetic/FISH was performed on enriched plasma cells using immunomagnetic separation. For MFC analysis, BM samples were stained using a four-color direct immunofluorescence technique that allowed the identification and characterization of the BMPC compartment. *Results.* We found that 88 cases (11%) had >5% N-PC/BMPC. Interestingly, patients with >5% N-PC/BMPC show a favourable profile at diagnosis compared with cases with ″5% N-PC/BMPC, with lower age (median of 58 vs. 60 years; $p=0.05$), lower frequency of IgA myeloma subtype (23% vs. 28% of patients; $p=0.04$), lower levels of serum

β2M (>3.5 mg/L: 33% vs. 46%; p= .01) and Monoclonal Component (≥3 g/dL: in 44% vs. 75%; p<0.001), lower BMPC infiltration by optical microscopy (>30% PC: in 17% vs. 45%; p<0.001) and by MFC (>5% PC: in 17% vs. 75%; p<0.001) and absence of immunoparesis (58% vs. 16%; p=.003). Also, they have higher levels of hemoglobin (>100 g/L: 80% vs. 56%; p<0.001), albumin (>3.5 g/dL: 62% vs. 50%; p=0.05), and platelet count (>200: 72% vs. 52%; p<0.001), and higher frequency of ISS stage I (48% vs. 33%; p=0.02). Interestingly, cases with >5% N-PC/BMPC showed lower frequency of IgH translocations (13% vs. 43%; p<0.001), del(13q) (5% vs. 41%; p<0.001) and del(17p) (3% vs. 8%; NS), and considering together high risk cytogenetics [t(4;14), t(4;16) or del(17p)] differences were highly significant (3% vs. 26%; p=0.006). Finally, patients with >5% N-PC/BMPC had better prognostic outcome than patients with ″5% N-PC/BMPC, with significantly longer progression-free survival (PFS; median 51 vs 39 months, p<0.001; Figure, Panel A) and overall survival (OS; median not reached vs 73 months, p=0.004; Panel B), and PFS and OS rates at 5 years of 40% vs. 30% (p<0.001), and 70% vs. 58% (p=0.004), respectively. *Conclusions.* Our findings uncover the existence of a particular good prognostic group of symptomatic MM patients defined by the presence of >5% of N-PC/BMPC assessed by MFC at diagnosis, stressing the clinical relevance of baseline routine BM evaluation by MFC in MM patients.

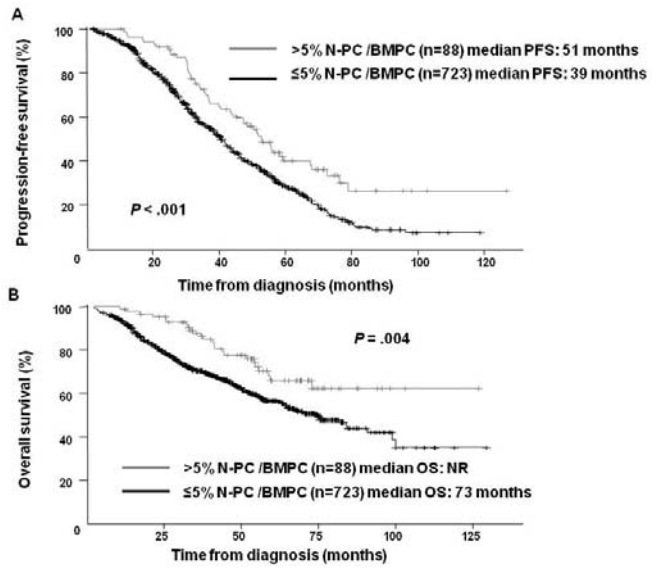

Figure.

0387

COMBINED VORINOSTAT, LENALIDOMIDE AND DEXAMETHASONE THERAPY IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: A PHASE I STUDY

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Background. Multiple myeloma (MM), the second most common hematologic malignancy, remains incurable despite recent therapeutic advances. Treatment of patients with relapsed and refractory MM is extremely challenging and represents a specific unmet medical need. However, novel treatment combinations have the potential to improve patient outcomes. Vorinostat, an oral inhibitor of Class I and II histone deacetylase enzymes, enhances the anti-MM activity of other pro-apoptotic agents, providing potential synergy in combination with lenalidomide and dexamethasone. This Phase I, multicenter, open-label, non-randomized, dose-escalation study evaluated vorinostat plus lenalidomide and dexamethasone in patients with relapsed or refractory MM. *Aims.* The primary objective was to determine the maximum tolerated dose (MTD); secondary objectives included overall safety and tolerability, and evaluation of clinical activity. *Methods.* Patients aged ≥18 years with relapsed or refractory MM were enrolled sequentially into 1 of 5 escalating dosing levels (Table) using a standard 3+3 design for ″8 cycles. In the absence of dose-limiting toxicities (DLTs) in the first cycle, dose escalation continued until the MTD was established. Response to treatment was assessed using modified European Group for Blood and Marrow Transplantation (EBMT) criteria with the overall response rate (ORR) defined as minimal response or greater, and all adverse events (AEs) recorded. *Results.* Of 18 patients assessed for safety to date, 16 (89%) experienced ≥1 AE, with 13 (72%) patients experiencing a total of 32 drug-related AEs, the majority of which were mild or moderate in severity. The most common drug-related AEs were diarrhea (n=8, 44%), fatigue (n=6, 33%), neutropenia (n=6, 33%) and thrombocytopenia (n=5, 28%). A total of 4 serious AEs (SAEs) were reported in 3 (17%) patients: pneumonia (n=1, 6%) and septic shock (n=1, 6%) in the same patient; syncope (n=1, 6%), and diarrhea (n=1, 6%). One SAE (diarrhea) was considered related to study treatment. No patients discontinued due to AEs or SAEs. The MTD has not yet been reached and to date no DLTs have been observed in patients enrolled in the study. DLT evaluation is ongoing at dose level 5. Of 15 patients evaluable for efficacy, 11 (73%) experienced clinical benefit while on treatment. Best responses to vorinostat combined with lenalidomide and dexamethasone, defined by EBMT criteria, include: 1 complete response, 4 partial responses (PR), 1 minimal response, 5 stable disease (SD) and 4 progressive disease (PD), for an ORR of 40%. Nine of the ten patients who have received prior lenalidomide therapy were evaluable for response; best responses in these patients included PR (n=2), and SD (n=3); while 4 of these patients progressed. Of the 12 patients who remain on study, 6 out of 9 evaluable patients have responded (67%). To date, 6 out of 18 patients have discontinued therapy due to PD. *Conclusions.* These preliminary data suggest that vorinostat combined with lenalidomide and dexamethasone may represent a convenient oral combination therapy that is active and generally well tolerated in the treatment of relapsed or refractory MM. Dose escalation continues for determination of the MTD.

Table.

Dose level	Dosing escalation: Vorinostat dose (mg qd) 7 days on 7 days off (Days 1-7 and Days 15-21)	Lenalidomide dose (mg qd) x 21 days (Days 1-21)	Dexamethasone dose (mg qd) on Days 1, 8, 15, and 22	Number of patients evaluable for DLTs	Number of treatment cycles to date
1	300	10	40	4	≤8
2	400	10	40	4	≤7
3	400	15	40	3	≤4
4	400	20	40	3	≤2
5	400	25	40	4	≤1

Cycles were repeated every 28 days and use of concomitant aspirin was recommended

0388

SURVIVAL AND YEARS OF LIFE LOST IN DIFFERENT AGE CATEGORIES OF PTS WITH MM

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Background and Aims. A systematic analysis of survival in different age cohorts (<40 to ≥80 years) by conventional or high dose therapy and information about the years of life lost of patients of different age categories is not available as yet. Here, we report results compiled in a large patient cohort. *Methods.* 10.549 patients (conventional therapy: 7.765, autologous transplantation: 2.784, median age: 60 years) were analysed. Estimates of relative overall survival were produced using to adjust for life expectancy depending on age, sex, and year of diagnosis. Years of life lost were calculated by subtracting the actual survival of individual patients since diagnosis from the patient's life expectancy at the time of diagnosis based on life tables from the country of the treating institution.

Results. Median relative survival decreased steadily from 5.2 years in patients <50 years by 10-year age categories to 2.6 years in those aged >80. Relative excess risk of death (RER) differed significantly between most 10 year age categories in the total cohort of patients, in males and females and those on conventional therapy. In patients with autologous transplantation, RER was only significantly increased in the age category 60-69 compared those aged 50-59 (1.263, p=0.0058). Average years of life lost years increased steadily over the various age categories from 4.6, 8.1, 13.7, 19.9, 26.9, to 36.1 years. *Conclusions*. Median relative survival decreased 2 fold (5.2 vs. 2.6 years) from patients <50 years to those ≥80 years of age, but the average years of life lost was almost 8 fold greater (36.1 vs. 4.6 years) in the youngest compared to the oldest cohort.

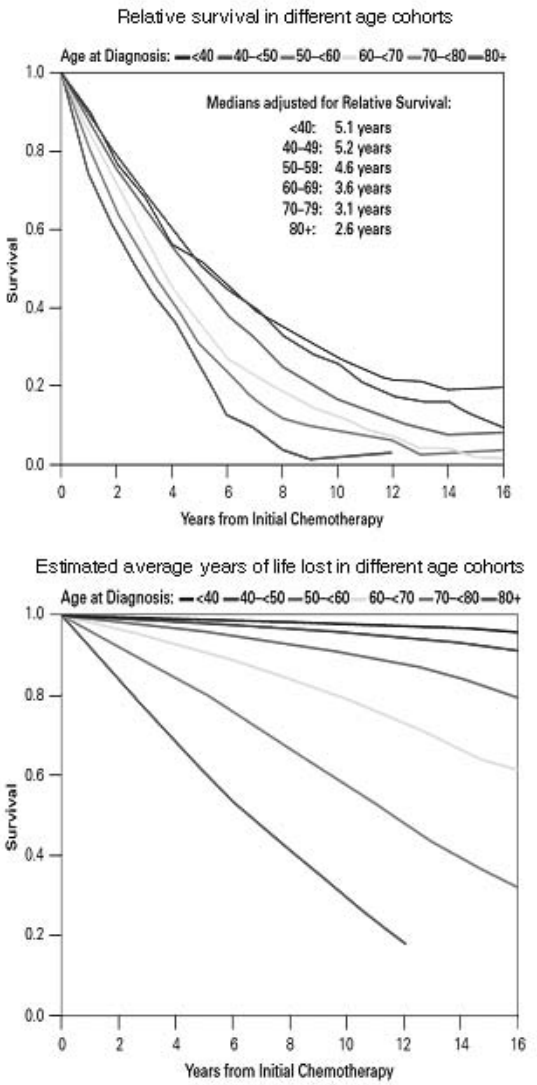

Figure.

0389

BORTEZOMIB , PEGYLATED LIPOSOMAL DOXORUBICIN AND DEXAMETHASONE (B-D-D) AS THERAPY FOR ELDERLY PATIENTS WITH RELAPSED REFRACTORY MULTIPLE MYELOMA: A WEEKLY BORTEZOMIB SCHEDULE REDUCES TOXICITIES MANTAINING THE SAME EFFICACY

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Background. There is a synergism between the proteasome inhibitor bortezomib and anthracyclines. In addition, several *in vivo* data show synergic-additive effect of bortezomib and pegylated liposomal doxorubicin (Peg LD). Recently these findings were confirmed by the results of a large phase III study. However the majority of patients treated in this study were younger then 65 years old. Nobody so far described the results of this combination regimen in an oldest group of patients. *Methods.* Patients. 40 patients (F:M 20:20), median age 68 years, (range 45-81) with a disease resistant to or relapsing after high or conventional dose chemotherapy have been treated. 24 of the 40 patients treated were elderly patients with a median of 75 years (71-81). 30 pts had IgG k/l (20/10); 3 pt had IgA/k, 1 IgA/l, 6pts had a Bence Jones k/l (3/3). β2-microglobulin was 3.1 (range 1.4-17.2). Treatment was started at a median of 31 months from diagnosis (range 2-108). Patient distribution according to disease status and previous therapy was as follows: 6 patients were refractory to the first regimen (primary refractory ,PR); 15 patients had an untreated relapse (UR); and 19 a refractory relapse (RR). A median of 3 lines of chemotherapy were received (range 1-6), with 9 patients with more then 5 lines of CHT. 15 patients were relapsing after autologous stem cell transplantation (ASCT) . Interestingly 5 patients had a disease previously refractory to antracyclines (VAD therapy), and 13 patients had an extramedullary localization of myeloma. *Therapy*. Bortezomib was given 1.3 mg/m^2 as a bolus IV injection on days 1,4,8 and 11 every 3 weeks PegLD was given IV at a dose of 30 mg/m^2 on day 4 every 3 weeks, desamethasone was given IV 40 mg on days 1-4 every 3 weeks, until a plateau phase was reached. After the age of 70 years bortezomib was given with the same schedule as previously described for the first two cycles and then weekly on days 1,7, 14,21 with a 10 days rest period until a plateau phase was reached. PegLD was given on day 4 every 3 weeks for the first two cycles and then was given on day 7 every 31 days cycle. Desamethasone was given with same schedule as previously reported for the first two cycles and then 20 mg IV on days 1,7,14,21 every 31 days. *Statistical analysis*. Overall survival (OS) and progression free survival (PFS) were calculated using the Kaplan -Meier method. Statistical analysis were performed using chi square or Fisher's exact tests. Logrank test comparison of survival curves was done. *Results*. Patients received a median of 4 cycles (range 2-6). B-PeghLD-D therapy resulted in 29/40 objective responses for an overall response rate (ORR) of 70% according to EBMT criteria. In particular we observed 8CRs and 2 nCRs (25%); 11 VGPRs (27%) and 5 PRs (12%), 3 MR (1%). Seven patients received less then 4 cycles (4NR, 3 for toxicity). Height patients had bortezomib dose reduction to 1mg/m^2. Median duration of response (PFS) was 6 months (range 1-36) and all 13 patients with less then PR relapsed. By contrary 17/26 (65%) patients with ≥VGPR still maintain a response at median of 8 months (range 1-36) (p=0.001). 20 patients that received more then 3 lines of previous chemotherapy had a median survival of 6 months compared to 13 months of patients that received less then 3 cycles (p=0.12). PFS in the same group was 3 vs. 10 months (p=0.5). 4/5 VAD refractory obtained a response. 5/13 patients with extramedullary disease responded. Toxicities were mild to moderate in most of the patients and maneageble. Grade 3-4 thrombocytopenia occurred in 7/40 (17%) patients and grade 3-4 neutropenia occurred in 7/40 patients (17%), but only 2 developed febrile neutropenia. 13 patients (32%) complained grade 1-2 paresthesias, 4 patients had grade 3-4 paresthesias. 6 pts (15%) had HSV reactivation, 1 hand-foot syndrome. Response in elderly patients. Interestingly in the oldest group we observed an ORR of 75% [7/24 CR/nCR (30%), 7 VGPR (30%), 1 PR, 3 MR and 6 NR]. Median OS was 15 months and PFS was 8 months. 10/24 patients with ≥ VGPR still maintain a response at 8 months vs 2 months of pts with ″ VGPR (PFS, OS p=0.0001). Toxicities were comparable to the youngest group. *Conclusion*. Bortezomib-PegLD-D combination is highly effective in resistant-relapsing MM with an ORR of 70% and 25% CR,nCRs, also in antracyclines refractory patients. Best and more durable responses are seen in patients treated with less then 3 lines of previous chemotherapy and in patients achieving at least a VGPR. Oldest patients with more then 70 years have fast and durable responses with acceptables and maneagebles toxicities . This regimen can be used also in the elderly MM patients.

0390

BETA-2 MICROGLOBULIN IS AN INDEPENDENT PREDICTOR OF PROGRESSION IN ASYMPTOMATIC MULTIPLE MYELOMA

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Background. The rationale of the study stems from three considerations: i) to date, few clinical predictors of asymptomatic multiple myeloma (MM) progression are available; ii) no study has assessed the role of β-2-microglobulin (B2M) on risk of asymptomatic-MM progression; iii) β2M is a serum marker of tumor burden/disease kinetics and represents

a key variable of the ISS staging system for symptomatic-MM. *Aim.* To assess the impact of B2M on risk of asymptomatic-MM progression. *Methods.* The study was based on a consecutive series of 148 asymptomatic-MM diagnosed according to the IMWG. Cumulative probability of progression to symptomatic-MM was calculated from diagnosis of asymptomatic-MM to progression to symptomatic-MM according to IMWG. The best cut-off for β2M, percentage of bone marrow plasma cells (BMPC%), serum monoclonal component (sMC) and urinary monoclonal component (uMC) were selected according to Youden's index using progression as state variable. Survival analysis was performed by Kaplan-Meier method using log-rank to test for associations. Cox proportional hazard regression was used to build a multivariate model. *Results.* Clinical features at asymptomatic-MM diagnosis were as follows: median age 67 years, male:female ratio 1.02, previous MGUS 32/148 (21.6%), IgG MC 113/148 (76.4%), IgA MC 32/148 (21.6%), light chain MC 3/148 (2.0%), median sMC 1.1 g/dL, positive urinary immunofixation 27/148 (18.2%), median uMC 98 mg/24h, median BMPC% 15%, median B2M 2.0 mg/L, median albumin 4.3 g/dL, median C reactive protein 0.3 mg/L. After a median follow-up of 36 months, 24/148 asymptomatic-MM progressed to symptomatic-MM, accounting for a 30.5% 5-year probability of progression. According to Youden's index, best cut-off values were 2.5 mg/l for B2M, 1.5 g/dl for sMC, 500 mg/24h for uMC, and 20% for BMPC%. Univariate analysis identified B2M>2.5 mg/L(5-year risk: 64.5%; HR=3.85; $p<0.001$), sMC>1.5 g/dL (5-year risk: 49.1%; HR=5.76; $p<0.001$), uMC>500 mg/24h (5-year risk: 68.7%; HR=6.60; $p<0.001$) and BMPC%>20% (5-year risk: 50.2%; HR=5.77; $p<0.001$) as predictors of progression to symptomatic-MM. Clinical variables not associated with progression to symptomatic-MM ($p>0.01$ in all cases) were age, sex, sMC type, polyclonal Ig reduction, albumin, C reactive protein, Hb, calcium, creatinine, and previous MGUS. Multivariate analysis identified B2M>2.5 mg/L (HR=3.57; $p=0.001$) as an independent predictor of progression to symptomatic-MM, along with sMC>1.5 g/dL (HR=4.07; $p=0.003$), uMC>500 mg/24h (HR=3.66; $p=0.015$) and BMPC%>20% (HR=3.20; $p=0.007$). We next combined B2M, sMC, uMC, and BMPC% into a model for predicting progression to symptomatic-MM. This model stratified patients into four risk groups: very low risk (0 risk factors: 5-year risk 0%), low-intermediate risk (1 risk factor: 5-year risk 19.6%), high-intermediate risk (2 risk factors: 5-year risk: 60.7%), and high risk (3 or 4 risk factors: 5-year risk 80.7%) (Figure 1). *Conclusions.* The implications of our results are twofold: i) B2M predicts progression of asymptomatic-MM to symptomatic-MM independent of conventional risk factors (sMC, BMPC%, light chain burden); ii) B2M refines the conventional model for predicting progression of asymptomatic-MM by allowing the identification of a very low risk group of patients who never progress to symptomatic-MM and a high risk group of patients who are virtually all projected to progress.

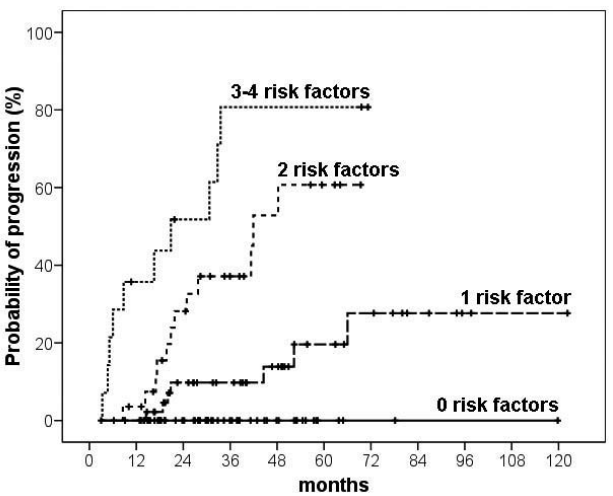

Figure 1. Risk of asymtomatic MM progression.

0391

PATTERNS OF SURVIVAL AND CAUSES OF DEATH FOLLOWING A DIAGNOSIS OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE: A POPULATION-BASED STUDY

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Background. Patients with monoclonal gammopathy of undetermined significance (MGUS) have an increased risk of lymphoproliferative malignancies. However, there are limited data on survival patterns among MGUS patients. *Aims.* To assess pattern of survival among MGUS patients diagnosed in clinical practice compared to the general population, and to classify causes of death in MGUS patients compared to matched controls. *Methods*. We included, from all major hematology outpatient units in Sweden, 4,259 MGUS patients (diagnosed 1986-2005) and compared survival to the general population by computing relative survival ratios (RSRs). We also compared causes of death with 16,151 matched controls using Cox's proportional hazards regression models. *Results*. One-year, 5-year, 10-year, and 15-year RSR were 0.98 (95% CI 0.97-0.99), 0.93 (0.91-0.95), 0.82 (0.79-0.84), and 0.70 (0.64-0.76), respectively (Figure). Younger age at MGUS diagnosis was associated with a significantly lower excess mortality compared to older ($p<0.001$). The excess mortality among MGUS patients increased with longer follow-up ($p<0.0001$). We found IgM (versus IgG/A) MGUS to be associated with a superior survival ($p=0.038$). MGUS patients had an increased risk of dying from MM (hazards ratio (HR)=553; 95% CI 77-3946), WM (HR= °), other lymphoproliferative malignancies (6.5; 2.8-15.1), other hematological malignancies (22.9; 8.9-58.7), amyloidosis (HR= °), bacterial infections (3.4; 1.7-6.7), ischemic heart disease (1.3; 1.1-1.4), other heart disorders (1.5; 1.2-1.8), liver diseases (2.1; 1.1-4.2), benign hematological disorders (6.9; 2.7-18), and renal diseases (3.2; 2.0-4.9). *Summary and conclusions.* Our findings of a decreased life expectancy in MGUS patients, most pronounced in the elderly population, explained by both malignant transformation and non-malignant causes are of importance in the clinical management of MGUS patients.

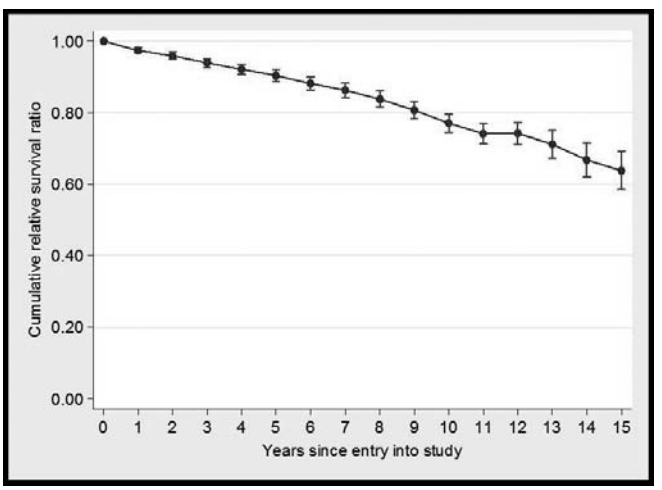

Figure. Relative survival ratios for MGUS patients.

0392

BORTEZOMIB IN COMBINATION WITH DEXAMETHASONE OVERCOMES THE NEED FOR ADDITIONAL CHEMOTHERAPY PRIOR TO HIGH-DOSE THERAPY WITH AUTOTRANSPLANT IN PATIENTS WITH MULTIPLE MYELOMA

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Background. The introduction of high-dose therapy (HDT) followed by stem cell rescue has significantly changed the approach of multiple myeloma (MM) patients, becoming the gold standard as initial therapy in patients younger than 65 years. Patients who most benefit from HDT are those achieving a complete response (CR) or a very good partial response (VGPR) after transplant. Velcade in combination with dexamethasone (Vel-Dex) has proven to be effective in inducing high rate of good quality response (CR, VGPR). *Aims.*To evaluate the role of DCEP chemotherapy after induction with Vel-Dex in untreated younger MM patients candidate to a HDT program. *Methods.* We conducted a phase II multicenter trial including four Vel-Dex courses as induction, followed by 2 courses of DCEP (Dexamethasone, Cyclophosphamide, Etoposide, cysPlatinum) as intensification and stem cell mobilization, and single autologous transplant (Tx) with melphalan 200 mg/m^2 as conditioning regimen. Fifty-seven patients were studied. Patient characteristics were: male/female 35/22 (60%/40%), median age 58 years (37-65), IgG/IgA/light chain 33/12/12 (56%/22%/22%), D&S stage I in progression/II/III 3(5%)/5(9%)/49(84%), ISS I/II/III 24(43%)/13(22%)/20(35%), cytogenetic abnormalities absent/present 48%/52% (del 13 21%, t (4;14) 23%, t (11;14) 7%) *Results.* Forty-three patients completed the program (75%). Fourteen patients (25%) went off-study: 7 after Vel-Dex (3 for progression, 3 for toxicity, 1 patient withdrew consent), and 7 after DCEP (5 for progression, 1 for toxicity, 1 withdrew consent). All patients collected an adequate number of peripheral stem cells after DCEP. Table 1 reports response after Vel-Dex and after DCEP. After 2 cycles of DCEP, 5 patients (10%) improved, 35 (70%) remained unchanged, and 15 (20%) worsened. High-dose melphalan further improved response. In fact, after transplant, ORR reached 96% (with 80% ≥VGPR). On an intention to treat basis, the 86% ORR achieved after Vel-Dex, decreased to 76% after DCEP, and to 73% after transplant. After a median follow-up of 15.3 months from Tx, of 43 responding patients 16 (37%) progressed. The median duration of response after Tx was 13 months (1.4-24 months). Patients achieving CR had lower progression rate (p=0.02) and longer duration of response (p=0.04) compared to patients with VGPR or PR (progression rate 12%/42%/60% and median duration of response 18/13/9 months for patients with CR/VGPR/PR). *Conclusions.* Bortezomib in combination with dexamethasone is a safe and effective induction program for MM patients candidates to high-dose therapy. Consolidation with DCEP is unable to improve response to Vel-Dex, while high-dose melphalan further increases the ORR. Shortening the interval between induction and high-dose melphalan may help in maintaining the level of response, preventing progressions that may be observed during the post-induction/consolidation phase.

Table 1. Breakdown of response after Vel-Dex and a.

Response after vel-dex	Response after DCEP					
	CR	VGPR	PR	SD	PD	Total
CR	12	2	-	-	1	15
VGPR	5	14	2	-	2	23
PR	-	-	8	1	-	9
MR	-	-	-	1	2	3
PD	-	-	-	-	-	0
Total	17	16	10	2	5	50

0393

SURVIVAL EFFECT OF ERYTHOPOIETIC AGENTS AND VENOUS THROMBOEMBOLISM (VTE) IN MULTIPLE MYELOMA PATIENTS TREATED WITH LENALIDOMIDE AND HIGH-DOSE DEXAMETHASONE

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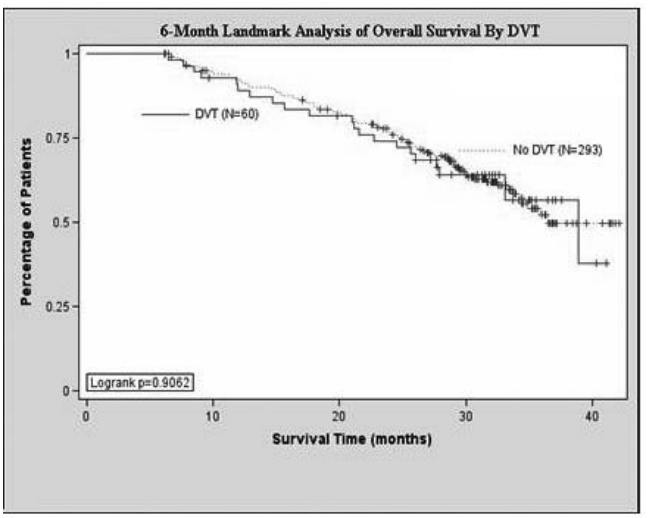
Background. Thromboembolism in cancer patients at diagnosis or during treatment has been associated with a shorter survival. Long-term follow-up studies in patients with thrombosis have shown that those with cancer have a 4- to 8-fold higher risk of dying after an acute cardiovascular event than patients without cancer. Concern has also been raised with regard to cancer-associated VTE during treatment with erythropoietin, either epoetin alfa or darbepoetin. Head and neck cancer patients treated with chemoradiotherapy and epoetin alfa experienced a 10-fold higher risk of VTE compared with patients treated only with chemotherapy. *Aims and Methods.* Retrospective analysis of the effect of erythropoietic agents and VTE development on patient survival. Two identically designed, multi-center, double-blind, Phase III clinical trials were conducted in Europe and the US to assess the effect of lenalidomide in combination with dexamethasone versus dexamethasone plus placebo in patients with relapsed or refractory multiple myeloma (MM), after failing at least one prior line of treatment. 353 patients were randomized to receive 25 mg of lenalidomide on days 1-21 of a 28-day cycle, plus 40 mg of oral dexamethasone on days 1-4, 9-12 and 17-20 for the first four cycles; after the fourth cycle, 40 mg of dexamethasone was administered on days 1-4 only.



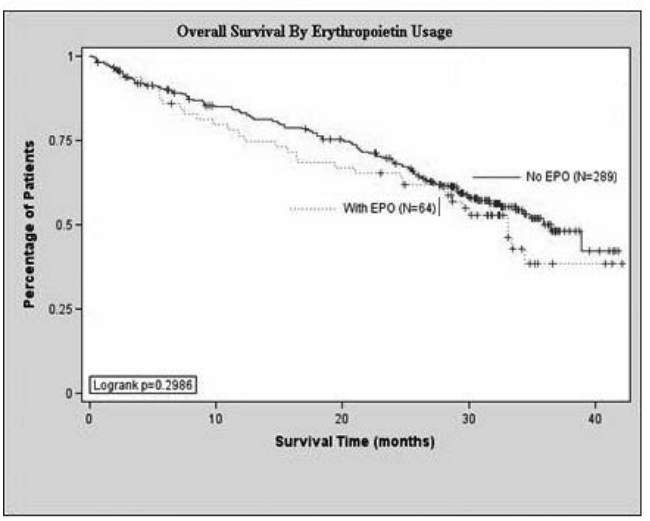


Figure 1 and 2.

Results. A total of 353 patients treated with lenalidomide and dexamethasone were included in this analysis; the median age was 63 years, 59.5% were male, 20.4% were IgA myeloma. With a median follow-up of 17.5 months, a total of 60 (17.0%) patients experienced a DVT during treatment. A total of 64 patients received erythropoietic agents during the treatment course (18.3% received EPO among DVT and 18.1% among non-DVT patients, p=0.96). Erythropoietin treated patients have been more exposed to thalidomide, had a significantly lower albumin, and higher β2 microglobulin. Figure 1, 2 show overall survival of patients by erythopoietic use and DVT development. The development of VTE did not significantly affect overall survival (p=0.40) or time to progression (p=0.43). No significant impact was observed in the subgroup of patients who received erythropoietic agents (overall survival p=0.85, time to progression p=0.26). *Conclusions.* Patients with relapsed or refractory MM treated with the combination of lenalidomide plus high-dose dexamethasone that developed a VTE, or received erythopoietic agent, did not experience shorter overall survival or time to progression.

0394

SERUM DKK1 IN MULTIPLE MYELOMA PATIENTS

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Introduction. Lytic bone destruction is a hallmark of multiple myeloma, caused by enhanced osteoclast activity and decreased bone formation. The Wnt-signaling antagonist Dickkopf (DKK) 1 has been identified as an important factor involved in osteoblast inhibition by myeloma cells. We therefore evaluated serum DKK1 in 184 untreated myeloma patients and correlated the results with disease stage and the extent of bone disease. In a second step, DKK1 was quantified in 101 patients undergoing anti-myeloma therapy for symptomatic disease, and correlated the results with disease activity, the kind of therapy and the response to treatment. *Results.* Mean serum DKK-1 significantly correlated with myeloma stage according to Durie and Salmon (2223 pg/mL vs. 15,209 pg/mL in stage I and II/III, p=0.005), with the presence of lytic bone disease (mean 3114 pg/mL vs. 17,915 pg/mL in patients with or without lesions, p=0.003), and the number of bone lesions (0 vs. 1-3 vs. >3 lesions: 3114 pg/mL vs. 3559 pg/mL vs. 24,068pg/mL; p=0.002). Pretreatment DKK1 levels in symptomatic MM patients were increased compared to individuals with MGUS (mean 3768 pg/mL vs. 1993 pg/mL). A significant decrease of DKK1 during therapy was seen in the following treatment groups: adriamycin/ dexamethasone (1668 pg/mL vs. 1241 pg/mL, p=0.016), HDCT + ASCT (2446 pg/mL vs. 1082 pg/mL, p=0.001), bortezomib (4059 pg/mL vs. 1862 pg/mL, p=0.016) and lenalidomide (11837 pg/mL vs. 4374/mL, p=0.039), whereas treatment with thalidomide led to a nonsignificant decrease in DKK1 (1705 pg/mL vs. 1269 pg/mL, p=0.081). Within all groups, a significant decrease of DKK1 was only seen in responders (i.e. patients achieving a complete or partial remission). Pretreatment serum levels of DKK1 were not predictive for response, and there was no significant difference in pretreatment DKK1 levels in responders and nonresponders (p=0.696). Furthermore, DKK1 did not correlate with the number of prior therapies given, and there was no difference between previously untreated patients with symptomatic multiple myeloma and patients at relapse regarding serum DKK1. These data suggest that myeloma cells are the main source of circulating DKK1 protein and provide a framework for clinical trials on anti-DKK1 treatment in multiple myeloma.

0395

LENALIDOMIDE-BASED THERAPY LEADS TO IMPROVEMENT OF HUMORAL IMMUNE SYSTEM IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA PATIENTS WHO RESPOND TO THE THERAPY

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Background. Recurrent bacterial infection is the most frequent cause of death in patients with advanced multiple myeloma (MM). Infections result primarily from the marked depression of normal immunoglobulins production that occurs in more than 75% of patients. Pneumococcal vaccination has been suggested as an option, however most MM patients respond poorly to bacterial antigenic stimulation. All MM patients including those with plateau-phase have an increased risk of life-threatening bacterial infections and polyclonal humoral immune suppression. Preliminary results from Borrello *et al.* demonstrated that the use of lenalidomide prior to pneumococcal vaccination significantly augments its efficacy, suggesting lenalidomide improves the humoral immune system. *Aim.* We evaluated the effect of lenalidomide plus dexamethasone (Len/Dex) therapy on IgA levels in relapsed or refractory MM patients from two large randomized pivotal phase III registration studies (MM009/010) comparing Len/Dex to placebo/Dex, as well as the large phase 2 study evaluating single agent lenalidomide (30 mg x 21 days in each 28-day cycle) in relapsed or refractory MM patients MM-014). These studies have shown that lenalidomide based therapy significantly improved response rate and progression free survival in patients with MM. *Methods.* The baseline level of residual polycolonal IgA, IgG and IgM levels for the different myeloma subtypes were evaluated and monitored monthly. Comparisons were made between responders and non-responders per the Blade criteria. Humoral improvement was defined as an increase from the baseline level of residual polycolonal immunoglobulin to at least the lower limit of normal and a 25% increase in value. *Results.* Of the 353 patients randomized to receive Len/Dex in MM009/MM010 (median age 63 years; median of 2 prior therapies) and 222 patients enrolled in MM014 who received Len (median age 63 years; median of 5 prior therapies), residual IgA was the only residual polyclonal protein to show recovery during the monitoring period of the studies. Patients with non-IgA MM type were included in this analysis (159 responders, 115 non-responders from MM009/MM010; 36 responders, 60 nonresponders from MM014). At baseline, residual IgA level was normal in (30% and 17%) in responders and (17% and 42%) in non-responders for patients on MM009/010 and MM014, respectively. Only patients responding to therapy showed significant improvement and normalization of residual IgA levels. 56% and 50% of patients on MM009/010 and 014 respectively normalized their IgA by cycle 7 and 5 of therapy. Baseline patient characteristics were similar in both groups. As expected patients showing normalization of the residual IgA levels experienced a longer progression free survival (PFS; 29-77 weeks; p=0.0001) and overall survival (OS; 121-220 weeks; p=0.0001). *Conclusions.* Lenalidomide-based therapy leads to normalization of the residual polyclonal IgA levels in a significantly number of relapsed/refractory MM patients who responded to the therapy. These patients also achieved longer PFS and OS.

0396

UP-FRONT SALVAGE TREATMENT WITH MELPHALAN 100 MG/M² IN FULMINANT PROGRESSION OF MULTIPLE MYELOMA AND CONSOLIDATION WITH NOVEL DRUGS

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Background. Patients (pts) with a fulminant progression (FPG) of multiple myeloma (MM) after autologous transplantation have poor prognosis. The treatment options are limited, pancytopenia and/or extramedullary disease and/or renal impairment are often presented. The treatment with melphalan 100 mg/m^2 (MEL 100) as a up-front salvage therapy with support of autologous peripheral blood stem cells (PBSC) can stop progression of MM and provides a opportunity for the application of novel drugs as a consolidation treatment. *Methods.* We have retrospectively evaluated 31 pts with FPG of MM after autologous transplantation (AT) treated with MEL 100 mg/m^2 with PBSC support and consolidation chemotherapy with novel drugs (thalidomide+dexamethasone+cyclophosphamide in 16 cases, bortezomib+dexamethasone+cyclophosphamide in 15 cases). Fulminant progression of MM was specified as a rapid progression during 4-8 weeks with one of these features: peripheral pancytopenia due to bone marrow myeloma infiltration, new presence of extramedullary disease, new renal impairment. Clinical stages according to ISS were the following: stage 1 in 22% of pts (7/31), stage 2 in 39% of pts (12/31), stage 3 in 39% of pts (12/31). Pancytopenia was presented at 52% of cases (16/31), renal impairment at 22% of cases (7/31), extramedullary disease at 32% of pts (10/31). Median time from diagnosis of MM to FPG was 18 months, median time from AT to FPG was 12 months, median number of previous therapy lines was 1 (range 1-3). The median age at MEL 100 was 58 years. *Results.* There was no treatment-related mortality. Mucositis grade 3-4 developed in 29% of pts (9/31), febrile neutropenia occurred in 39% of pts (12/31) after MEL 100. Overall response rate (ORR) was 58% (18/31). After MEL

100, one patient (3%) achieved complete remission, 29% of pts (9/31) were in partial remission, 26% of pts (8/31) had very good partial remission, and 42% of pts (13/31) had stable disease. Median number of consolidation chemotherapy cycles with novel drugs was 4 (range: 1-6); 40% of pts (12/31) interrupted therapy earlier for progression of MM, 6% of pts (2/31) ended therapy earlier for neurological toxicity grade 3, no other significant toxicity grade 3 or 4 was observed. Rapid progression within 3 months after MEL 100 occurred in 35% of pts (11/31). The median follow-up from MEL 100 was 8 months (range 3-23). The median TTP was 5.0 months (range: 2-15) and the median OS was 8.0 months (range: 3-23). *Conclusions.* MEL 100 is a safe salvage up-front regimen for fulminant progression of MM with good response rate (ORR 58%) and acceptable toxicity. This treatment strategy with combination of novel drugs can prolong survival over 12 months in 33% of pts with this fatal form of MM.

This work was supported by grants IGA NR9317-3 and NR9225-3.

0397

SPEED OF RESPONSE WITH LENALIDOMIDE AND DEXAMETHASONE IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: FIRST RESULTS OF THE MM-019 GERMAN COMPASSIONATE USE PROTOCOL

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Background. The combination of lenalidomide and dexamethasone has been well established as therapy for patients with relapsed or refractory multiple myeloma. However, the speed of obtaining response has not yet been sufficiently described. *Patients and Methods.* Before marketing approval patients could be recruited into this phase IIIb, open-label, non-comparative clinical trial if suffering from multiple myeloma with at least one previous therapy. All patients received lenalidomide, 25 mg/d, d1-21, and dexamethasone 40 mg/d, d1-4 (and for the first four courses d 9-12, 17-20). Courses were repeated every 28 days and continued until progression, toxicity or withdrawal from physician or patient. Dose reductions were made according to renal function or toxicity following standard guidelines. The EBMT response criteria were used and assessed every 28 days. However, confirmatory bone marrow aspirations required for CR are not available for all patients with respective M protein reductions. In addition, M protein and serum free light chains (sFLC) were analysed every 14 days for the first two courses. *Results.* Of 150 included patients, validated data are available for 122 at this time, the median age was 65 years (interquartile range: 58-71). ECOG performance scale was 0 in 32%, 1 in 55% and 2 in 13%. Previous transplantation therapy was noted in 61%, previous bortezomib in 57% and thalidomide in 40% of patients. Fifteen patients (12%) had all three regimens, 43 (35%) had two, 42 (34%) had one of these regimens. Best response information was available in 113 patients: CR in 4 (4%; 95% confidence interval: 1-9%), PR in 80 (71%; 62-78%), SD in 28 (25%; 18-33%) and PD in 1 patient (1%; 0-5%). Previous transplantation, bortezomib or thalidomide (≥ PR: no thalidomide 73% vs. previous thalidomide 77%) did not influence the remission rate. Time to a 50% reduction of M-protein or sFLC (in the absence of progression at other sites) was analysed in 77 patients (missing values in 3): Median time was 28 days (interquartile range 14-42, range 12-84 days). Thirty-two (39%) patients demonstrated this reduction in 12-15 days, 27 (33%) patients in 20-32 days, 14 (17%) in 40-49 days and 10 (12%) in 56-84 days. *Conclusions.* Despite considerable previous treatment at least a partial response was reached by 74% of patients; previous thalidomide, bortezomib or transplant did not influence the response rate. A 50% M protein/sFLC reduction was reached by half of the patients within 28 days and by 72% of the patients within 32 days.

0398

USE OF MAINTENANCE THERAPY WITH BORTEZOMIB AND DEXAMETHASONE (VD) IN ADVANCED MULTIPLE MYELOMA

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Background. The efficacy and safety of bortezomid therapy ± dexamethasone in patients with relapsed or refractory multiple myeloma (MM) was confirmed by several studies but no data are available regarding the maintenance therapy (MT) after salvage treatment. *Aims.* In this trial we tested safety and efficacy of bortezomib/dexamethasone MT in patients with refractory/relapse MM who responded to salvage therapy. We also assessed the impact of MT on progression-free survival (PFS) and overall survival (OS). *Methods.* We included 49 MM patients with advanced MM who responded to salvage therapy. As MT, bortezomib was administered by intravenous bolus on days 1 and 15 at 1.3 mg/mq; dexamethasone was given orally at the dose of 20 mg/d on days 1-2 and 15-16 every 28-day cycle for a total of 6-8 cycles. PFS was defined as the time from start of MT to progression or death from any cause. The cumulative incidence of death for PD was calculated accounting for competitive death or other causes. *Results.* From October 2004 until April 2008, 49 MM patients have been enrolled. The characteristics of the patients were the follows: 28 males and 21 females, median age was 71 years (IQR:66-75). Median haemoglobin value was 11.3 (IQR:10.4-18.8) and 8 (16,3%) patients had a renal failure. The median number of prior therapies was 2 (2-3). All patients were in PR after salvage therapy that included bortezomib as single agent or in combination with steroids and/or thalidomide in 39 patients (79.6%). Median time from diagnosis to the first dose of MT was 39 months (IQR:26-56). The median number of bortezomib infusion was 8 (7-12). After a median follow up of 19 months (IQR:13-22), 12 patients died for PD, 1 for IMA and 6 patients for infections. The MT improved the quality of response after salvage therapy as follows: 4 CR, 3 VGPR, 10 PR, 19 SD whereas 13 patients experienced PD. The median time to progression was 17 months (95%CI: 7-38) with a progression free-survival at 1 year of 63% (95%CI: 48-75) (Figure 1). The overall survival at 1 years was 79% (95%IC: 75-88) and the cumulative incidence of death due to PD adjusted for competitive risk event was 12% (95%IC:2-19). In a univariate analysis the response rate to MT was not significantly affected by age, sex, number or type of previous therapy and haemoglobin concentration. Non-dose-limiting toxicities included neuropathy grade 1 (15 pts), herpes zoster reactivation (2 pts), pulmonary infections (2 pts), and gastrointestinal affections (3 pts). Three patients developed a neuropathy grade 2 which required a dose reduction (1.0 mg/mq) of bortezomib. *Conclusions.* The combination bortezomib/dexamethasone as a maintenance therapy in relapse/refractory MM is effective and well tolerated. These preliminary data suggest that bortezomib/dexametasone MT can improve remission duration and also quality of response with an acceptable toxicity.

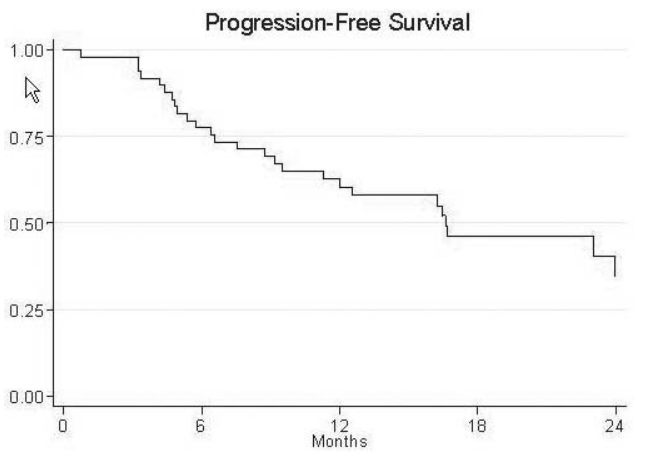

Figure 1.

0399

HALOFUGINONE, AN ORALLY-BIOAVAILABLE AGENT, HAS *IN VITRO* AND *IN VIVO* ANTIMYELOMA ACTIVITY, ASSOCIATED WITH UP REGULATION OF C-JUN, JNK, AND P-53, PROAPOPTOTIC PROTEINS

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Halofuginone, a synthetic derivative of quinazolinone alkaloid, previously has been shown to have anti-cancer effects in various solid and hematological malignancies. Halofuginone inhibits mainly collagen type I synthesis, and extracellular matrix formation, via the inhibition of TGFβ signaling, matrix metalloproteinase 2(MMP2), and angiogenesis. Last year, we first reported, that Halofuginone in a low doses (IC50 of 50-100 nM) induces cytotoxicity in multiple MM cell lines, including cells resistant to conventional (e.g., dexamethasone, alkylating agents, and anthracyclines) or novel (e.g. thalidomide and bortezomib) anti-MM agents and overcomes the survival and growth advantages conferred by interleukin-6, insulin-like growth factor-1 and by bone marrow stroma cells. Halofuginone induced apoptosis in a caspase 3, 8, and 9 dependent mechanisms, reduced mitochondrial membrane potential, and down regulated MCL1 protein. We now assessed the cytotoxic effect of Halofuginone in primary MM patient cells *in vitro* and, its effect on tumor growth and survival in *in vivo* models. We found that Halofuginone also induces growth inhibition and cell death in primary MM cells (n=4, IC_{50}: 100-200nM). Importantly, Chou-Talalay analysis of the effects of combinations of Halofuginone with anti-MM drugs (dexamethasone, melphalan and Lenalidomide) revealed that all combinations have synergistic or additive MM cytotoxicity. In addition, Halofuginone inhibits IL6 production in the supernatant of a co-culture of MM.1S cells with HS-5 stromal cell line. Mechanistically, Halofuginone induces MM cell death, which involves the up-regulation of c-jun NH2-terminal kinase signaling (JNK), c-Jun, as well as the p-53 proapoptotic proteins, as shown by Multiplex analysis of phoshorylation signaling pathways, using the Luminex system. Additionally, the *in vivo* anti-MM activity of Halofuginone was evaluated in 2 separate *in vivo* models, a xenograft model in SCID mice (subcutaneous injection of MM1S cells), and a model of diffuse MM lesions in SCID-beige mice (generated by i.v. injections of OPM-2 cells). In both models, mice were first sublethally irradiated (200 rads), injected s.c or i.v., respectively, with 1x106 MM cells and then randomly assigned to receive, either treatment with 0.75 mg/kg halofuginone (IP or by oral gavage, respectively; n=10) or vehicle only (n=10) on a cyclical schedule of 5 days-on/2 days-off. In a very low dose(0.75/mg/kg), Halofuginone inhibited MM tumor growth and improved survival, 70% vs 40% at 220 days (p=0.07) in the treated vs control group respectively (Figure), longer follow up are needed. Clinical evidence of adverse events (weight loss, vomiting) were not observed. Halofuginone may, thus represents a promising novel orally bioavailable anti-MM agent that needs further evaluation for possible clinical trials in MM.

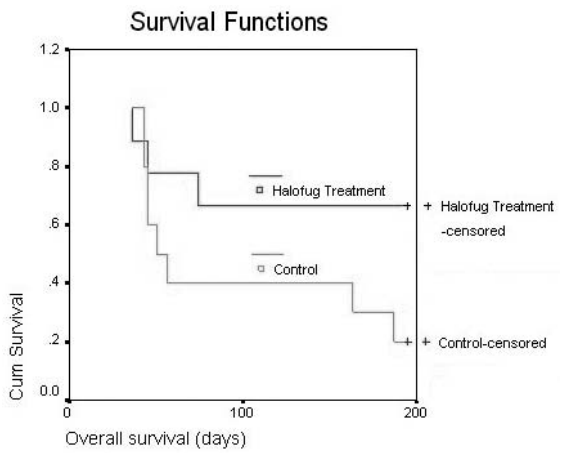

Figure. Diffuse MM lesions in SCID-beige mice.

0400

SERUM LEVELS OF TOTAL-RANKL IN MULTIPLE MYELOMA

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Background. Receptor activator of nuclear factor-κB ligand (RANKL) plays a key role in osteoclast activation in myeloma bone disease. RANKL is a member of the tumor necrosis factor (TNF) superfamily and is produced mainly by osteoblastic lineage cells and stromal cells as a membrane-bound protein. It also exists in a soluble form, which is cleaved from the cellular protein or released as a primary secreted isoform. The increased expression of RANKL in the bone marrow microenvironment was demonstrated in several studies, but there are only rare data on circulating RANKL levels in multiple myeloma (MM) patients. *Aims.* In the present study we investigated the clinical significance of serum RANKL levels in patients with newly diagnosed MM, in individuals with monoclonal gammopathy of undetermined significance (MGUS) and in a control group of healthy donors. *Methods.* Circulating RANKL concentrations were measured by a commercially available ELISA-test (Immundiagnostik, Bensheim, Germany), which detects both free and osteoprotegerin (OPG)-bound RANKL (total-RANKL, tRANKL). The two-site sandwich assay is constructed by a monoclonal human anti-RANKL capture antibody to bind OPG-bound RANKL combined with a recombinant human OPG for capturing free RANKL and a polyclonal anti-OPG detection antibody. Serum samples were collected from 93 patients with newly diagnosed MM, 20 individuals with MGUS and 20 healthy donors.

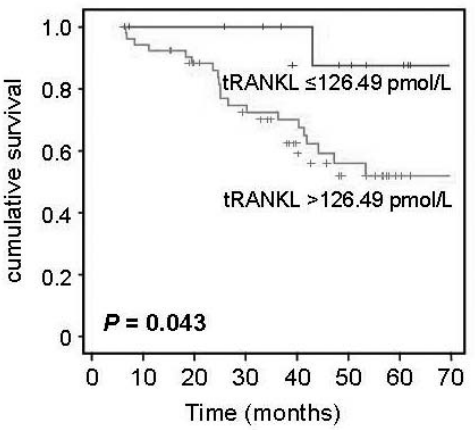

Figure 1. Prognostic value of tRANKL for OS in symptomatic MM.

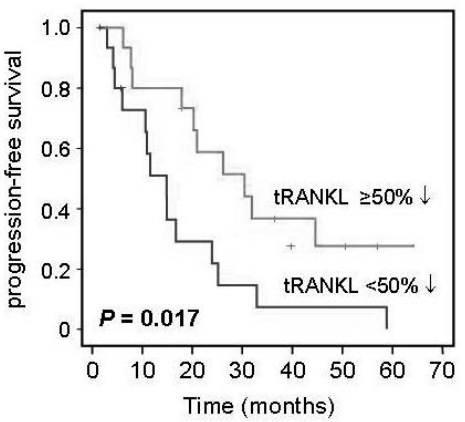

Figure 2. Improved progression-free survival in patients with post-treatment tRANKL decrease >50%.

Results. Circulating serum tRANKL was significantly elevated in MM patients compared to controls (p<0.001) or MGUS (p<0.001). Furthermore, tRANKL levels were higher in smoldering MM versus MGUS

(p=0.031) and in symptomatic versus smoldering MM (p 0.001). Serum tRANKL increased parallel to ISS stages I to III (p=0.031) and correlated with the presence of lytic bone lesions (p<0.001). Total-RANKL was a prognostic factor for overall survival in symptomatic MM (p=0.043, Figure 1) with a 5-fold elevated risk in patients with high (>126.49 pmol/L) tRANKL values. In the subgroup of symptomatic MM patients with elevated tRANKL, there was a significant survival benefit (p=0.018) of high-dose therapy (HDT), while this was not seen in the group with low tRANKL levels (p=0.431). A significantly longer progression-free survival was observed in patients with a greater than 50% decrease in tRANKL levels after 3 months of combined chemotherapy and bisphosphonate treatment (Figure 2). *Summary and Conclusions.* Our study shows for the first time, that serum tRANKL reflects advanced disease, lytic bone destruction and poor prognosis in MM.

Non-Hodgkin lymphoma - Clinical I

0401

QUALITATIVE AND QUANTITATIVE ASSESSMENT OF BONE MARROW INVOLVEMENT IN THE ENTIRE SPECTRUM OF NON-HODGKIN'S LYMPHOMAS: COMPARISON BETWEEN HISTOLOGY AND FLOW CYTOMETRY IN 572 PATIENTS

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Background. Bone marrow (BM) examination is essential in the staging of non-Hodgkin's lymphoma (NHL) pts. Few studies have compared BM histologic findings with results of flow cytometric (FC) analysis. Overall, rates of concordance between the two techniques are usually good. However, detailed data about correlation of the results of these two methods in large series comprising different NHLs are lacking. *Aim.* To correlate histologic features of staging BM biopsies with corresponding FC data in a large series of consecutive NHL pts. *Methods.* We analysed the incidence and patterns of histologic BM involvement in a series of 822 pts with NHL diagnosed at Division of Hematology of Pavia from 1998 to 2008. BM specimens were plastic- or paraffin-embedded and both staining with hematoxylin-eosin and Giemsa and immunohistochemistry were carried out. For 572 pts a concurrent FC analysis on BM was available. FC analysis was performed accordingly to Bethesda International Consensus guidelines (2006). *Results.* We studied BM biopsies of 253 pts with follicular lymphoma (FL), 220 diffuse large B-cell lymphoma (DLBCL), 82 splenic MZL (SMZL), 69 extranodal marginal zone lymphoma of MALT (EMZL), 23 primary nodal MZL (NMZL), 30 lymphoplasmacytic lymphoma (LPL), 33 mantle cell lymphoma (MCL), 29 small lymphocytic lymphoma (SLL), 11 primary cutaneous follicle center lymphoma (PCFCL), 11 peripheral T-cell lymphoma (PTCL) and 16 cutaneous T-cell lymphoma (CTCL); 72 cases showed a isolated leukemic picture and were classified as B-cell chronic lymphoproliferative disorders (B-CLPD). Overall, histologic BM involvement was detected at diagnosis in 373/801 (44%) pts. In 21 pts a lymphoid infiltrate was detected without reaching minimal criteria for histologic involvement. By FC analysis, BM involvement was detected in 207/572 (36%) available samples. In the group with suspected histologic infiltrate, FC analysis was positive in 7 out of 16 pts. Detailed results are summarized in Table 1.

Table 1. Comparison betweem histological and flow citometry findings.

	BM histology		BM flow cytometry		Concordance	BM+ FC+	Discordance	BM+ FC-	BM- FC+
	N cases	N cases pos (%)	N cases	N cases pos (%)	%	%	%	%	%
All cases	801	373 (46)	572	207 (36)	85	33	15	12	3
FL	245	110 (45)	163	40 (25)	78	22	22	19	3
DLBCL	211	35 (16)	156	18 (12)	88	8	12	8	4
SMZL	75	69 (92)	40	33 (83)	92	84	8	8	0
EMZL	61	16 (26)	46	6 (13)	82	12	18	15	3
NMZL	23	12 (52)	14	7 (50)	86	43	14	7	7
LPL	30	23 (77)	17	10 (59)	76	53	24	18	6
MCL	32	18 (56)	26	14 (54)	84	48	16	8	8
SLL	27	25 (93)	24	23 (96)	85	85	15	5	10
PCFCL	11	0 (0)	8	0 (0)	100	0	0	0	0
B-CLPD	65	62 (96)	62	56 (90)	88	88	12	8	4
PTCL	10	2 (20)	6	0 (0)	83	0	17	17	0
CTCL	11	1 (9)	10	0 (0)	100	0	0	0	0

BM bone marrow, FC flow cytometry, DLBCL diffuse large B-cell lymphoma, FL follicular lymphoma, LPL lymphoplasmacytic lymphoma, EMZL extranodal marginal zone lymphoma of MALT, SMZL splenic marginal zone lymphoma, MCL mantle cell lymphoma, SLL small lymphocytic lymphoma, NMZL nodal marginal zone lymphoma, PCFCL primary cutaneous follicle center lymphoma, B-CLPD B-cell chronic lymphoproliferative disorder, PTCL peripheral T-cell lymphoma, CTCL cutaneous T-cell lymphoma

A concordance between 2 methods (Spearman's R=0.7, p<0.001) was detected in 459 (85%) cases (33% BM+/FC+; 52% BM-FC-) and a discordance was present in 82 (15%); 64 cases (12%) were BM+/FC- and 18 (3%) BM-/FC+ (with a small clonal population at FC; median 9%). Discordance was more frequent in FL (19% BM+/FC- and 3% BM-/FC+) and in LPL (18% BM+/FC- and 6% BM-/FC+). The rate of false negative FC exams resulted inversely related to the extent of histologic infiltrate for the whole series (p<0.001) and, specifically, for FL (p=0.01), LPL

(p=0.04) and B-CLPD (p=0.03). Patterns were analyzed in 316 BM biopsies. Nodular paratrabecular pattern was associated with FL (p<0.001), sinusoidal with SMZL (p<0.001), interstitial with SLL (p<0.001), diffuse with DLBCL (p<0.001) and with LPL (p=0.02). FC better overlapped with histology in case of interstitial (p<0.001), sinusoidal (p=0.03) and nodular centrolacunar pattern (p=0.01). False negative FC results correlated inversely with% of CD19+ cells (p=0.04). The quantitative assessment of infiltrate detected by the 2 methods resulted significantly overlapping (p<0.001). *Conclusions.* Our data demonstrate that FC is comparable with histology in qualitative and quantitative assessment of BM involvement for the entire spectrum of NHL, with the exception of FL and LPL.

0402

CLIPI: A NEW PROGNOSTIC INDEX FOR INDOLENT CUTANEOUS B-CELL LYMPHOMA PROPOSED BY THE INTERNATIONAL EXTRANODAL LYMPHOMA STUDY GROUP (IELSG 11)

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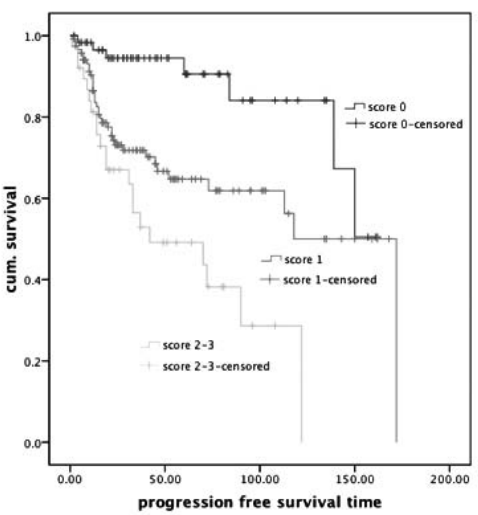
Low grade primary cutaneous B-cell lymphomas (PCBCL) are a distinct group of NHLs, which are generally associated with a good prognosis. However, they frequently relapse and in some cases disseminate, leading to a short survival. The aim of this study was to develop a model for predicting their clinical outcome. From 1980 to 2006, 19 cancer centres in 7 countries recruited 463 PCBCL patients. The median age was 55 years (range, 16-92 years) with a M/F ratio of 1.5. According to the WHO-EORTC classification the main histological subtype was FCL (n=341), followed by MZL (n=122). The sites of presentation were: trunk/arms (56%), head/neck (31%) and legs (7%). Seven percent had a generalized disease (>1 site) with a maximal lesion diameter of >4 cm in 27% of cases, while >2 lesions in one site occurred in 27%. Nodules prevail (75%), followed by plaques (17%), ulcers or 2 distinct coexisting lesion types. Few patients had Ann Arbour Stage II (11%), poor ECOG-PS (9%) and/or elevated LDH (5%).

Table 1. Impact of the CLIPI on treatment outcome.



10 - years	OS (P=0.309)	PFS (P<0.001)	DFS (P<0.001)
Low risk	92%	84	85
Intermediate risk	86%	54	53
High risk	66%	29	30
Total	83%	57	58

The majority of patients (50%) underwent local treatment, mainly consisting in radiotherapy and/or surgery, while 40% were given chemotherapy + local treatment, 7% received rituximab + other treatments, whereas 3% remained untreated. CR rate was 89%, PR 10% and SD 1%. Among 398 responders, 122 (33%) eventually relapsed, 65% in the skin, 26% in extracutaneous sites and 9% in both. After a median follow-up of 56 months (range, 2-333 months), 10-year estimate of OS was 87%, PFS 56% and DFS 59%, respectively. Disease caused 27/48 deaths (56%) and disease specific survival at 10 years was 91%. Taking account of the possible risk factors identified by univariate log-rank test we developed a prognostic model by bootstrap resampling of the original group of patients and applying Cox's proportional hazard model with backward elimination procedure on survival. A complete dataset was available in 240/463 patients. Elevated LDH, nodular lesion, and > 2 lesions proved to have a significant and independent impact on survival. Based on these four factors, weighting every factor with one point, the following prognostic index has been developed: 0 points, low risk (LR); 1 point, intermediate risk (IR) and 2 or 3 points, high risk (HR). We showed that this score significantly influences PFS (Figure 1) and DFS, while showed a trend in OS (Table 1). This retrospective multicenter study confirmed that PCBCLs have an high relapse rate and half of deaths depends on disease progression. The CLIPI proves an important impact on the clinical course of this disease, independently on treatment. Therefore, it could provide a risk adapted treatment strategy to be assessed in prospective clinical studies.

0403

RITUXIMAB-CHOP (R-CHOP) IN THE TREATMENT OF 441 DIFFUSE LARGE B-CELL LYMPHOMA PATIENTS: OUTCOME AND PROGNOSTIC FACTORS FOCUSING TO THE REVISED INTERNATIONAL PROGNOSTIC INDEX (R-IPI)

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Background. The improved outcome of iffuse large B-cell lymphoma (DLBCL) patients with R-CHOP and the biological mechanism of action of rituximab may overcome the adverse prognostic significance of various prognostic factors, identified prior to the introduction of rituximab. Although IPI is still applicable under R-CHOP, a minor modification may enhance its discriminative ability (Rituximab-IPI; R-IPI, Sehn LH, Blood 109: 1857-61, 2007). Furthermore, it is not clear whether a high-risk group of young DLBCL patients, suitable for front-line stem cell transplant, still exists under R-CHOP. *Aim.* The determination of the outcome of patients with DLBCL under treatment with immunochemotherapy in a multicenter retrospective study and the analysis of the corresponding prognostic factors. *Patients and Methods.* We analyzed 441 patients with DLBCL treated with R-CHOP or similar anthracycline-based combinations. The 5 components of the IPI, gender, B-symptoms, serum albumin levels and hemoglobin levels were evaluated as potential prognostic factors. The value of IPI, age-adjusted IPI (aaIPI) and R-IPI was determined. The analyzed endpoints were failure free survival (FFS) (including early toxic deaths as events) and overall survival (OS). *Results.* The median age of the patients was 65 years (18-88), 59% were older than 60 years, and 57% were males. The distribution of patients according to the IPI was as follows: Low Risk (L) 47%, Low-Intermediate (LI) 21%, High-Intermediate (HI) 19% and High Risk (H) 12%. According to the R-IPI, 21% were classified as Low Risk (L), 47% as Intermediate Risk (INT), and 32% as High Risk (H). The 3-year FFS and 5-year OS for all patients were 77% and 80% respectively. According to the IPI, the 3-year FFS was 91%, 84%, 53% and 54% for L, LI, HI and H risk patients respectively (p<0.0001). The corresponding 5-year OS rates were 93%, 77%, 59% ÎαÈ 62% (p<0.0001). According to the R-IPI, the 3-year FFS was 97%, 85% and 53% for L, INT and H Risk patients respectively (p<0.0001). The corresponding 5-year OS rates were 100%, 83%, and 60% (p<0.0001). All 5 IPI components, B-symptoms, low albumin and haemoglobin levels were predictive of FFS in univariate analysis. In multivariate analysis, serum albumin <3.5 g/dL and R-IPI were independent prognostic factors (p=0.01 and <0.001 respectively). In the group of 43 patients ≥60 years with aaIPI 2-3, the 3-year FFS and 5-year OS were

67% and 84%. Only the 12 patients with aaIPI=3 (7% of all younger patients) had indeed a poor 3-year FFS of 38%, with 5-year OS of 59%. *Conclusions.* Our data fully confirm the British Columbia study, which introduced the R-IPI as a better prognostic tool in R-CHOP-treated DLBCL. R-IPI may be further improved by the addition of other conventional prognostic factors, such as serum albumin levels. The role of intensified therapy in younger patients with aaIPI 2-3 is questionable in the era of R-CHOP, since only the minor subgroup of patients with aaIPI=3 appears to carry an indeed poor FFS. The concomitant evaluation of more conventional and biological prognostic factors in even larger series of patients treated with R-CHOP may improve the prognostic stratification.

0404

FINAL RESULTS OF THE PHASE II MULTICENTER ITALIAN STUDY OF IMMUNOCHEMOTHERAPY WITH FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB (FCR) FOR SYMPTOMATIC WALDENSTROM'S MACROGLOBULINEMIA

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Background. Rituximab is an active and well tolerated agent in the treatment of Waldenstrom's Macroglobulinemia (WM), furthermore, there is evidence that the association of Rituximab (R) with chemotherapy may improve the quality of responses. In WM the combination of Fludarabine (F) + Cyclophosphamide (C) induces response rates of 85% and 55-89% in previously untreated and relapsed/refractory pts, respectively. Aims In February 2005 we started a multicenter phase II study on the effectiveness, tolerability and safety of FCR in symptomatic WM pts previously untreated or relapsed/refractory to one line of chemotherapy. *Methods.* Treatment consisted of: R 375 mg/sqm d1, F 25 mg/sqm and C 250 mg/sqm iv d 2-4 every 4 weeks. The first assessment of the disease, including bone marrow evaluation, was performed after the third FCR course. Pts with progressive disease (PD) were considered as failure, responding pts or those with SD went on to receive up to 3 further courses. *Results.* Accrual of the planned 43 pts, median age 65 years, ended on March 2008. Median time from diagnosis to FCR was 25.5 mos; 65% of pts were in first line treatment, 7% and 28% in relapsed and refractory disease, respectively. According to the International Scoring System for WM (ISSWM) 33% of pts were low risk, 38% intermediate and 29% high risk. Median number of courses administered was 6 (range 2-6), 65% pts received the planned 6 courses. Thirty-nine pts (91%) received ≥4 courses of therapy and have been considered valuable for response. The overall response rate was of 87% and categorized as follows: 13% CR, 69% PR, 5% MR. Nine of the 27 pts in PR could be considered as in *near CR* as they fulfilled criteria for CR except for the persistence of a positive immunofixation. None of the patient and disease variables (age, sex, light chain, disease status, Hb, PLT and Ig level,% BM infiltration, β2m, albumin, creatinine, ISSWM) was significant for the achievement of a response. A comparison was performed between responses achieved after the third course and after the end of treatment. A significant improvement in the achievement of a major response has been observed (49% vs. 62%) in the group of pts receiving the planned 6 courses. Extrahematological toxicity was manageable and mostly limited to grade 1-2 . Neutropenia occurred in 63% of courses and was the main cause for not completing the planned 6 courses of treatment (23%). A late improvement of responses was observed during follow-up: 3 PR converted to CR and 1 SD to PR. Median DFS and OS have not been reached after a median follow-up of 14 and 20.2 mos, respectively. *Conclusions.* FCR produced a high ORR with good quality of responses, which improved over time. However we observed a high incidence of neutropenia with long lasting neutropenic episodes limiting the administration of the planned therapeutic program. According to the analysis of our population it seems reasonable in order to avoid myelotoxicity to reduce the number of planned FCR courses, administering, Rituximab as consolidation/maintenance to.

0405

LNH-PRO-05 STUDY: EXCELLENT CLINICAL AND MOLECULAR RESPONSES IN FLIPI=2 FOLLICULAR LYMPHOMA PATIENTS WITH RITUXIMAB PLUS CVP (BAGLEY) AND 12 WEEKS OF INTERFERON ALFA

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Background. The use of immunomodulator agents, such as Rituximab (R) and Interferon (IFN) in Follicular Lymphoma (FL), has improved progression free survival (PFS) and overall survival (OS). Since 1990, our FL patients receive CVP (Bagley, 1972) with IFN-α tiw for 12 weeks. In intermediate-high risk FLIPI patients overall response rate (ORR) was 90% (75% CR) with a median PFS of 6 years. Published data have shown that R and IFN have synergistic antitumor activity, so we designed a trial to test this treatment in association with R. *Aims.*Evaluate the efficacy (ORR, CR, PFS and MRD response) and toxicity of R +CVP+ IFN in newly diagnosed FL patients with FLIPI ≥2. *Methods.* Treatment was R 375 mg/m^2 + CY 400 mg/m^2 D1-5 (po) + VCR 1.4 mg/m^2 D1 + PRD 100 mg/m^2 D1-5 + IFN (3MU/m^2 tiw x 12 wks) x 8 cycles, with G-CSF support. Doses of CY and IFN were adjusted according to grade 3-4 toxicity. Patients were re-evaluated after the 4th cycle and at the end of treatment. Follow-up studies were performed every 4 months thereafter. Patients were removed from the study if no response after cycle 4. Molecular studies to detect MRD (classic and real-time PCR for Bcl-2/IgH and rearrangements of IgH, FR2-FR3) were performed at diagnosis, after 4th and 8th cycle, and during the follow-up period. *Results.* Since November 2006, 26 patients have been enrolled. Herein we communicate the results of 21 monitorized patients. Median age is 51 years old (range 33-74), FLIPI 3-5: 43%, ≥2 extranodal involvement: 57% and bulky disease: 29%. No molecular marker was detected in 4 patients in spite of bone marrow infiltration. Seventeen patients had molecular marker, 15 (88%) with Bcl-2/IgH and 4 (22%) IgH rearrangements. Clinical Response at the end of treatment, is available from 17 patients. At 4th cycle 82% were in CR and at 8th cycle all patients achieved CR/CRi. Molecular response data was available in 12 patients, with 87% response after the 4th and 100% at the end of treatment. Toxicity was evaluated over 124 cycles. Grade 3-4 neutropenia was observed in 33% cycles. Lymphopenia <400/mm^3 was present in 58% patients during treatment. Non-hematologic toxicity was low, 8% (3 cases with flu-like syndrome, 3 peripheral neuropathy, 2 diabetes, 1 diarrhea, 1 osseous pain). Dose intensity for CY was 77% (range 60%-86%) and 59% for IFN (range 31%-85%). Ten SAEs (7% of all cycles) were reported due to febrile neutropenia or infection, although in 6 patients were associated with protocol deviation. None of the patients experienced SUSAR. *Conclusions.* In patients with FLIPI ≥2 follicular lymphoma, R + CVP + IFN schema achieves excellent clinical and molecular responses with a very good toxicity profile.

0406

POSTTRANSPLANT LYMPHOPROLIFERATIVE DISORDER (PTLD): CHARACTERISTICS AND OUTCOME IN A BELGIAN UNIVERSITY HOSPITAL

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Background. PTLD is a life-threatening complication of both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT). Data regarding incidence, treatment and outcome are mainly retrospective and are derived from smaller single center studies and larger registration databases. *Aims.* We undertook a retrospective analysis of all patients diagnosed with PTLD between January 1989 and December 2008 at the University Hospitals of Leuven, aiming to obtain information about incidence, pretreatment characteristics, treatment and outcome. *Methods.* Medical records of all patients were used for this retrospective observational study. Information was obtained with regard to baseline patients characteristics, transplant related characteristics, PTLD characteristics, treatment administered and response to treatment. Histological subtyping was based on the medical records, but we still plan a revision of all samples. *Results.* 112 patients were included in this study. PTLD was biopsy proven in 107 patients, whereas in 5 patients, all following HSCT, diagnosis was made based on rapid increase of EBV viral

load combined with a positive PET/CT scan. Overall incidence for all transplant types was 1.96%. Highest incidence was reported in heart-lung transplantation (7.5%), followed by heart (4.9%), lung (2.9%), liver (2.67%), stem cell (1.45%) and kidney transplantation (1.34%), whereas no PTLD was seen in intestinal transplantation so far. Most PTLD were monomorphic (78.6%), with DLBCL being the most frequent subtype. The majority of cases occurred later than 1 year post-transplantation (67%). Recognized lymphoma and PTLD specific prognostic scores were calculated for all patients. At the moment of PTLD diagnosis immunosuppressive therapy included calcineurin inhibitors (93.8%), antimetabolites (67.9%), low dose steroids (68.5%) and proliferation signaling inhibitors (<1%). RIS was performed in 91% of the cases. Other first line treatment modalities included rituximab (49.1%), chemotherapy (26.8%), surgery (11.6%), radiotherapy (6.3%), antiviral therapy (4.7%) and high dose steroids (3.6%). Following first line therapy overall response rate was 69% (60% CR, 9% PR). At last follow up 54.5% of the patients were alive whereas 12.8% of the patients lost their graft during follow up. In univariate analysis complete response was associated with male gender (p=0.04), normal LDH (p=0.0003), use of high dose steroids (p=0.019), lower ECOG performance state (p=0.015), lower Ann Arbor stage (p<0.0001) and lower number of involved extranodal sites (p=0.019) , whereas overall response rate was associated with male gender (p=0.009), normal LDH (p<0.0001), absence of bone marrow involvement (p=0.04), lower ECOG performance state (p=0.003) and lower Ann Arbor stage (p=0.0063). Overall survival Kaplan Meier curves with regard to all prognostic scores are shown in Figure 1. Conclusions. We report a retrospective analysis of 112 cases of PTLD following SOT or HSCT. Overall incidence was 1.96%, with the highest incidence reported in heart-lung recipients. Interestingly, no PTLD was observed following intestinal transplantation, which might be related to the use of a specific tolerance promoting regimen. Rituximab therapy was not associated with a higher response rate. Although the prognostic role of the IPI score in PTLD has been questioned, we were able to confirm its value in our analysis.

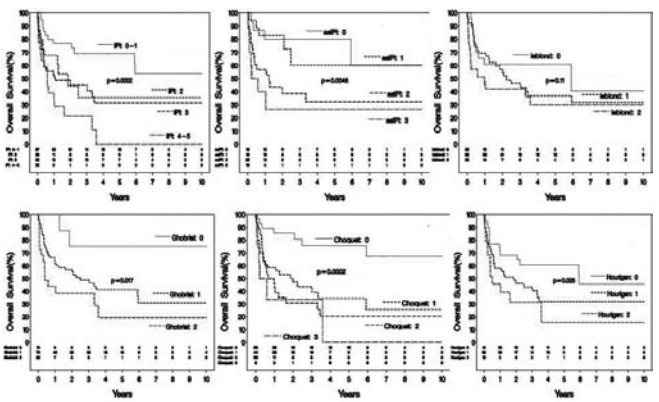

Figure 1. Kaplan Meier overall survival curves.

0407

SAFETY AND CLINICAL ACTIVITY OF THE ANTI-CD22 IMMUNOCONJUGATE INOTUZUMAB OZOGAMICIN (CMC-544) IN COMBINATION WITH RITUXIMAB IN RECURRENT/REFRACTORY FOLLICULAR LYMPHOMA OR DIFFUSE LARGE B-CELL LYMPHOMA

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Background. CMC-544 is a humanized anti-CD22 antibody conjugated to calicheamicin, a potent cytotoxic agent. CMC-544 targets CD22 which is expressed in the majority of B cell non-Hodgkin's lymphomas (NHL). In a phase 1 study of patients (pts) with CD22+ B-cell NHL, the maximum tolerated dose (MTD) for single agent CMC-544 was determined to be 1.8 mg/m^2 administered IV every 28 days. CMC-544 showed clinical activity in pts with follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL). *Aims.* A short dose-escalation study was performed to determine the MTD of CMC-544 in combination with rituximab. An expanded cohort at the MTD assessed the safety and efficacy of the drug combination in recurrent/refractory FL or DLBCL. *Patients and Methods.* Pts were eligible if they had CD20$^+$/CD22$^+$ FL or DLBCL, which had not responded to, or progressed after, 1 or 2 therapies of probable clinical benefit including at least 1 regimen containing rituximab, but could not be refractory to rituximab (arm 1 for FL and arm 2 for DLBCL). A third arm for rituximab-refractory aggressive lymphoma is currently enrolling with no upper limit on the number of prior therapies. Pts received 375 mg/m^2 of rituximab IV on day 1 followed by CMC-544 on day 2 of each 28-day cycle for up to 8 cycles, provide that there was no evidence of progression. *Results.* The MTD of CMC-544 given with rituximab was confirmed as 1.8 mg/m^2. The study is currently enrolling, and as of Feb 2009, 96 pts were entered at CMC-544 doses of 0.8 mg/m^2 (n=5), 1.3 mg/m^2 (n=3) and 1.8 mg/m^2 (n=88). Median number of doses received was four. Among the pts treated at the MTD, the median age was 66 y (range: 20-85), and 60% were male; 38% had 1 prior chemo/immunotherapy regimen, 49% had 2 regimens and 9% had ≥3 regimens; 69% had stage 3/4 disease, 35% elevated LDH, and 15% bulky disease (>7.5 cm). Pts in the relapsed DLBCL arm were generally older with a median age of 72 y. All 96 pts were evaluable for safety. The most common drug-related adverse events (AEs), all grades, were nausea (40%), fatigue (37%), increased AST (31%) and thrombocytopenia (37%). Grade 3/4 drug-related AEs occuring in ≥5% of pts were thrombocytopenia (22%), neutropenia/neutrophil decreased (13%/8%) and lymphopenia (6%). A total of 26 (27%) pts discontinued treatment due to an adverse event, most commonly hematologic AEs (14 pts) or elevation of ≥1 liver function test (10 pts). Table 1 shows the tumor response for patients in arms 1 and 2 treated at the MTD. Both groups had an objective response rate of 81%. The 6-month progression-free survival rate was 95% for FL pts and 66% for DLBCL pts. *Conclusions.* The safety profile of CMC-544 plus rituximab is similar to that previously reported for CMC-544 monotherapy. The main toxicity was manageable, self-limiting thrombocytopenia. The response rates indicate promising efficacy in pts with recurrent/refractory FL and DLBCL. These data warrant continued development of CMC-544 in combination with rituximab for the treatment of NHL.

Table. Best overall response for patients treated at MTD.

Response, n (%)	FL (N=37)	DLBCL (N=31)
Complete response (CR)	15 (41)	13 (42)
Complete response unconfirmed (CRu)	4 (11)	3 (10)
Partial response (PR)	11 (30)	9 (29)
Objective response (OR)	30 (81)	25 (81)
Stable disease	5 (14)	5 (16)
Progressive disease	2 (5)	1 (3)

Note: OR = CR + CRu + PR

0408

CLINICAL SIGNIFICANCE OF THE PRESENCE OF OCCULT CNS INVOLVEMENT ASSESSED BY FLOW CYTOMETRY IN PATIENTS WITH NON HODGKIN'S LYMPHOMA AT HIGH RISK OF CNS RELAPSE

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Background and Aim. Several studies have demonstrated that flow cytometry (FCM) improves the sensitivity of conventional cytology (CC)

in cerebrospinal fluid (CSF) for the identification of leptomeningeal disease in aggressive lymphomas with high risk of central nervous system (CNS) involvement. A previous report from our group showed infiltration assessed by FCM study in 27 out of 123 (22%) newly diagnosed aggressive B-cell lymphomas, while CC was positive in only 7 (6%) cases (Quijano *et al.*, J Clin Oncol 2009, in press). However, the clinical significance of occult disease in CNS (FCM positive and CC negative) remains unknown. *Patients and Methods.* At the time of the analysis, 105 out of 123 (85%) patients of the previous study had adequate follow-up (median follow-up for patients remaining alive [n=67] of 20 months). In this cohort of patients the presence of CNS involvement during follow-up was correlated with the CC and FCM results in CSF at diagnosis. *Results.* Three groups were defined according to the results of CC and FCM: Group 1, patients without CNS involvement (CC and FCM negative, n=83); Group 2, patients with occult CNS disease (FCM positive and CC negative, n=15); Group 3, patients with CNS disease (CC and FCM positive, n=7). The groups were comparable for the main clinical and biological characteristics, except for histological subtypes of NHL (23% of Burkitt's lymphoma in Group 1 vs 71% in Group 3, p=0.01). In Group 1, 58 patients received intrathecal (IT) prophylaxis, 2 received CNS therapy due to presence of neurological symptoms and 23 did not receive any prophylaxis. In Group 2, CNS prophylaxis was administered to 7 patients, 7 received active CNS therapy, whereas the remaining patient did not receive any prophylaxis. All 7 Group 3 patients received active CNS therapy. Overall, 6 patients showed CNS relapse/progression, 2 in Group 1 (2.4%) (both with diffuse large B cell lymphoma [DLBCL]), 2 in Group 2 (13%) (one with DLBCL and the other with Burkitt's lymphoma) and 2 in Group 3 (29%) (both with Burkitt´s lymphoma). Patients from Groups 2 and 3 showed a significantly increase in CNS relapses compared to those from Group 1 (Group 2 vs. 1, p=0.04; Group 3 vs. 1, p<0.001). However, the incidence of CNS relapse in patients from Groups 2 vs. 3 was not significantly different. In addition, patients with FCM+/CC+ showed a significant worse survival compared to those with FCM-/CC- (p=0.02), while no differences were observed for survival in patients with FCM+/CC+ vs FCM+/CC- (p=0.23) and FCM+/CC- vs FCM-/CC- (p=0.40). *Conclusions.* The presence of occult CNS disease in our cohort of patients with NHL at high risk of CNS disease was associated with a higher risk of CNS relapse. In addition to classical clinical and biological risk-factors, FCM could be an useful diagnostic tool to identify those NHL patients at higher risk of CNS relapse and to improve the CNS prophylaxis/therapy.

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0409

A PHASE 2 STUDY OF VORINOSTAT (SUBEROYLANILIDE HYDROXAMIC ACID, SAHA) IN RELAPSED OR REFRACTORY INDOLENT NON-HODGKIN'S LYMPHOMA. A CALIFORNIA CANCER CONSORTIUM STUDY

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Background. The indolent (follicular, marginal zone and mantle cell) lymphomas tend to recur with decreasing intervals of remission after standard chemotherapy. New modalities of treatment are necessary. Vorinostat (SAHA) is an orally administered hydroxamic acid histone deacetylase inhibitor with activity against class I and II deacetylases with preclinical and clinical activity against various forms of lymphoma. *Methods.* We report the results of a phase II study of oral vorinostat in patients with relapsed or refractory (to chemotherapy and/or rituximab) follicular, marginal zone, or mantle cell lymphoma. Vorinostat is given at 200 mg PO twice daily for 14 consecutive days on a 21 day cycle. CT scanning and/or FDG-PET are performed after every three cycles, as is marrow biopsy for those with marrow involvement at time of entry into study. Patients may have received up to four prior chemotherapy regimens including tositumomab or ibritumomab; previous transplant is allowed. 37 patients (14 female, 23 male) were accrued (2 ineligible patient due to wrong histology are excluded from the analysis). The median age at treatment was 65 (32-79) years. Histologies represented: mantle cell (MC)-8, marginal zone (MZL)-7, and follicular lymphoma (FL)-20. Treatment was well tolerated. Grade 3-4 toxicities possibly attributable to study drug were thrombocytopenia, neutropenia, anemia, diarrhea, anorexia, myalgia (1 patient), hypokalemia, hypophosphatemia, hyponatremia, thrombus (1 patient), febrile neutropenia (1 patient), INR (1 patient), mucositis (1 patient) and fatigue. *Results.* The overall response rate is 29%, for patients with FL and MZL the response rate is 37%, with no responses seen in mantle cell. 19 patients were taken off study due to progression. Three pts came off study due to toxicities (fatigue and diarrhea after 10 cycles, diarrhea and dizziness after 2 cycles, DVT after 5 cycles), 1 stopped therapy due to intercurrent illness, 1 came off for alternate therapy and 1 due to patient refusal. Eight remain on therapy, and two completed therapy per protocol. By the current Cheson criteria, 5 patients achieved complete remission (CR), and 5 patients achieved partial remission (PR). Three patients with PR as best response subsequently progressed (at 6, 15 and 16 months), the other two remain on therapy (25+, and 32+ months). One CR was achieved after 2 years of stable disease on therapy. By histology, 10 formal responses were seen in patients with follicular (8) or marginal zone lymphoma (2) whereas no responses were seen in mantle cell lymphoma. The median progression-free survival for the 35 patients is 11 months; the median follow-up of alive patients is 11 months; 5 patients are progression-free for more than 18 months. *Conclusions.* The histone deacetylase inhibitor vorinostat is well tolerated over long durations of therapy, and shows promising activity (10 CR+PR out of 27 patients) against relapsed/refractory follicular and marginal zone lymphoma.

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0410

EARLY EVALUATION OF 18-FDG-POSITRON EMISSION TOMOGRAPHY/COMPUTED TOMOGRAPHY (PET) DOES NOT PREDICT THE OUTCOME IN DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) PATIENTS TREATED WITH R-CHOP

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Background. Unlike Hodgkin Disease, the predictive value of early PET in DLBCL patients is controversial. Published data are often based on retrospective studies, including miscellanea of subtypes and therapies. Moreover, the dichotomous interpretation of PET findings as positive or negative is often difficult to apply in clinical evaluation. Aim of the study. To determine the predictive value of the early PET (PET-2) during treatment of DLBCL pts and of the final PET (PET -3) on Progression Free Survival (PSF). *Patients and Methods.* From April 2004 to December 2008, 81 newly diagnosed DLBCL or follicular grade IIIb pts were included into the study. Clinical characteristics were as follows: 49 males and 32 females; median age 56 years (22-82); 18 pts stage I-II and 63 stage III-IV; according to IPI 52 pts were at low/low-intermediate risk, 28 at intermediate-high/high risk. All pts were treated according to the planned treatment, not modified according on PET-2 *Results.* 55 with 6-8 R-CHOP and 26 with 4 R-CHOP courses followed by high-dose chemotherapy with autologous stem cell transplantation (HDC+ASCT). All pts had PET scan performed at the diagnosis, after 2-4 courses of therapy (PET-2) and at the completion of treatment (PET-3): all PET results were defined as positive or negative with visual dichotomous consensus response criteria. Results. 70 pts completed planned treatment and were evaluable for response. PET-2 was perfomed after 2 R-CHOP in 41 pts, after 3 courses in 15 and after 4 in 22. At the end of treament 64 pts (91%) achieved a CR and 6 (9%) were non responders. Forty-nine pts (62%) were negative and 30 (38%) positive at the PET-2 and 64 pts(91%) were negative and 6 (9%) positive at the PET-3. The concordance between clinical evaluation of CR and PET-3 negativity was 98.6%: one CR pt was false PET-3 positive due to parothid carcinoma. Correlation between PET results and outcome was evaluated. No correlation between PET-2 results and CR rate was found: CR 95% in PET-2 negative pts vs 81% in PET-2 positive (p=ns). With a median FU of 17 months, PFS was 76%. PET-2 did not correlate with PFS (p=.88), conversely PET-3 strongly predicted PFS (p<0.0001). (Figure 1). In univariate analysis IPI, time of PET-2 evaluation, plan of therapy had not a statistically significative influence. Mainly, the lack of predictive value of PET-2 was observed both in the subgroup of pts treated with/without HDC+ASCT. 2y-PFS rates PET-2 negative vs PET-2 positive pts were: HDC+ASCT group 77% vs. 91%; no HDC+ASCT group 81% vs. 79%. 2y-PFS rates PET-3 negative vs PET-3 positive pts were: HDC+ASCT group 86% vs. 0%; no HDC+ASCT group 88% vs. 33%. Conclusions.

In contrast to prior studies regarding prognostic value of interim PET in DLBCL untreated pts, our results indicate that in R-CHOP treated pts early PET does not predict outcome. Conversely PET results at the end of treatment strongly correlate with PFS. Future prospective larger studies will be need to establish the real role of interim PET to predicting outcome in this subset of patients.

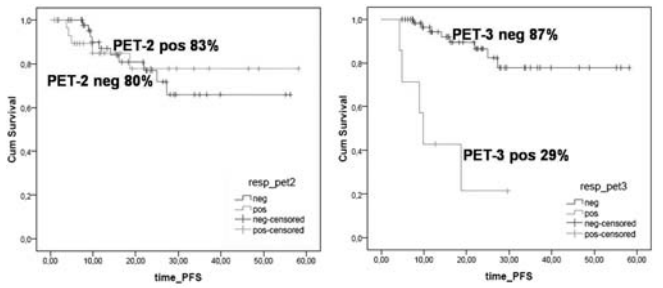

Figure 1. PFS at 17 months by PET-2 and PET-3 evaluation.

0411

ACTIVITY AND SAFETY OF BORTEZOMIB AND RITUXIMAB IN RELAPSED/REFRACTORY INDOLENT NON FOLLICULAR AND MANTLE-CELL NON HODGKIN LYMPHOMA: A PHASE II MULTICENTER STUDY BY INTERGRUPPO ITALIANO LINFOMI

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Background. Combination of Bortezomib (B) and Rituximab (R) has been shown *in vitro* synergistic apoptosis and enhanced NFkB depletion in Mantle Cell Lymphoma (MCL) and Marginal Zone Lymphoma (MZL) cells. Aim of the study. To evaluate safety and efficacy of the association of R+B in relapsed/refractory indolent non follicular and mantle cell lymphoma patients not eligible to high dose chemotherapy and ASCT. The primary endpoint was Overall Response Rate (ORR) >40%. *Patients and methods.* From September 2006 to March 2008, 54 patients were enrolled into the study. Clinical characteristics were as follows: 29 males and 25 females; median age was 68 years (range 50-74); 20 lymphocytic/lymphoplasmacytic (LL), 10 MZL and 24 MCL; 45 stage III-IVdisease; according to IPI 36 patient were at low-intermediate risk and 18 at intermediate-high/high risk; 10 patients had 1 and 44 >2 prior lines of therapy; 17 were R-naïve patients and 37 R-pretreated, 23 patients had refractory disease (<1 yr from the last therapy) and 31 were in relapse (>1 yr). The treatment plan was: one course of 4 weekly doses of R (375 mg/mq) and B (1.6 mg/mq IV bolus) followed by 2 courses of 4 weekly IV bolus of B (1.6 mg/mq) as single agent. Responding (CR+ PR) and stable disease patients were planned to be given further 3 courses at the same schedule. Histological diagnosis was centrally reviewed. *Results.* ORR was 25/54 (46%, 95%CI: 34-59). Overall responses were: 14 CR+ CRu (26%) and 11 PR (20%). ORR by histology was: 7/20 (35%) in LL, 5/10 (50%) in MZL and 14/24 (58%) in MCL respectively. Pretreatment with Rituximab did not adversely influence the ORR: 18/36 (50%) in R-pretreated patients and 6/17 (35%) in R-naïve patients. ORR was better in relapsed patients compared with refractory ones: 19/30 (63%) and 5/23 (22%) (p=0.002). Data regarding PFS and OS are on course. A total of 251 courses were delivered with a median of 4.6 courses per patient. Thirty-two patients completed the treatment plan and 22 withdrawn from the study because of: 15 PD during treatment and 7 AE (1 pleural effusion, 1 neurotoxicity grade II, 2 pneumonia, 1 MOF, 1 concomitant gastrointestinal adenocarcinoma and 1 toxic death due to interstitial pneumonitis). Grade III-IV CTC haematological toxicity was rare with neutropenia in 4% of the courses and thrombocytopenia in <2%. The most frequent extra-haematological toxicity was: neurotoxicity grade II in 13 pts and grade III in 5 with recover or return to grade I in all of them but one; infections were detected in 3 patients (pneumonitis), constipation grade III in 2 and diarrhea grade >III in 5. *Conclusions.* This study suggests that the combination of R plus B is feasible and effective in relapsed/refractory indolent non follicular lymphoma and MCL. This combination treatment based on *in vitro* data of synergistic effect of R and B may be a good therapeutic option without chemotherapy mainly in MCL and MZL. The combination of R plus B should be explored in further and larger studies.

0412

LYMPHOMA - EMERGING REALITIES IN SUB-SAHARA AFRICA

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Background. Substantial geographical differences exist for Hodgkin and other lymphoproliferative disorders with these having previously been documented in a report from the lymphoma reclassification project. *Aims.* In the light of rampant human immunodeficiency syndrome, largely centred in sub-Sahara, this experience is updated in a further 512 consecutive individuals treated over an eight-year period in a privately based academic centre. *Methods.* Consecutive registered cases were clinically evaluated. Diagnosis based on nodal histopathology and classification according to World Health Organisation criteria. Staging on traditional CT imaging and bone marrow trephine biopsy: performance status defined by international prognostic indices. Treatment was risk-stratified to standardized protocols with informed consent and outcome statistically analyzed using conventional methods. *Results.* Median age was 55.2 years 61% were males, 10% in Hodgkin lymphoma and, overall, constitutional symptoms were present in 20%. Prior to referral 19% had received chemotherapy and a further 20% some form of irradiation. Median survival in hairy cell leukaemia (n=14), chronic lymphocytic leukaemia-small lymphocytic lymphoma (n=103), Hodgkin (n=41) and follicular lymphoma (n=59) was not reached at the time of analysis and exceeded 36 months. This was followed by 32 months for those with mantle cell (n=7), splenic (n=2) and extranodal marginal cell (n=11), 24 months for T-cell lymphomas (n=24), 20 months for diffuse large B-cell variants (n=88) but only 12 months for the aggressive tumours exemplified by Burkitt (n=7) and lymphoblastic subtypes (n=6). The remaining 36 patients had to be excluded because numbers were too small for statistical analysis or unreliable staging. Adverse factors were constitutional symptoms, prior treatment with chemotherapy, intermediate or high-risk scores as defined by the International Prognostic Index, histologic grading and certain anatomical sites of primary tumour. In contrast gender, staging by Rye or Rai classification, prior treatment with radiotherapy and, notably, retroviral infection were without effect. Overall survival at three years in each category was compared to the curve for the entire cohort and was 100% in hairy cell leukaemia receiving two chlorodeoxyadenosine and greater than 88% in Hodgkin lymphoma treated according to the German study group protocols (p=0.0004). Corresponding figures for chronic lymphocytic leukaemia - small lymphocytic lymphoma were 82% (p=0.0006), follicular lymphoma 71% (p=0.060), peripheral T-cell lymphoma 43% (p=0.0156), diffuse large B-cell lymphoma 39% (p<0.0001), aggressive tumours 25% (p=o.0002) and for the indolent categories including mantle cell, splenic and extra nodal marginal cell lymphomas 22% (p=0.2023). *Summary and conclusions.* Outcome argues in favour of patient management by a multidisciplinary team implicit in which is use of standardised protocols for diagnosis, staging and treatment. Under these circumstances the well recognized centre effect applies and here results approximate those from first world reference centres. Conversely any deviation from such a disciplined approach is unlikely to achieve comparable benefit and therefore to be strongly discouraged. Relevantly effective antiretroviral has blunted the impact of this infection on outcome thereby significantly changing older practices in under resourced areas of the world.

0413

FIRST RESULTS OF LONG TERM RITUXIMAB MAINTENANCE TREATMENT IN FOLLICULAR LYMPHOMA: SAFETY ANALYSIS OF THE RANDOMIZED PHASE III TRIAL SAKK 35/03

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Background. Rituximab maintenance has been shown to be effective in patients with follicular lymphoma. *Aims.* To ascertain a more optimal duration of Rituximab maintenance in this indication. *Methods.* We

prospectively registered 270 patients with untreated, chemotherapy resistant or relapsed follicular lymphoma. All patients received rituximab induction consisting of 4 weekly doses (375 mg/m^2). Responding patients (PR and CR) were randomized to a short maintenance consisting of four doses of rituximab (375 mg/m^2) every two months (arm A) or prolonged maintenance consisting of rituximab every two months for a maximum of five years or until progression or unacceptable toxicity (arm B). The primary endpoint was event-free survival. All patients gave informed consent. Here we present the safety analysis results. *Results.* From October 2004 to November 2007 165 patients were randomized; 82 in arm A [25 (30%) previously relapsed/progressed, 57 (70%) untreated] and 83 in arm B [24 (29%) previously relapsed/progressed, 58 (70%) untreated and one stable]. Of the non-randomized patients, 49% (51 patients) were previously untreated and 50% (52 patients) were previously relapsed/progressed, the remaining 2 patients were previously resistant or stable. The median follow up is 22.7 months. A total of 442 hematological and non-hematological adverse events were observed, 27 of grade 3 and 6 of grade 4. Five subsequent cancers and 9 grade 3 and 4 infections were reported. Grade 3 and 4 neutropenia occurred in 5 patients of which the grade 4 neutropenia occurred in a previously untreated patient. Decreased levels of IgG were observed in 19 patients, 60% of who were previously untreated. Four grade 3 infections occurred after 2 years of maintenance. In arm B, maintenance was stopped due to unacceptable toxicity (fever) in 1 patient after 18 months and due to subsequent breast cancer in 1 patient after 20 months. One patient died 4 months after randomization because of ileus and consecutive peritonitis; considered to be unrelated to therapy. All three of these mentioned patients that stopped therapy were previously untreated. Twenty-nine patients are on maintenance for two or more years of which 6 patients are on for three or more years. In this analysis, median duration of the prolonged maintenance is 23.7 months. The trial has been closed for accrual but there are still patients on treatment. *Conclusions.* Rituximab maintenance beyond two years is feasible. Previous treatment seems not to influence occurrence of adverse events. However, close follow up of patients under prolonged rituximab maintenance is necessary.

0414

VALIDATION OF ABSOLUTE LYMPHOCYTE COUNT / REVISED IPI (ALC/R-IPI) SCORE MODEL, AS A PROGNOSTIC INDEX FOR DIFFUSE LARGE-B-CELL LYMPHOMA IN RITUXIMAB ERA

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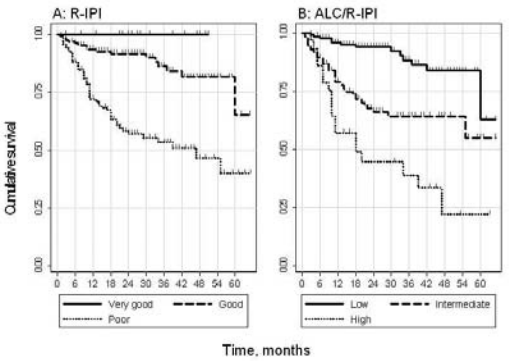
Background. The International prognostic index (IPI), since its publication in 1993, became the primary prognostic tool for patients with DIFFUSE LARGE B-CELL LTMPHOMA (DLBCL). However, the introduction of rituximab (R) has improved patients outcomes and has partially changed the predictive capacity of IPI. In 2007, in the R era, a most accurate prognostic index, termed revised IPI (R-IPI) was proposed by Sehn *et al.* More recently, Cox *et al.* showed that absolute lymphocyte count (ALC), at diagnosis has a prognostic impact and it is independent from R-IPI. Thus, they have built-up a score, termed ALC/R-IPI, incorporating both parameters (low risk=ALC≥0.84 109/L and R-IPI very good or good, intermediate risk= ALC<0.84 109/L or R-IPI poor; high risk= ALC<0.84 109/L and R-IPI poor). Aim of our research, utilizing Gruppo Italiano Studio Linfomi (GISL) database is to validate the ALC/R-IPI model on a second larger independent patients data set like the GISL database. *Methods and Results.* From GISL database we collected data of 831 DLBCL patients, of which 560 treated with chemotherapy alone (1988-2003) and 271 treated with R-containing chemotherapy regimens (2003-2007). We have used the Kaplan-Meier curves, c-Harrell concordance index and the Cox proportional hazard regression to evaluate the ability of ALC/R-IPI to discern patients having good or poor survival. The median age of the 271 cases treated with R was 69 years and 51% were male. At diagnosis, 62% were in stage III/IV, 49% had LDH>UNL, 16% a PS>1, 26% had extranodal sites>1 and 26% presented with ALC<0.84 109/L. The distribution of patients in R-IPI was 7%, 48% and 45% in very good (VG), good (G) and poor (P) groups, respectively. The distribution in ALC/R-IPI was 47%, 42% and 11% in low (L), intermediate (I) and high (H) risk groups, respectively. The OS at 3-years was 88%, 64% and 39% in L, I and H risk groups, with significative differences (p<0.001). The HR were: I vs L = 3.7 (p<0.001); H vs I = 2.1 (p=0.012), while the c-Harrell was = 0.71 (CI95% 0.65-0.76). *Conclusions.* The ALC/R-IPI showed a good ability to discriminate the prognosis of patients in term of OS in our data base. ALC was confirmed as a specific prognostic factor for DLBCL in R era.



R-IPI: Very Good = IPI 0, Good = IPI 1-2 and Poor = IPI 3/5.
ALC/R-IPI: 1, Low risk = R-IPI VG AND ALC ?0.84·10^9/L ; 2, Intermediate risk = R-IPI Poor OR ALC<0.84·10^9/L; 3, High risk = R-IPI Poor AND ALC<0.84·10^9/L.

Figure. Overall survival stratified by revised prognostic models of our 271 patients. A) R-IPI; B) ALC/R-IPI.

0415

SPLENECTOMY VERSUS RITUXIMAB IN THE TREATMENT OF SPLENIC MARGINAL ZONE LYMPHOMA

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Background. Splenic marginal zone lymphoma (SMZL) treatment is not standardized. Splenectomy has traditionally been considered as the treatment of choice. Recent data however suggests that Rituximab is a promising agent for the treatment of SMZL. *Aims.* To evaluate the activity of splenectomy as well as Rituximab monotherapy in the treatment of SMZL and to compare these two treatment modalities regarding response rate, progression free survival, overall survival and toxicity. *Methods.* 64 SMZL patients, diagnosed and followed in our Departments, were retrospectively analyzed. Among them, 36 (56%) received rituximab monotherapy (induction therapy at a dose of 375 mg/m^2/week for 6 weeks and maintenance therapy every 2 months for one year), while 28 patients underwent splenectomy. Treatment was administered to patients with symptomatic splenomegaly, cytopenias and/or autoimmune phenomena. *Results.* Clinical and laboratory characteristics between the two treatment groups are presented on Table 1.

Table 1. Characteristics of 64 SMZL patients.

Parameters	Splenectomized Patients		Rituximab treated Patients	
	#	(%)	#	(%)
Patients	28	44	36	56
Median age (years)	63		58	
Male gender	13	46	13	36
Median spleen size (range)	10 (0-18)		10 (0-20)	
B-symptoms	8	28	3	8
Lymphadenopathy	0		2	5
Anaemia (Hb<12gr/dl)	16	57	19	53
Median Lymphocyte count (10^9/L)	4.8		2.8	
Thrombocytopenia (<100 X10^9/L)	6	21	7	19
IPI				
Low Intermediate	21	75	27	75
High Intermediate	7	25	9	25
Median follow up (months)	57		30	

All splenectomized patients except one (96%) achieved complete resolution of splenomegaly-related symptoms along with restoration of cytopenias to normal, although lymphocytosis persisted. One toxic death was recorded one month after splenectomy. Moreover, 7 patients underwent bone marrow (BM) evaluation a year after splenectomy which revealed an increase in lymphocytic infiltration along with a change in the pattern of BM involvement in 5 of them. The overall response rate of rituximab treated patients was 92% (CR 45%, PR 25%, unconfirmed CR 22%) after induction therapy. Maintenance therapy further improved the response in 4 patients. Among the complete responders 38% achieved a molecular response. Rituximab treatment was well tolerated with no grade 3 or 4 toxicities. No differences were observed in 3-year overall survival and progression free survival between splenectomized and rituximab treated patients (84% vs. 94% and 79% vs. 90% respectively. *Conclusions.* The present study demonstrates that splenectomy and rituximab are among the most effective treatment strategies in SMZL. The limited toxicity of Rituximab along with a high complete response rate clearly indicate that it can replace splenectomy, especially in patients with advanced age, comorbidities, or an underlying autoimmune disorders.

0416

PHASE II STUDY OF INTRATHECAL LONG ACTING LIPOSOMAL CYTARABINE (DEPOCYTE®) IN THE PROPHYLAXIS OF LYMPHOMATOUS MENINGITIS IN HIV-RELATED NON-HODGKIN'S LYMPHOMA

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Around 5% of patients with aggressive non-Hodgkin's lymphoma (NHL) develop central nervous system (CNS) progression or relapse during the course of their disease. Patients with human immunodeficiency (HIV)-related NHL often develop CNS progression despite the use of adequate prophylaxis. Liposomal cytarabine has show a significant activity in lymphomatous meningitis but there are limited data in the prophylactic setting. Between May 2006 and December 2008, we performed a prospective phase II study of intrathecal liposomal cytarabine (Depocyte®) at the dose of 50 mg in 28 patients with HIV-NHL with the aim to evaluate the feasibility and activity of this drug in the prevention of lymphomatous meningitis. Twenty-three patients were males and the median age was 42 years (range 18-64 yrs). As far as the histological subtype of NHL, 64% of patients had a diffuse large B-cell (DLBC) NHL and 36% Burkitt NHL. Stage III-IV was diagnosed in 75% of patients and 79% of DLBC were age-adjusted IPI 2 or more. An extranodal involvement was diagnosed in 64% of patients (gastrointestinal 29%, bone marrow 18%, spleen 18%, bone 14%). Liposomal cytarabine was well tolerated with headache grade I to III being the most frequent side effect in 46% of patients. Less common toxicity (all grade I) included cortical changes (7%), fever (4%), vomiting (4%), hypertension (4%), chills (4%). With a median follow up of 10 months only one patient (4%) with Burkitt lymphoma developed a combined systemic and meningeal relapse. Moreover, in our experience previously the present study, we used methotrexate as practical use in 267 HIV-NHL with a meningeal progression or relapse of 10% (p=0.32). The use of a liposomal formulation allowed to significantly reduce the number of lumbar injections in comparison to the standard schedules (approximately of 50%) with an improvement of quality of life of patients and with a reduction of professional exposure risk for health care staff. In conclusion, in this first prospective study on prophylaxis of lymphomatous meningitis in HIV-NHL reported in the literature, liposomal cytarabine seems safe and active and it reduces of approximately 50% the number of lumbar punctures and exposure risk for health staff as well.

0417

INTEREST OF 18-FLUORO-DEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY (FDG-PET) IN INITIAL STAGING, RESPONSE ASSESSMENT AND PROGNOSTIC VALUE OF MANTLE CELL LYMPHOMA

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Background. FDG-PET is a non-aggressive imaging modality commonly used in the management of patients with malignant lymphoma. FDG-PET is recommended for initial staging and response assessment of diffuse large B-cell lymphoma and Hodgkin lymphoma. There are few published data on the interest of FDG-PET in the initial staging, response assessment and prognostic value in MCL. *Aims.* This multicentric retrospective study investigate FDG-PET imaging at initial staging, response assessment and its prognostic value in the setting of MCL patients. *Methods.* From 2003 to 2008, 44 previously untreated MCL patients were recruited in six GOELAMS centers and evaluated by both conventional method and FDG-PET for initial staging. After completion of therapy, response was assessed according to IWC and IWC-PET criteria. *Results.* FDG-PET uptakes at diagnosis were abnormal in all cases. Compared to conventional evaluation, some additional nodal and extra-nodal sites were only detected by FDG-PET, especially splenic involvement. FDG-PET was not able to detect bone marrow (BM), central nervous system and gastro-intestinal (GI) involvement. Due to frequent visceral involvement in MCL (BM and GI), initial staging was not modified by FDG-PET. At end-treatment assessment, response according to IWC and IWC-PET shown discrepancies in six cases. Statistical analysis demonstrated that response assessment according to IWC-PET was not associated with others prognostic variables (IPI, MIPI, treatment, blastoid variant, SUV, Ki67). The 2-year event-free survival rate was influenced by IWC response (p=0.01), IWC-PET response (p=0.002), maximal SUV (p=0.005) and the 2-year overall survival was influenced by IWC response (p=0.03), IWC-PET response (p<0.001), maximal SUV (p=0.04) and blastoïd morphology (p=0.002). *Conclusions.* FDG-PET is a relevant tool at initial staging, complementary to conventionnal evaluation BM and GI biopsies remains mandatory in MCL. Concerning final assessment, the IWC-PET system identifies patients at high risk of relapse and could be more effective than standard IWC system. However, prospective studies with a longer follow-up are warranted before to draw final conclusions about the clinical impact of FDG-PET in MCL.

0418

LONG-TERM FOLLOW-UP OF RITUXIMAB AND INFUSIONAL CYCLOPHOSPHAMIDE, DOXORUBICIN, AND ETOPOSIDE IN COMBINATION WITH HAART IN HIV-RELATED NON-HODGKIN'S LYMPHOMAS

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Background. The combination of Rituximab plus chemotherapy (CT) is more effective than CT alone in the treatment of high grade NHL. *Objective.* To report the long-term follow-up of etoposide (CDE) plus Rituximab in HIV-NHL. *Methods.* In June 1998, we started a phase II study using infusional CDE (Cyclophosphamide 187.5 mg/m^2/day, Doxorubicin 12.5 mg/m^2/day and Etoposide 60 mg/m^2/day) administered by continuous intravenous infusion for 4 days every 4 weeks and Rituximab 375 mg/m^2 i.v. on day 1. HAART was given concomitantly with CT. *Results.* Seventy-four patients (pts) have been enrolled. The median CD4$^+$ cell count was 161 (range 3-691) and the median Performance Status was 1 (range 0-3). Diffuse large B-cell NHL was diagnosed in 72% of pts and Burkitt in 28%. Seventy per cent of pts had advanced stage (III-IV) disease and 57% of pts had an age-adjusted international prognostic index >2. Fifty-two out of 74 pts (70%) achieved a complete remission (CR), 4/74 (5%) had a partial remission and 18 pts progressed. With a median follow-up of 61 months, only 17% of CRs have relapsed and 41/74 pts are alive. The overall survival, disease free survival and time to treatment failure (TTF) at 5 years were 56%, 81% and 52%, respectively. Only one secondary tumor (acute leukemia) has been observed. No case of late pulmonary or cardiac toxicity has been reported. *Conclusions.* The combination of Rituximab and CDE in HIV-NHL treated concomitantly with HAART is very active. CR rate (70%) and TTF at 5 years (52%) are comparable to those observed in high grade NHL of the general population. Our data confirm that in HAART era a high proportion of HIV-NHL can be cured.

Non-Hodgkin lymphoma - Clinical II

0419

HISTOLOGIC TRANSFORMATION OF FOLLICULAR LYMPHOMA INTO DIFFUSE LARGE B-CELL LYMPHOMA IN THE IOSI EXPERIENCE

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Background. Follicular lymphoma displays an indolent clinical course characterized by a continuous pattern of relapses, with no clear evidence of cure. A proportion of patients ultimately experiences a histological transformation into aggressive large cell lymphoma. Patients and *Methods.* We retrospectively analyzed the outcome of 281 patients with FL treated at the Oncology Institute of Southern Switzerland (IOSI) since 1979 to 2007, with respect to clinical features at diagnosis, therapeutic approaches, survival patterns, with special reference to the impact of histologic transformation on disease course. Median survival times, Kaplan-Meier survival curves and relative survival rates were calculated. *Results.* The median age of the entire group at time of diagnosis was 58 years (range 21-92 years). Median overall survival was 11 years (95% CI, 8.8-14 years). After a median follow-up from diagnosis of 10 years, histologic transformation into aggressive lymphoma was observed in 39 patients (14%, 95%CI:10-18%). The median time to transformation was 5 years from diagnosis. Risk of transformation at 5, 10 and 15 years was 13% (95%CI, 9-18%), 16% (95%CI, 12-22%), 27% (95%CI, 19-38%), respectively. The histologic transformation adversely impacts cause specific survival (CSS) (p=0.0003): in the population who did not experience histologic transformation the 10-years CSS was 74% (95%CI:66-80), in the group of patients who had histologic transformation, the 10-years CSS was 44% (95%CI:26-61). The median survival after transformation was 3 years. The risk of histologic transformation was higher in the group of patients diagnosed before 1989 compared to the subsequent period (p=0.03). An initial watchful waiting approach was associated to a lower risk of subsequent histologic transformation (p<0.05). No other therapeutic approach seems to impact the risk of histologic transformation. Among the clinical variables at diagnosis evaluated, a trend toward a significant association has been observed for an hemoglobin level lower than 12,0 gr/dL (p=0.06) and for the presence of at least a single bulky site of disease at diagnosis (p=0.07). *Conclusions.* Our findings are in keeping with other reports showing a negative impact of histologic transformation on the clinical outcome but we were not able to confirm the recently reported increased risk of transformation in follicular lymphoma patients initially followed with a watchful waiting policy. The impact of specific therapeutic strategies on histologic transformation needs to be further explored.

0420

FOLLICULAR LYMPHOMA IN LEUKEMIC PHASE AT DIAGNOSIS: CLINICAL AND BIOLOGICAL FEATURES IN A SERIES OF 24 PATIENTS AND COMPARATIVE ANALYSIS WITH NON-LEUKEMIC PATIENTS

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Background. According to the WHO classification of tumours of lymphoid tissues, apart from common nodal presentation, bone marrow, spleen, Waldeyer´s ring and peripheral blood (PB) are possible sites of involvement, among others. However, presentation of follicular lymphoma (FL) with morphologically evident leukemic phase at diagnosis is rare. It is well known that prognosis of this type of lymphoma is closely related to the extent of the disease. As pointed out by other authors, if leukemic expression is considered as an extranodal involvement, it might serve as prognosis factor in FL. However, differential characteristics of patients with this specific form of presentation are poorly studied. *Aims.* To analyze the main clinical and biological features of patients with FL in morphological leukemic-phase at diagnosis, and to compare them with those of patients without peripheral expression. *Methods.* We retrospectively analyzed the records of 24 patients with FL in leukemic phase at onset, collected from three third-level hospitals in our community. Optical microscopic evidence of lymphoma cells in blood smear was the primary condition to recruit our cases. Among others, studied variables included: sex, age, constitutional B symptoms, FL grade, Ann Arbor stage, Follicular Lymphoma International Prognostic Index (FLIPI), spleen and liver involvement, hemogram, serum LDH and β 2-microglobulin, bone marrow biopsy findings, therapeutic approaches (specifically if rituximab and/or autologous stem cell transplantation were included), best achieved response, time to obtain a complete remission (CR), and survival. Immunophenotypic analysis of lymphomatous cells from blood samples was performed in the group of patients with peripheral expression. Randomly chosen from our database, a group of 30 patients with FL without peripheral blood involvement was used as control-group for comparisons. *Results.* There were not differences in sex, age, histological grade, and serum LDH, between both groups. Of interest, patients with leukemic phase at diagnosis, presented more frequently with B symptoms (p=0.024), and also with a higher incidence of splenomegaly (p=0.009), a higher serum level of β 2-microglobulin (p=0.007), and a lower haemoglobin level and platelet count (p=0.0004 and p=0.006, respectively). All the patients with peripheral expression had bone marrow involvement, while only 63.3% in the other group (p=0.001). According to the FLIPI, 50% of the patients in leukemic phase were classified as high risk, and 33% in the control group (trend to statistical significance, p=0.08). In the group of patients in leukemic phase at diagnosis, lymphocytosis was found in 71% of cases, peripheral lymphocyte count ranging widely from 6.7 to 365×10^9/L. Percentage of FL cells oscillates from 6% to 96% of lymphoid cells in blood (mean 59%). Lymphomatous cells in blood expressed CD19, CD20 and CD79b (100% of analyzed cases), CD10 (91%), CD22 (73%), FMC7 (65%), CD11c (43%), CD38 (42%), and CD23 (30%), and they were consistently negative for CD5 and CD103. Both groups of patients were therapeutically equivalent concerning the use of rituximab and autologous stem cell transplantation. Percentage of cases that reach CR and average time to achieve this CR were similar. Finally, patients with peripheral expression at onset showed a trend to a worse prognosis in terms of survival. *Conclusions.* In our study, patients with FL in leukemic phase at diagnosis presented with features of a greater tumor burden, bone marrow involvement in all cases included. In accordance with other authors, our results suggest that leukemic status may be associated with a worse prognosis in patients with FL. Thus, as indicator of tumour burden, the role of peripheral expression might be reconsidered as prognostic factor, although further studies are needed to confirm these findings.

0421

ORAL VORINOSTAT COMBINED WITH BEXAROTENE IN ADVANCED CUTANEOUS T-CELL LYMPHOMA: A PHASE I STUDY

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Background. Vorinostat, an oral inhibitor of Class I and II histone deacetylase (HDAC) enzymes, is registered in the US for treatment of cutaneous manifestations of T-cell lymphoma (CTCL) in patients with progressive, persistent or recurrent disease on or following two systemic therapies. Preclinical studies suggest that vorinostat may synergistically enhance the activity of chemotherapeutic or biological anticancer agents in a variety of solid and haematologic malignancies. This Phase I, multicentre, open-label, non-randomised, dose-escalation study evaluated the combination of vorinostat and the retinoid bexarotene in patients with advanced CTCL. *Aims.* The primary objective was to determine the maximum tolerated dose (MTD) of this combination; secondary objectives included assessment of overall safety and tolerability, and estimation of clinical activity. *Methods.* Eligible patients were aged ≥18 years with stage ≥IB progressive, persistent or recurrent CTCL refractory to ≥1 systemic therapy. There were two parts to the Phase Ia portion of the study. In Part I, patients were enrolled sequentially to receive escalating doses of vorinostat (200-400 mg/day) and bexarotene (150-300 mg/m^2/day). In Part II, the dose of vorinostat was fixed at 400 mg/day and the dose of bexarotene was escalated (150-450 mg/day). Cycles were repeated every 28 days for a maximum of ″6 cycles. A continuation phase of the study was available for patients who experienced

potential clinical benefit after completing six cycles of treatment. *Results.* To date, all 23 patients who received ≥1 dose of study medication have been evaluated for safety. Three patients experienced dose-limiting toxicities (DLTs) at dose level (DL) 2 (Table). No DLTs were observed in the first cycle of DL 2a and 2b. The MTD for Part I was established at vorinostat 200 mg/day plus bexarotene 300 mg/m^2/day. The MTD for bexarotene dose escalation (Part II) was not reached as the study was discontinued early due to low enrolment. One DLT was observed at DL 7; DL 7 was the highest level tested. The most common drug-related adverse events (DRAEs) were hypothyroidism (35%), fatigue (30%), and hypertriglyceridaemia (30%). No Grade 4 or 5 DRAEs were reported and four patients had serious DRAEs (Grade <3). Eighteen patients have discontinued: five due to AEs, six due to progressive disease, and seven withdrew consent. Of 23 patients evaluated for efficacy (prior to the continuation phase), four patients (17%) had an objective response and seven patients (30%) derived clinical benefit (pruritus relief). *Conclusions.* These preliminary data suggest that vorinostat in combination with bexarotene is feasible and requires further investigation in patients with advanced CTCL; however, the dosage of either drug must be reduced to avoid unacceptable side effects.

Table.

Dose level (n=23)	Vorinostat (mg/day)	Bexarotene (mg/m^2/day)	Dose-limiting toxicity No. of patients	Objective response[a,b] n (%)	Pruritus relief[c] n (%)	Best response by individual physician's assessment[a] n (%) Confirmed response	Unconfirmed response	Stable disease	Progressive disease
Part I									
1 (n=3)	200	150	-	0	1 (33)	0	0	2 (67)	1 (33)
2 (n=5)	300	150	Grade 3 fatigue (1), Grade 3 hypertriglyceridaemia (3)[d]	0	1 (20)	0	1 (20)	4 (80)	0
2a (n=3)	200	225	-	0	0	0	1 (33)	2 (67)	0
2b (n=3)	200	300	-	1 (33)	1 (33)	1 (33)	0	2 (67)	0
Part II									
6 (n=5)	400	150[e]		2 (40)	3 (60)	2 (40)	0	2 (40)	1 (20)
7 (n=4)	400	225[e,f]	Grade 3 neutropenia (1)	1 (33)[g]	1 (33)[g]	1 (25)	0	3 (75)	0

[a]Measured using the modified Severity Weighted Assessment Tool (mSWAT); [b]>50% reduction in mSWAT score from baseline maintained for ≥4 weeks; [c]assessed using questionnaire; [d]one patient experienced both fatigue and hypertriglyceridaemia; [e]mg/day; [f]bexarotene 150 mg/day (cycle 1) then 225 mg/day (cycles 2-6); [g]n=3.

0422

CLINICAL IMPACT OF SIX-COLOR FLOW CYTOMETRY IN LYMPHOPROLIFERATIVE DISORDERS: IDENTIFICATION AND EXTENDED CHARACTERIZATION OF COMPOSITE LYMPHOMAS

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Background. Composite lymphomas (CLs) are relatively frequent neoplasms among B-cell chronic lymphoproliferative disorders (CLPDs). Sanchez *et al.* (Blood, 2003) validated the role of three-color flow cytometry for detection of biclonality in single specimens. *Aim.* In this study we used, for first time, six-color flow cytometry for the diagnosis of composite lymphomas with the aim to demonstrate the reliability of this technique in indentification and extended characterization of different clonal populations. *Methods.* In our clinico-diagnostic activity, we studied, form November 2006 to September 2008, 250 samples including peripheral blood (PB), bone marrow asiprates (BM) and fine needle aspiration cytology (FNAC) collected from patients with B-CLPDs. The specimens analyzed for the diagnosis of CLs were 4 PB, 2 BM, and 2 FNAC samples. A total of fifteen antigens were studied (see the Table) in every specimens, introducing in our antibody panel some less used antibodies such as anti-CD43, anti-CD25, anti-CD81 and anti-CD200. The two clones detected in every specimen were named as clone A and clone B. Analysis was performed using FACSCanto II cytometer (Beckton Dickinson, BD, San Jose, USA) and the FACSDiva software (BD). *Results.* Among 250 lympoproliferative disorders, six cases (4,8%) were classified as CLs. Two cases were composed by a marginal zone lymphoma (MZL) clone plus a chronic lymphocytic leukemia (CLL) clone, two cases showed the simultaneous presence of a mantle cell lymphoma (MCL) clone plus a MZL clone, one case was characterized by the presence of a MCL clone plus a follicular lymphoma (FL) clone and one was composed by two different clones of FL. Detection of biclonality was univocal and easy, as well as the extended characterization of clones. Complete immunophenotypes are shown in the Table. The two clonal population found in every specimen are reported as Clone A and Clone B. *Discussion.* In this study two or three flurochromes were exploited to apply high-quality gates and remaining fluorochromes were used to define other cytometrical specificities in each clone, making sure the cytometrical diagnosis of CLs. In four cases cytometrical and histopathological reports did not overlap. Complex architectural patterns in histological and immunohistochemical slides may not enable microscopical identification of different clones. On the other hand, six-color flow cytometry seems to overcome this obstacle capitalizing on analysis of several parameters that cannot be studied with less sophisticated flow cytometry techniques as well as light microscopy. Introduction of CD43 in our panel played a fundamental role in one case. Indeed, in one specimen we were able to completely split two clones only on a CD43-CD10 dot plot. All other investigated antigens gave us no chances to clearly separate two distinct populations. Multicolor techniques are becoming more diffuse day after day and 6-color flow cytometry is already present in a large number of laboratories of hematology. In this study we demonstrated feasibility and simplicity of 6-color flow cytometry in composite lymphomas diagnosis. We also emphasized advantages of this more sophisticated approach that can overcome problems related to a 3-color approach and can allow a cytometrical diagnosis of composite lymphoma even through a single tube analysis.

Table.

Patient	1		2		3		4		5		6	
Clone	A	B	A	B	A	B	A	B	A	B	A	B
Kappa	+	-	+	-	-	+	-	-	+	+	-	+
Lambda	-	+	-	-	+	-	+	+	-	-	+	-
CD19	+	+	+	+	+	+	+	+	+	+	+	+
CD5	-	-	-	+	+	-	-	+	-	+	-	+
CD10	+	+	-	-	-	-	+	-	-	-	-	-
CD20	+	+	+	+	+	+	+	+	+	+	+	+
CD22	+	+	+	+	+	+	+	+	+	+	+	+
CD23	-	-	-	+	-	+	-	-	-	+	-	+
CD43	-	-	-	+	-	-	-	-	-	-	-	+
CD38	+	+	-	-	-	+	-	+	-	+	-	-
CD11c	-	-	na	na	-	-	-	-	-	-	+	+
CD103	-	-	-	-	-	-	-	-	-	-	-	-
CD81	+	+	na	na	na	na	na	na	na	na	na	na
CD25	na	na	na	na	+	+	na	na	-	-	-	+
CD200	-	-	na	na	na	na	na	na	na	na	na	na

0423

LONG-TERM FOLLOW-UP OF TANDEM HIGH-DOSE THERAPY WITH AUTOLOGOUS STEM CELL SUPPORT FOR ADULTS WITH HIGH-RISK AGE-ADJUSTED INTERNATIONAL PROGNOSTIC INDEX AGGRESSIVE NON-HODGKIN'S LYMPHOMAS: A GOELAMS PILOT STUDY

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Background. Single high-dose therapy followed by autologous peripheral blood stem cell support improves the complete response and overall survival in untreated aggressive non-Hodgkin's lymphoma. However, patients with a high age-adjusted international prognostic index retain a poor prognosis with a complete response and 5-year overall survival rates at 46% and 32%, respectively. *Aims.* In an attempt to improve complete response in these patients, the GOELAMS conducted the phase II 073 pilot trial to evaluate upfront tandem high-dose therapy with peripheral blood stem cell support in aggressive non-Hodgkin's lymphoma with high-risk age-adjusted international prognostic index. This study was planned before the era of monoclonal antibodies. *Methods.* All patients aged from 15 to 60 years with previously untreated histologically proven aggressive non-Hodgkin's lymphoma (F, G, H according to the Working Formulation), a high age-adjusted international prognostic

index (equal to 3) provided written informed consent. Two courses of the CEEP regimen (cyclophosphamide, epirubicin, vindesine, prednisone) with two weeks intervals, were planned. Responders underwent tandem high-dose therapy conditioned by high-dose mitoxantrone plus cytarabine for the first high-dose therapy and total body irradiation, carmustine, etoposide and cyclophosphamide for the second. Patients were eligible for the second high-dose therapy if they reached at least a partial response after the first. *Results.* Thirty-one patients out of forty-one evaluable patients completed the program. A total of four toxic deaths occurred during treatment: one after the first CEEP, three after the second high-dose therapy. The intensity of the TBI-containing regimen was subsequently reduced to total body irradiation + cyclophosphamide, resulting in the absence of additional lethal toxicity. The complete response and overall response rates were 49% and 61% respectively. With a median follow-up time of 114 months, 10-year overall survival and progression-free survival are estimated by 51% [38-69%] and 53% [38-73%], respectively. On multivariate analysis, overall survival was significantly affected by serous effusion (p=0.043) and by histology, with a favorable prognosis of the G and H histologies (p=0.026) while progression-free survival was affected by histology (p=0.0064) only. All patients, except one, who survived after ten months were relapse-free. The patient who relapsed with a non aggressive histology (follicular) was successfully treated using rituximab alone. To date, two non-fatal secondary malignancies have been observed (54 and 113 months after inclusion). Of note, no myelodysplastic syndrome or secondary acute myeloblastic leukemia have occurred. *Summary and Conclusions.* With a complete response rate of 49% and a 10-year overall survival estimate of 51%, the 073 compares favorably with other studies. Moreover, the occurrence of a plateau after ten months suggests that a subgroup of patients with aggressive non Hodgkin's lymphoma and with high age-adjusted international prognostic index might be cured by tandem autologous high-dose therapy.

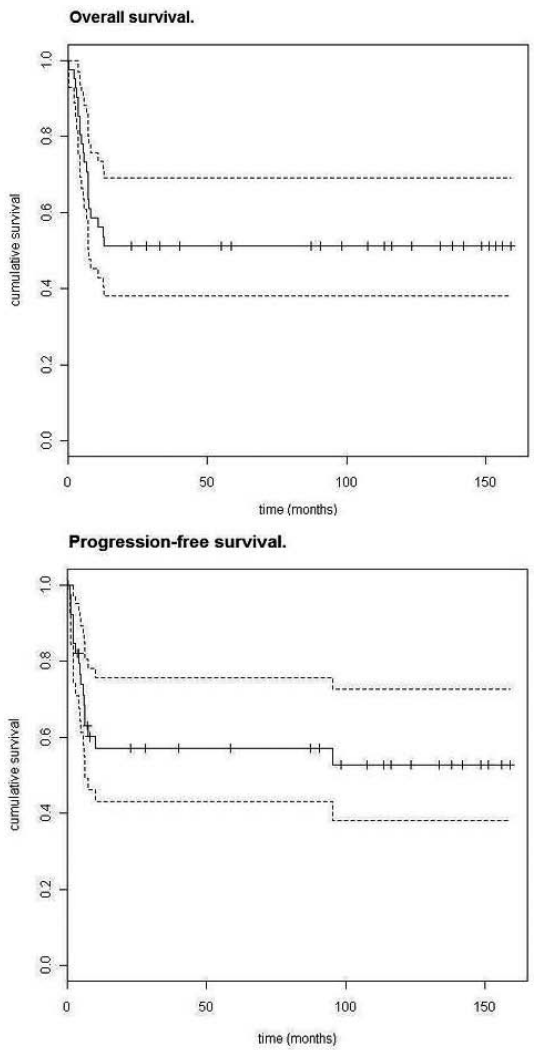

Figure. OS and PFS.

0424

RAPID INFUSION OF RITUXIMAB IS WELL TOLERATED AND ENABLES MORE EFFICIENT USE OF HAEMATOLOGY DAY WARD RESOURCES

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Background. Rituximab is the only currently approved monoclonal antibody for the treatment of CD20 expressing B-cell malignancies with indications and usage continuously extending. Rituximab is generally well tolerated, however, infusion related toxicity remains a concern. Therefore, the recommended infusion rate is slow, resulting in infusion times of 4-6 hours. This has major implications on the resources of a chemotherapy day ward where a treatment chair usually remains occupied for the whole day by a patient receiving rituximab at the approved infusion rate. *Aim.* We sought to establish a simple, easy to follow schedule as previously described for infusion of rituximab at an increased infusion rate (Rapid Mabthera®) at our institution and in a second step quantify the benefits achieved in resource utilisation. *Methods.* We prospectively collected data from two patient cohorts, the first consisting of patients with B-cell NHL only, without circulating malignant cells, the second cohort included other diseases as well. All pts received their second or subsequent rituximab infusion after having tolerated previous rituximab without grade 3 or 4 adverse events (AEs). All patients received standard premedication including corticosteroids. Pts received 20% of the infusion over 30 mins and the remaining 80% over 60 minutes. All pts were monitored during the rituximab infusion and 30 min thereafter, AEs were recorded and graded according to NCI CTCAE version 2. For the 2nd cohort, the number of patients additionally treated as a result of liberated treatment space was determined. *Results.* Over a period of 14 months, 102 pts (58M, 44F), median age 59 years (range; 16-88) received a total of 284 Rapid Mabthera® infusions. The underlying diseases treated were DLBCL (51%), FL (24%), CLL (13%), other indolent B-cell NHL (10%), and autoimmune diseases (2%). The majority of pts (82/102, 80%) received Rapid Mabthera® in conjunction with chemotherapy (209/284 infusions, 74%). The rapid infusion of rituximab was very well tolerated with no AEs grade 3 or 4 observed. Only 2/284 (0.7%) of Rapid Mabthera® administrations were associated with infusion-related toxicities grade 1 (pruritus and chest pain), both resolved with interruption of the rituximab infusion for 30 min and additional anti-histamine and corticosteroid administration. The infusion was completed at conventional rates without further toxicities. The first patient cohort of 32 pts received a total of 83 Rapid Mabthera® infusions resulting in 208 hrs nursing time saved over a period of 7 months. A total of 201 Rapid Mabthera® infusions were given to the 76 pts of the 2nd patient cohort, resulting in an estimated 503 hrs of additional time the respective treatment spots became available. These liberated resources were used for 15 additional treatments. *Conclusions*. Our data confirm that Rapid Mabthera® administered over 90 min is well tolerated when given as second or subsequent administration and results in considerable saving of nursing time. However, this translated into modest additional patient treatment opportunities, most likely due to conventional allocation of treatment slots. Greater confidence in the ability to deliver rapid infusions will allow more efficient resource utilisation.

0425

A NOVEL GERMLINE MUTATION IN MSH6 - IMPLICATIONS FOR THE CLINICAL CARE OF CHILDHOOD LEUKAEMIA/LYMPHOMA PATIENTS

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Background. Constitutional mismatch repair deficiency is caused by homozygous or compound heterozygous germline mutations in mismatch repair (MMR) genes, e. g. MLH1, MSH2 and MSH6, leading to a severe cancer susceptibility syndrome designated as mismatch repair cancer syndrome (MMRCS, MIM). Compared to Lynch syndrome (LS) caused by heterozygous germline mutations in the same genes, MMRCS is characterized by a more severe clinical phenotype with childhood onset of leukaemias / lymphomas or brain tumours, early onset of Lynch syndrome-associated malignancies, and phenotypic features of neurofibromatosis type 1. To illustrate the clinical implication of this syndrome for the management of childhood leukaemia/ lymphoma patients, we report here on a female index patient with MMRCS caused by a novel homozygous germline mutation in MSH6. *Subjects*. The female index

patient, having several café-au-lait spots, developed a mediastinal T-cell lymphoblastic lymphoma at age 6. Following chemotherapy, she relapsed and underwent haematopoietic stem cell transplantation from a matched sibling donor. By the age of 13, she was referred for genetic counselling due to the diagnosis of colorectal cancer. Her consanguineous parents reported on colorectal cancer at age 42 in a paternal uncle married to the sister of the index patient's mother, but LS or MMRCS was not yet considered. *Results.* The index patient's colon cancer showed high-grade microsatellite instability (MSI). Immunohistochemistry demonstrated a loss of MSH6 both in normal and cancer cells. Sequence analysis of MSH6 detected the homozygous germline mutation c.691delG (p.Val231TyrfsX15). Subsequently, the parents of the index patient and the affected paternal uncle, whose synchronous colorectal carcinomas also displayed MSI and loss of MSH6, were shown to be heterozygous carriers of the frame shift mutation. *Conclusions.* We report here on a novel MSH6 mutation and provide clinical information on a further family with LS and MMRCS. Although LS is well known, up to now there are only few reports on MMRCS. This report further emphasizes how important it is to be aware of MMRCS in pedigrees with Lynch syndrome-associated cancer, e.g. early-onset colon cancer, and childhood leukaemia / lymphoma and / or brain tumour. The synchronous colorectal carcinomas of the affected uncle, retrospectively the first evidence of a mismatch repair defect in the reported family, were diagnosed one year before the lymphoma relapse of the index patient and six years before she was diagnosed with colorectal cancer. Up to now, no evidence-based screening recommendation exists for patients with constitutional mismatch repair deficiency. Nevertheless, early diagnosis of this severe cancer susceptibility syndrome may improve the clinical management of affected individuals and their relatives at risk.

0426

DOSE-DENSE THERAPY WITH NON-PEGYLATED LIPOSOMAL DOXORUBICIN, CYCLOPHOSPHAMIDE, VINCRISTINE, PREDNISONE AND RITUXIMAB (R-COMP) IS FEASIBLE AND EFFECTIVE IN ELDERLY PATIENTS WITH NEWLY DIAGNOSED AGGRESSIVE B-CELL NON-HODGKIN LYMPHOMA: R-COMP 14 VS R-COMP 21. INTERIM ANALYSIS FROM AN ITALIAN MULTICENTE

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The toxicity and efficacy of nonpegylated liposomal doxorubicin (MyocetTM) when substituted for conventional doxorubicin in the CHOP 14 or 21 regimen (Doxorubicin, Cyclophosphamide, Vincristine, Prednisone given every 2 or 3 weeks) were evaluated in the treatment of elderly patients with newly diagnosed aggressive B-cell non-Hodgkin's lymphoma. Forty-eight patients with aggressive B cell non-Hodgkin lymphoma at diagnosis were enrolled so far in the study. Patients were split in 2 groups according to the Multidimensional Geriatric Assessment (MDGA). Patients with an Activities of Daily Living (ADL) = 6 were addressed to receive dose-dense R-COMP every 2 weeks (14), whereas patients with an ADL > 7 were addressed to receive R-COMP every 3 weeks (21). Starting from this background, 30 patients were enrolled in the R-COMP 21 arm and 18 in the R-COMP 14 arm. The study was planned as a double phase II according to a single-step Fleming design using 3 years event-free survival as primary endpoint. The characteristics of patients enrolled in the study were as follows: the median age was 72 years (range: 67-80) in the R-COMP 14 group and 75 years (range: 66-89) in the R-COMP 21. At baseline 13/18 (72%) patients had stage IV disease in the R-COMP 14 group, whereas 13/30 (43%)in the R-COMP 21. Median performance status and median number of comorbities were comparable between the 2 groups. Thirteen out of 18 (72%) patients had an intermediate or high risk International Prognostic Index score in the R-COMP 14 group, compared to 12/30 (40%) in the R-COMP 21. The median left ventricular ejection fraction (LVEF) before starting chemotherapy was comparable between the two groups (59% vs 60%). A total of 261 cycles of chemotherapy were administered (96 R-COMP 14 and 165 R-COMP 21). Of the cycles administered, 9 (9%) were delayed by haematological toxicity in the R-COMP 14 group and 11 (7%) in the R-COMP 21 group, with a relative dose intensity for the regimens of 91% and 93%, respectively. Toxicity was mainly haematological in both groups. Grade 3/4 neutropenia occurred in 10% and 24% of cycles in the R-COMP 14 and 21 groups respectively, with an incidence of febrile neutropenia of 3% and 5% respectively. It is of note that patients addressed to receive dose-dense chemotherapy were treated with pegfilgrastim on day +2 during the entire study treatment, with a notable reduction in the incidence of both severe and febrile neutropenia. Regarding cardiotoxicity, only 1/18 patients presented a grade II-IV WHO toxicity (atrial fibrillation) in the R-COMP 14 group, whereas 4/30 in the R-COMP 21 group (one congestive heart failure, two ischemic heart failure, one reduction of 20% in the LVEF). All patients were evaluable for response between the two groups. In the R-COMP 14 group, 15/18 patients (83%) obtained a CR, 2/18 (11%) achieved a PR, and 1/18 (6%) did not respond to therapy and rapidly died due to progressive disease. In the R-COMP 21 group, 20/30 patients (67%) obtained a CR, 7/30 (23%) achieved a PR, and 3/30 (10%) did not respond to therapy and rapidly died due to progressive disease. With a median follow-up of 7 months (range 2-12) and 10 (range 4-24) as of January 2009, 15/18 patients (83%) and 25/30 (83%) are alive and disease free in the R-COMP 14 and in the R-COMP 21 group, respectively. In conclusion, 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 favorably impact on response rate and survival. The increased response rate obtained with dose-dense R-COMP 14 might impact on long-term event-free survival. A longer follow-up is warranted to better define the impact of dose-dense R-COMP regimen on overall survival of patients with high-intermediate or high IPI score.

0427

ALLOGENEIC STEM CELL TRANSPLANTATION IN NON-HODGKIN'S LYMPHOMA PATIENTS WHO PROGRESSED AFTER HIGH DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION: A RETROSPECTIVE STUDY BASED ON DONOR AVAILABILITY

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Background. There are few treatment options in non-Hodgkin's lymphoma (NHL) patients who progressed after high dose chemotherapy (HDC) with autologous stem cell transplantation (auto-SCT). The role of allogeneic stem cell transplantation (allo-SCT) in these patients has not yet been clarified. *Aims.* The objective of this study was to investigate the role of allo-SCT in NHL patients who progressed after HDC with auto-SCT. *Methods.* Retrospective analyses were performed on NHL patients who underwent human leukocyte antigen (HLA) typing after failure of HDC with auto-SCT between February 1998 and November 2008. We compared patients who received salvage allo-SCT (allo-SCT group) with those who did not have a suitable donor and received salvage chemotherapy only (chemotherapy group). Analyses of clinical outcomes of allo-SCT were also performed. *Results.* A total of 30 patients (male, 20) were included. Median age was 38 years (range, 17-61). During a median follow-up of 43.3 months (range, 3.9-103.4), 20 patients (allo-SCT group) received allo-SCT from a suitable donor: 15 HLA-identical siblings (75.0%) and 5 matched unrelated donors (25%). The other 10 patients (chemotherapy group) did not have a donor and received salvage chemotherapy only. In allo-SCT group, 11 patients received conventional conditioning (CC) and 9 patients received reduced intensity conditioning (RIC). Median overall survival (OS) from the time of failure after HDC with auto-SCT was 11.9 months (95% confidence interval (CI), 3.2-20.6). Allo-SCT group (21.5 months (95% CI, 7.9-35.1)) had significantly longer OS than chemotherapy group (3.4 months (95% CI, 0.5-6.3); p=0.002). In allo-SCT group, median event-free survival (EFS) and OS from allo-SCT were 2.8 (95% CI, 0.0-6.5) and 19.0 months (95% CI, 4.0-34.0), respectively. Estimated 5-year survival rate of allo-SCT group was 33.8%. There was no significant difference in EFS and OS between CC group and RIC group. Median number of $CD34^+$ cells infused was 4.36×10^6/kg (range, 1.26×10^6-10.37×10^6). Median time to recovery of neutrophil (>500/microliter) and platelet (>20,000/microliter) was 17 and 27 days, respectively. Median duration of hospitalization after allo-SCT was 26 days (range, 2-54). Acute graft-versus-host disease developed in 4 patients (20.0%). There were 5 transplant-related deaths (25.0%). Incidence of transplant-related mortality (TRM) in CC group tended to be higher than that in RIC group (36.4% vs. 11.1%; p=0.319). Patients with low baseline serum albumin level (<3.0 g/dL) had significantly higher TRM (75.0% vs. 12.5%; relative risk 21.0 (95%

CI, 1.4-314.0); *p*=0.027) as well as shorter EFS (0.3 months (95% CI, 0.0-1.4) vs. 4.9 months (95% CI, 2.0-7.8); hazard ratio (HR) 7.4 (95% CI, 1.4-38.0); *p*=0.017) and OS (0.3 months (95% CI, 0.0-1.4) vs. 22.1 months (95% CI, 14.6-29.6); HR 11.9 (95% CI, 1.9-74.9); *p*=0.008) after allo-SCT. *Summary and Conclusions.* Despite high TRM, allo-SCT is a viable option in NHL patients who progressed after HDC with auto-SCT. Low baseline serum albumin level (<3.0g/dL) was related to higher TRM and shorter EFS and OS in this group.

0428

IFOSFAMIDE, EPIRRUBICIN, AND ETOPOSIDE (IEV) AS MOBILIZATION-THERAPY REGIMEN FOR PATIENTS WITH NON-HODGKIN LYMPHOMA AND HODGKIN DISEASE: COMPARISON WITH OTHER CHEMOTHERAPY REGIMENS

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*Background and Aims.*High dose chemotherapy and autologous peripheral blood stem cell (PBSC) rescue is a treatment modality for lymphoma patients presenting with high risk features or relapse/refractory disease. PBSC can be obtained using growth factors alone or in combination with chemotherapy. The latter approach could be more effective to mobilize stem cells with an additional anti-lymphoma effect. Among these regimens, Ifosfamide, epirrubicin and etoposide (IEV) has been described as effective providing a high response rate in refractory/relapse patients and excellent mobilizing characteristics, but comparison with other chemotherapy regimens is lacking. In this study, we analyze the efficacy of IEV to mobilize stem cells and the impact on outcome after transplant compared with other chemotherapy regimens. *Patients and-Methods.* We included 74 patients who underwent autologous PBSC transplant for Non-Hodgkin lymphoma (NHL) and Hodgkin Disease (HD) in two institutions. 35 patients (19 HD and 16 NHL) in first complete remission (n=7), relapse (n=25) or refractory (n=3) disease received IEV regimen (Ifosfamide 2.500 mg/m^2, days 1-3, epirrubicin 100mg/m^2 day 1, etoposide 150 mg/m^2 days 1-3). 39 patients matched for diagnosis and status at transplant who received other chemotherapy schedules (OCS) (Cyclophosphamide 23.1%. ESHAP 20.5%, Etoposide 12.8%, ABVD/MOPP 7.7%, DexaBEAM 5.1% and Hiper-CVAD 5.1%), were used as cohort-control. Conditioning regimen was mostly BEAM (83.8%) and Total body irradiation + Cyclophosphamide (16.2%). *Results.* Number of CD34 positive cells obtained was higher with IEV regimen (8.2±1.5 vs. 5.4±0.7×10^{6}7Kg, *p*=0.08) compared to other regimens. The median of leukapheresis procedures was 1.5 (range 1-5) for IEV group and 2 (range 1-8) for OCS group (*p*=0.01). Meaningfully, we observed a earlier neutrophil (>500) engraftment day (10.5±1.4 vs. 12±4.5, *p*=0.08) and platelet (>20.000) engraftment day (11.2±3vs. 13. 6± 3.9, *p*=0.01) in IEV group. Disease free survival (DFS) for IEV group was 57.7±9.9% and 43.2 ±9.1% for OCS group (*p*=0.4). Overall survival (OS) was also higher in IEV group (55.8±10.7 vs. 36.1±12.1%) but these figures did not reach statistical differences. *Conclusions.* IEV regimen is highly effective in mobilizing PBSC for autologous transplant in patients with HD and NHL comparing to standard chemotherapy regimens, resulting in a faster hematopoiesis recovery. OS and DFS after transplant were also higher, although we did not find statistical differences.

0429

SMALL LYMPHOCYTIC LYMPHOMA: RETROSPECTIVE ANALYSIS FROM THE REGISTRY OF CZECH LYMPHOMA STUDY GROUP

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Background. Small lymphocytic lymphoma (SLL) accounts for 3-10% of Non-Hodgkin´s lymphomas (NHL). According to World Health Organisation classification of lymphoid neoplasms, SLL forms a common entity with chronic lymphocytic leukemia (CLL). SLL differs from CLL only by arbitrary cut-off of circulating malignant lymphocytes below 5000/mm^3. SLL is still a relatively poor-defined entity with few published data, partly because clinical studies in CLL usually exclude patients with SLL. This results in marked variability in diagnostic and especially therapeutic strategies; uniform guidelines for SLL do not exist. *Aims.*to perform a retrospective analysis of patients (pts) with SLL focused on diagnostic and therapeutic approach in real practice. Patients and *Methods.* As of April 2008, the Czech Lymphoma Study Group (CLSG) registry contained data from 161 patients (63% males, median age 65 years [range, 29-87]) who fulfilled diagnostic criteria for SLL, i.e. coexpression of CD5/C19/23 by flow cytometry or immunohistochemistry and peripheral blood lymphocytes <5000/mm^3. Cases of SLL comprised 3.4% of all NHL in CLSG registry. *Results.* Ann Arbor stage I/II/III/IV was present in 6/2/8/84%. Splenomegaly was detected in 18%, elevated lactate dehydrogenase (LDH) in 30% and bone marrow involvement in 79% of pts. Of note, 91% pts underwent computer tomography (CT) of thorax and abdomen as part of 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 percent of pts had B-symptoms. Age-adjusted International Prognostic Index (aa-IPI) score 0/1/2/3 was present in 7/49/38/6%. Treatment was initiated in 156 pts: chemotherapy in 94 and chemoimmunotherapy in 56 pts (rituximab, 55 pts; alemtuzumab, 1 case). Four patients were treated by radiotherapy and two by surgery. The most frequent regimens used in first line were CVP (18%), CHOP (17%), R-CVP (15%), chlorambucil (15%), FCR (11%), FC (10%) and R-CHOP (10%). Median follow-up was 37.7 months (mo). Median progression-free-survival (PFS) and overall survival (OS) were 35.3 and 63.3 mo. None of the prognostic factors (Ann Arbor stage, aa-IPI, elevated LDH, bulky lymphadenopathy) had significant influence on OS. Similarly, OS was not affected by the type of treatment (fludarabine-based vs. anthracycline-based vs. other; addition of rituximab to chemotherapy). *Conclusions.* Results from CLSG registry confirm previously published demographic 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. Bulky lymphadenopathy was detected in more than a third of pts. No prognostic factors influencing overall survival were identified. Therapeutic strategies varied greatly; two thirds of pts were treated by regimens commonly used in other indolent lymphomas. Our study underscores the need for standardized diagnostic and therapeutic approach to SLL. Supported by grant NR/ 9453-3 and research project MZO 00179906 from Ministry of Health, Czech Republic.

0430

PHARMACODYNAMIC ANALYSES OF PERIPHERAL BLOOD FROM RELAPSED B-NHL PATIENTS TREATED WITH ANTI-CD19/-CD3 BISPECIFIC BITE≥ ANTIBODY BLINATUMOMAB

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Introduction. Blinatumomab (MT103/MEDI-538), a BiTE® antibody bispecific for CD19 and CD3, redirects and activates T cells resulting in the lysis of B lymphoma and normal B cells. An ongoing phase 1 single-agent dose escalation study in patients with advanced B-NHL has shown sustained complete and partial objective responses for up to and exceeding 12 months. *Methods.* Peripheral blood of patients with relapsed B-NHL undergoing treatment with blinatumomab was analyzed for counts of B cells, T cells and T cell subsets as well as T cell activation markers and cytokine levels at defined time points during the course of continuous i.v. treatment. *Results.* Blinatumomab caused a rapid reduction of B cell counts, which was amplified and accelerated with increasing dose levels. This reduction was caused by B cell depletion mediated by apoptosis. Specific fluctuations of T cell counts with a high inter-patient variability were seen. After onset of continuous infusion of blinatumomab, a fast disappearance of CD4$^+$ and CD8$^+$ T cells in peripheral blood within hours was followed by reappearance within few days. The majority of reappearing T cells expressed activation marker CD69 and to a lesser extent CD25 and HLA-DR. In most cases, peripheral T cell counts exceeded after several weeks of treatment those at baseline. Analysis of T cell subpopulations indicated that the expansion of peripheral T cell counts was limited to the subset of CD8$^+$ and CD4$^+$ effector memory T cells as defined by the absence of CD45RA and CCR7 markers. Counts of other T cell subsets remained more or less constant during treatment with blinatumomab, including those of naïve T cells defined as CD45RA$^+$/CD28$^+$. No clinical signs of cytokine release syndrome have

been observed. Nevertheless, cytokines IL-2, IL-6, IL-10, TNF-α, and IFN-gamma were detected in serum samples of patients treated with blinatumomab doses of 0.030 mg/m²/24 h and higher. All cytokine signals showed maximum levels within less than 24 h after onset of infusion and mostly returned to baseline within 24 h for the remaining treatment period. Of note, no IL-4 levels were detected. *Conclusions.* Blinatumomab dose-dependently depleted B cells from peripheral blood for the entire treatment period. Both $CD4^+$ and $CD8^+$ T cells of patients undergoing treatment with blinatumomab showed a specific pattern of activation, rapid redistribution and a subsequent expansion of effector memory cells with high inter-patient variability. Dose-dependent cytokine signals were transient and occurred early during treatment.

0431

SAFETY AND EFFICACY OF BENDAMUSTINE WITH OR WITHOUT RITUXIMAB IN THE TREATMENT OF HEAVILY PRETREATED PATIENTS WITH LYMPHOMA OR CLL. 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. It was developed in 1960s but was never systematically studied in patients until 1990s. Recently this drug was introduced in Italy and it was used in pretreated patients to test its efficacy and safety. *Aims.* The aim of this study was to collect nationwide cases treated with bendamustin in order to evaluate response to therapy and haematological and extra-hematological toxicities. We analized all patients treated in eleven haematological Italian centers with Bendamustine alone or in combination with anti-CD20 antibody. *Methods.* Patients who have received at least one complete cycle were evaluated for response and toxicity, patients with ongoing therapy were not evaluated for response but were evaluated for toxicity. Treatment consisted of: Bendamustine 60-90 mg/m² days 2,3 alone or in combination with Rituximab 375 mg/m² day 1, every 21 or 28 days. *Results.* One hundred and twenty-two patients were analized, median age was 65 (range 31-87), 83 were male and 39 female, the diagnosis were: 40 chronic lymphatic leukaemia 28 indolent non-follicular lymphoma (small lymphocitic, marginal, lymphoplasmocitic), 20 diffuse large B cell lymphoma, 19 follicular lymphoma, 13 mantle cell lymphoma, 2 Peripheral T cell lymphoma. Patients were heavily pretreated and the median number of previous treatments was 3 (range 1-8). Fifthy patients have experienced more than three chemotherapy schemes. Ninety-six patients were previously treated with Rituximab and 21 had performed an autologous stem cell transplantation. The Bendamustine pre-treatment condition was: 53 relapsed patients, 28 with refractory disease and 41 with a progressive disease after partial response. The median number of Bendamustine cycles was 4 (range 1-11), 23 patients were on treatment. Ninety-seven patients were evaluable for response: 25 (26%) complete remission, 52 (54%) partial response or stable disease with an overall response rate of 81%. Nineteen patients were judged as non responders. No differences were observed according to Bendamustine dosage or scheduling. Interestingly, we observed that all evaluable patients with mantle cell lymphoma obtained a response (5 CR and 4 PR). Also, 22/23 (4 CR and 18 PR) indolent non follicular lymphoma and 13/15 (6 CR and 7 PR) follicular lymphoma obtained a response, 26/31 CLL obtained a response and 7/18 (3 CR and 4 PR) DLBCL obtained a response to therapy. With a median period of observation of 7 months (1-36), 78% of patients are alive. In this group of heavily pretreated patients, 498 cycles were performed: the extrahematological toxicity was acceptable in a subset of heavily pretreated patients (2 fatal sepis) and the hematological toxicity was trombocytopenia grade 3-4 in 7 patients and neutropenia grade 3-4 in 14 patients. *Conclusions.* In conclusion this retrospective study shows that treatment with Bendamustine alone or in combination with Rituximab is a safe and effective regimen in a subset of pluriresistent patients with lymphoma and CLL. These data show also that the best results could be obtained in indolent lymphoma and that data in mantle cell lymphoma are encouraging.

0432

CONTINUOUS, ORAL CYCLOPHOSPHAMIDE AND PREDNISOLONE AS A VALID TREATMENT OPTION FOR PERIPHERAL T CELL LYMPHOMA

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Background. Peripheral T cell lymphomas (PTCL) are associated with a poor clinical outcome, regardless of the different treatments employed. Although anthracycline-containing regimens like CHOP are still the most frequent treatment used, there is no evidence that anthracyclines are of benefit in PTCL and alternative options are needed. Since patients often showed recurrence of symptoms, particularly fever, during the intervals between CHOP cycles, we decided to use long term administration of low dose oral cyclophosphamide and corticosteroids to try to continuously keep the lymphoma under control. *Aim.* to retrospectively compare the outcome of the series of patients with PTCL treated either with aggressive anthracycline-containing therapy, or with a therapeutic plan consisting in chronic oral cyclophosphamide and prednisolone administration. *Patients and Methods.* Fifty-five patients with a diagnosis of unspecified PTCL (PTCL-U) or angioimmunoblastic T cell lymphoma (AILD-T), consecutively seen at our Institute from January 1993 to December 2008, were considered. Treatment included: polychemotherapy, particularly CHOP or CHOP-like regimens, or oral cyclophosphamide 2.5 mg/Kg for 4 months and 1.5 mg/Kg for the subsequent 2 months and prednisolone starting at the dose of 1 mg/Kg and tapering it slowly over one year. Cyclophosphamide dose was adjusted based on WBC count. Eleven patients were excluded: two for early death, two lost of follow up, 3 with stage I treated with radiation only, 4 receiving corticosteroids only. Twenty patients received polichemotherapy (group PKT) and 24 cyclophosphamide and prednisolone (group CyPD). *Results.* The overall RR was 60% vs 46% (p=0.38) in group PKT and CyPD respectively; 5 ys PFS and OS were 13% (+ 12) and 29% (+ 13) in group PKT vs 26% (+15) and 31% (+ 11) in group CyPD respectively with no difference between the two groups (p=0.73 and p = 0.9). PKT and CyPD groups did not differ for main clinical characteristics including Ann Arbor stage (stage III/IV: 75% vs 91.6%), IPI score (intermediate-high/high score: 60% vs 70.8%). However mean age was significantly higher in CyPD group (64.7 vs 55.2; p = 0.038) which also had a significantly higher number of patients with the PIT high risk score (42% vs 10%; p=0.039). Median follow up was 16 months for group PKT (range 1-111) and 17 months for group CyPD (range 1-111). Toxicity was acceptable in both groups; cause of death was lymphoma in all patients except in two patients in group PKT (pneumonia and pulmonary thromboembolism) and in three patients in group CyPD (one pneumonia and two CMV infection). *Conclusions.* Overall survival data confirm the bad prognosis of patients with PTCL; however our analysis shows that the same results obtained with CHOP/CHOP-like regimens can be also obtained using less intensive, chronic, outpatient treatment with oral cyclophosphamide and prednisolone even in older patients and in patients with bad prognostic characteristics.

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LONG TERM OUTCOME OF HIGH DOSE ARACYTINE, RITUXIMAB, AND DEXAMETHASONE CHEMOTHERAPY FOR AGGRESSIVE MANTLE CELL LYMPHOMA IN ELDERLY PATIENTS

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Mantle cell lymphoma (MCL) mainly affects elderly, and response to standard therapy is deceiving. High dose chemotherapy could ameliorate these results, but this is too toxic for elderly and fragile patients. We evaluate the safety and efficacy of chemotherapy associating High dose Aracytine, Rituximab and Dexamethasone (HARD) for the treatment of elderly patient with aggressive MCL. Patients & *Methods.* From November 2005, patients with aggressive MCL were eligible to receive HARD chemotherapy. All patients received Rituximab 375 mg/m²/cycle and Dexamethasone 40 mg/d x3 /cycle. The dose of Aracytine was modulated according to the general condition and accompanying comorbidities. Data were revised for all patients, and comorbidities were assessed according to medical records. *Results.* Eight consecutive patients, 5 males

and 3 females, median age 72.6 years (65-77) with aggressive MCL were treated by HARD based chemotherapy. All presented with an aggressive histological pattern, stage III or IV disease according to Ann Arbor classification. The majority had performance score >2 (6/8), high LDL (6/8), high β2-microglobulin (5/5), Hemoglobin level <12 g/dL (5/8). The FLIPI score was high-intermediate / high in most patients (7/8). The Charlson comorbidity score was >5 in most patients (7/8) and principal documented comorbidities were coronary heart disease (3/8), cardiac systolic dysfunction (3/8 with LVEF <55%), cerebrovascular diseases (3/8), chronic renal insufficiency (creatinine clearance <60 cc/min) (4/8)), and chronic respiratory disorders (3/8). A total number of 43 HARD based chemotherapy cycles were given. All patients received planned doses of Rituximab and dexamethasone. The median initial dose of Aracytine was 2.5 g/m2 (1.12 - 4 g/m^2), which was subsequently modified according to patient's tolerance. There were five episodes of thrombocytopenia and five episodes of anemia requiring transfusion. One patient developed acute transfusion reaction complicated by myocardial infarction with pulmonary edema and acute renal insufficiency that responded well to medical treatment. Only one cycle of chemotherapy was complicated by febrile neutropenia. There was no treatment associated mortality. Six patients responded favourably, 4 in complete remission (CR) that is still maintained for three, with a follow-up of 36, 26 and 17 months; while the forth patient relapsed 19 months later, and is currently in second CR after re-treatment with HARD, with 9 months of follow-up. Two patients had a partial remission that persisted for 15 and 6 months before disease progression to leukemic phase MCL and they died at 19 and 11 months. Two other patients were refractory and died rapidly from progressive disease. Of note that both patients had leukemic phase MCL at the time of treatment. *Conclusions.* HARD based chemotherapy is safe and effective in geriatric patients with MCL, providing that the dose is modulated according to patient comorbidities. Despite the modulation of the dose of Aracytine, the response rate is interesting and compares favourably with regimens adapted for young and physically fit patients.

0434

DOSE INTENSITY IN HODGKIN AND NON-HODGKIN'S LYMPHOMA

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Background. The major dose-limiting toxicity of anti-cancer chemotherapy (CT) is myelosuppression. The risk of neutropenic complications, frequency of dose reductions, and treatment delays in patients (pts) with Hodgkin (HL) and Non-Hodgkin's lymphoma (NHL) compromise Relative Dose Intensity (RDI) and consequently long-term outcome. *Aims.* Primarily to assess the risk factors for reduced RDI in pts treated with CT for HL and NHL in first line treatment regimens. Secondarily, to assess the incidence of grade 3 and 4 neutropenia and evaluate patterns of Granulocyte-Colony Stimulating Factors (G-CSF) use. *Methods.* Multicentre retrospective survey was conducted in 5 Portuguese oncology centres to evaluate CT treatments in 370 pts with HL and NHL that started CT between 2005-2007. Data from pts treated with ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine), R-CHOP 21 like (R-CHOP) (rituximab, cyclophosphamide, vincristine, doxorubicin/mitoxantrone, prednisolone) and CHOP 21 like (CHOP) regimens was analysed using Dose Intensity Evaluation Programme (DIEP®) software, developed to allow calculation of RDI and registry of demographic and clinical data. Univariate and multivariate analyses were performed to identify factors related to RDI ″90% of standard. *Results.* One hundred and six pts (29%) were diagnosed with HL (median age: 31 years) and 99% of them were treated with ABVD. R-CHOP (49.2%, median age: 62 years) and CHOP (26.1%, median age: 72 years) were the most common CT regimens for the 264 pts diagnosed with NHL (median age: 62 years). Although overall median RDI was 97%, 19% of pts had a RDI ″90%. Univariate analysis revealed that reduced RDI was predominant in pts older than 65 years, with secondary extranodal involvement, higher levels of creatinine, and lower levels of haemoglobin. Within CT regimens groups differences in proportion of pts with reduced RDI was also statistically significant (ABVD: 20%, CHOP: 33%, R-CHOP: 20%). Primary prophylaxis with G-CSF was associated to RDI > 90%. Incidence of hospitalization were also significantly different in RDI ″ 90% (21%) and RDI >90% (9%) groups, as well the duration of hospitalization (median: 18 days and 6 days, respectively) being febrile neutropenia the major reason (54%) for hospitalization. Multivariate analysis identified several independent predictors for reduced RDI, including age ≥65 years (OR=4.02, CI95%:2.1-8.1), presence of secondary extranodal involvement (OR=2.2, CI95%:1.2-4.2), hospitalization (OR=2.66, CI95%: 1.3-5.9) and CT regimen CHOP (OR=3.7, CI95%:1.6-9.1) when compared to R-CHOP. Grade 3 and 4 neutropenia incidence as well as description of patterns of G-CSF use are presented on Table 1. Highest frequencies of grade 3 and 4 neutropenia episodes occur during cycle 1 of CT. *Conclusions.* A considerable number of our HL and NHL pts, receiving first line treatments continue to experience RDI reductions. Presence of age ≥65 years, secondary extranodal involvement, NHL chemotherapy regimens, and hospitalizations were identified as independent risk factors for reduced RDI. Primary G-CSF prophylaxis could be an appropriate supportive care in these subgroups of patients allowing the delivery of full chemotherapy doses on schedule.

Table 1. Incidence of neutropenia grade 3 and 4 and patterns of G-CSF use.

	Type of lymphoma		p-value
	Hodgkin	Non Hodgkin	
Total no. cycles	595	1493	
Total no. patients	106	264	
Incidence of neutropenia n (%)			
Total of cycles with Grade 3/4 episodes	117(20%)	116(8%)	p < 0.001
Cycle 1	38 (33%)	37 (32%)	-
Total of pts with Grade 3/4 episodes	36 (34%)	71 (27%)	p = 0.22
Patterns of G-CSF use n (%)			
Total no. patients with G-CSF administration	81 (76%)	173 (66%)	-
Primary Prophylaxis	10 (12%)	43 (25%)	p = 0.02
Secondary Prophylaxis	65 (80%)	125 (72%)	p = 0.17
Febrile Neutropenia	4 (5%)	25 (14%)	p = 0.03
Neutropenia	73 (90%)	133 (77%)	p = 0.01

0435

T- AND B-CELL LYMPHOMAS IN CHILDREN: CLINICAL AND IMMUNOLOGICAL FEATURES, RESULTS OF TREATMENT

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We reviewed all cases of primary pediatric T- and B-cell lymphomas diagnosed and treated over a 10-year period. In this study 186 cases of newly diagnosed childhood T- and B-cell lymphomas were included. The patients ranged in age from 9 months to 16 years at the time of diagnosis, with a mean age of 10 years. There were 138(74.6%) boys and 48(25,8%) girls, boys/girls ratio - 2,8:1. Diagnosis was based on cytomorphologic analyses, histopathological evaluation, imaging methods (CT scan of chest, abdomen and pelvis, clinical ultrasound, MRI), flow cytometry and/or immunohistochemistry of tumor species. B-cell lymphomas were diagnosed in 104(56%) cases, T-cell in 81 (43.5%) and NK-cell in 1(0,5%) case. According to WHO Classification of Hematological Malignances(2001) B-cell lymphoblastic leukemia/lymphoma was diagnosed in 28(15%) cases. Pre-pre-B-variant was in 19(68%), pre-B in 3(11%) and pro-B in 6(21%) cases. Diffuse large B-cell lymphoma (DLBCL) was found in 26(14%), Burkitt lymphoma/leukemia (BL) in 49(26%). Precursor T-cell lymphoblastic leukemia/lymphoma was diagnosed in 44 patients (24%), anaplastic large-cell lymphoma (ALCL) in 34(18%). The rate of a quite rare types of lymphomas (blastic NK-cell lymphoma - 1 case, follicular lymphoma - 1 case, angioimmunoblastic T-cell lymphoma-1 case) was 3%. At the time of diagnosis 13 patients (7%) had Stage I disease (by S.Murphy), 19(10%) Stage II, 63(34%) Stage III and 91(49%) Stage IV. Patients with BL were treated with the B-NHL-mBFM-90 protocol, relapse-free survival (RFS) during 2 years was 88.2±5.6%. Patients with DLBCL were treated with NHL-BFM-90 protocol and we achieved RFS in 58±13% cases. RFS in patients with ALCL treated according to NHL-mBFM-90/95 protocol was 86±9%. Lymphoblastic leukemias/lymphomas were treated with ALL-mBFM-90/95 protocol and 5-year RFS was registrated in up to 66.7% cases. T- and B-cell lymphomas are the third most common cancer in children. Our data show that it comprises a heterogeneous group of tumors with distinct pathologic and clinical characteristics. Over the past three decades, significant advancements have been made in the immunologic characterization of these disorders. With the use of intensive multiagent chemotherapy, T- and B-cell lymphomas are now among the most successfully treated cancers in the pediatric population.

0436

THE NEW ALC/R-IPI SCORE HAS A GOOD ABILITY TO DISCRIMINATE PROGNOSIS IN DLBCL TREATED WITH IMMUNO-CHEMOTHERAPY

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Background. The advent of immuno-chemotherapy for the treatment of Diffuse-Large-B-Cell-Lymphoma (DLBCL) has resulted in a marked improvement in survival outcome, but has altered the paradigm of risk assessment and the IPI factors can no longer identify a group of patients with a less than 50% chance of survival. Absolute-Lymphocytic-Count (ALC), has been recently identified as an independent prognostic factor in DLBCL. We built up a new score ALC/R-IPI incorporating both ALC and Revised-IPI (R-IPI) that, in a preliminary work, was the most powerful predictor of EFS, PFS and OS. *Aims.* Up-dating our preliminary work and confirm the prognostic value of ALC and of the new ALC/R-IPI score in a prospective series of DLBCL treated with R-CHOP-like chemotherapy. *Materials and Methods.* We assessed prospectively the value of ALC at diagnosis and also after the completion of immuno-chemotherapy in 131 Diffuse-Large-B-Cell-Lymphoma(DLBCL). Analysis of prognostic factors with respect to Overall Survival(OS), Event Free Survival(EFS) and Progression Free Survival(PFS) was done by two-tailed log-rank test. The ALC cut-off value was calculated as $<0.84\times10^9$/L at diagnosis: this was a strong negative prognostic factor for OS(p=0.0003), EFS(p<0.00001) and PFS(p<0.00001) and in multivariate analysis was independent from the Revised-International-Prognostic-Index(R-IPI). ALC after chemo-immunotherapy was not of prognostic value. As R-IPI and ALC$<0.84\times10^9$/L, were the factors better discriminating poor prognosis, a new trichotomous score(ALC/R-IPI) was built up: 1] low risk: R-IPI=Very Good or Good and ALC$\geq0.84\times10^9$/L; 2] intermediate risk: patients with at least one risk factor (R-IPI=Poor or ALC$<0.84\times10^9$/L). 3]high risk: patients with both risk factors. This new prognostic score was highly significant in univariate analysis for OS(p=0.0002), EFS(p<0.00001) and PFS(p<0.00001). In multivariate analysis ALC/R-IPI was the most predictive factor for OS(OR=2.32; p=0.002) and EFS(OR=2.431; p<0.0001) and the only predictive factor for PFS(OR=4.252; p<0.00001). *Conclusions.* Our data, show that ALC at diagnosis has a strong prognostic relevance and is independent from the R-IPI. The new score including both values proved the most powerful predictor at multivariate analysis.

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PATIENTS REQUIRING INTERRUPTION OF LONG-TERM ORAL ANTICOAGULANT THERAPY: THE ADVANTAGE OF FIXED SUB-THERAPEUTIC DOSES OF LOW-MOLECULAR WEIGHT HEPARIN

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Introduction. The interruption of long-term Vitamin-K antagonist (VKA) therapy for surgery or other invasive procedures puts patients at increased risk of thromboembolic events. We conducted a study testing the hypothesis that fixed doses of Low-Molecular Weight Heparin (LMWH) is a safe and efficacious perioperative bridging therapy in patients on long-term VKAs. *Methods.* This prospective, cohort study included patients requiring bridging therapy due to major surgery (defined as surgery lasting at least 1 hour) or invasive procedures at increased risk for bleeding that require interruption of VKA. Patients were considered to be at low or at high-risk for thrombosis. In all patients, Warfarin was discontinued 5 + 1days prior to the procedure. In patients considered at low-risk for thrombosis, LMWH at prophylactic dosage (4.000 UI anti-FXa once daily) was commenced the night before the procedure. In patients considered at high-risk for thrombosis LMWH at fixed sub-theraputic doses (4.000 UI anti-FXa twice daily).) was started pre-operatively when INR was below 1.5 and continued until the night before the procedure. In the post-operative period, LMWH was reinitiated 12 hours post-procedure while Warfarin was restarted the day after procedure in case of adequate haemostasis. LMWH was given accordingly to patients' thrombotic risk: those at high-risk received fixed sub-therapeutic dosage (4.000 UI anti-FXa twice daily) while those at low-risk received prophylactic doses (4.000 UI anti-FXa once daily). Heparin was continued until a therapeutic INR value. The primary efficacy endpoint was the incidence of thromboembolism from warfarin cessation to 30+2 days post-procedure. The primary safety endpoint was the incidence of major haemorrhage from first dose of LMWH until 24 hours after the last dose. *Results.* Over a period of 4 years (2003-2008), a total of 328 patients were included in the study; among them, 182 (55.4%) belonged to low-risk group and 146 (44.6%) to high-risk group. In total, 103 (31.4%) patients underwent major surgery and 225 (68.6%) other invasive procedures requiring VKAs suspension. Thromboembolic events occurred in 5 patients (1.5%); 4 belonging to high-risk and 1 to low-risk group. Major bleeding occurred in 7 patients (2.1%); among them, 5 patients belonged to high-risk and 2 to low-risk group. Five out of 7 major haemorrhage occurred in patients undergoing major surgery; none was fatal, intracranial, retroperitoneal, intraocular or required re-surgery. *Conclusion.* Use of LMWH given at fixed sub-therapeutic doses is a feasible and safe approach for bridging therapy in chronic anticoagulated patients.

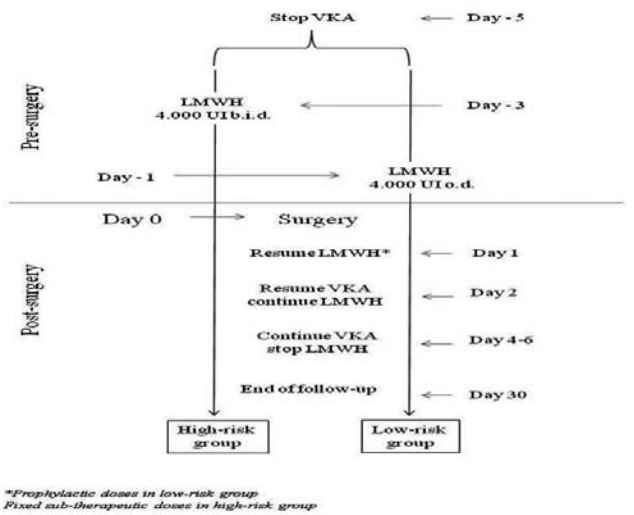

Figure.

0438

RESISTANCE TO CLOPIDOGREL DETECTED BY REAL TIME EVALUATION OF PLATELET THROMBUS FORMATION

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Background. Platelet adhesion, activation and aggregation play a key pathogenetic role in acute coronary syndromes (ACS), thus antiplatelet therapy with aspirin and clopidogrel is a mainstay of treatment. Individual response is variable, however, and patients with lesser platelet inhibition are at increased risk of adverse cardiovascular events. *Aims.* Assess the phenotype of clopidogrel *resistance*. *Methods.* we studied 36 ACS patients treated with antiplatelet therapy by perfusing heparinized blood over collagen fibrils under controlled flow conditions and measuring thrombus volume by real time confocal videomicroscopy. Measurements were obtained within 24 h from admission, when therapy was initiated, and at 5 days, 1 and 6 months. Antiplatelet treatment consisted of aspirin, 100 mg, and clopidogrel administered with a 300 mg loading dose followed by 75 mg daily. *Results.* in 30 patients, thrombus volume was inhibited >50% relative to control in all samples tested. In contrast, 6 patients (16.7%) exhibited no significant decrease of thrombus volume in spite of antiplatelet treatment. In 4 patients the lack of response was evident in the first samples tested, while in two patients thrombus volume was decreased >50% at day 5 but not at 1 month. Three of the 6 patients (50%) showing no inhibition of thrombus volume developed stent thrombosis; in two of them the 'resistance' to treatment was detected after the onset of symptoms. After switching to ticlopidine, 5 patients showed >50% inhibition of thrombus volume. In selected cases, we measured the platelet VASP phosphorylation index as a direct test of P2Y12 inhibition by clopidogrel, and found agreement with the results of thrombus volume measurement. Patients with <50% thrombus volume inhibition had a VASP index >50%. In one case, however, the VASP index was 21% but thrombus volume was not significantly decreased and the patient developed stent thrombosis. In one patient the VASP Index remained 71% after switching to ticlopidine in spite of >50% thrombus volume inhibition. *Conlclusions*. Our findings indicate that thrombus volume measurement in flowing blood exposed to collagen may provide a clinically relevant correlate of the effect of antiplatelet therapy.

0439

EFFECT OF THE NOVEL, ORAL, DIRECT FACTOR XA INHIBITOR RIVAROXABAN ON COAGULATION ASSAYS

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Background. Rivaroxaban is an oral, direct Factor Xa (FXa) inhibitor that is approved in the EU and several other countries for the prevention of venous thromboembolism after elective hip and knee replacement, and is in advanced clinical development for other thromboembolic disorders. There is no need for routine laboratory monitoring with rivaroxaban. However, a haemostasis assay may be valuable to provide physicians with a means to measure the pharmacodynamics of rivaroxaban. *Aims.* The aim of this study was to identify a widely available assay (clotting assay or colorimetric assay) that could be used in clinical practice. *Methods.* Increasing concentrations of rivaroxaban were spiked into citrated pooled human platelet-poor plasma. The global clotting assays prothrombin time (PT), dilute PT (dPT) and activated partial thromboplastin time (aPTT) were assessed, as well as the specific clotting assays HepTest® (heparin test), prothrombinase-induced clotting time (PiCT), dilute Russell's viper venom time (dRVVT) and the thrombin generation test (TGT). In addition, inhibition of FXa activity was measured using the FXa coagulometric assay Staclot®. *Results.* A concentration-dependent prolongation of PT, dPT and aPTT was observed with rivaroxaban, although the results varied depending on the reagents used. When testing PT over time using frozen plasma spiked with defined concentrations of rivaroxaban, PT values did not change during storage. Using a standard calibration curve, the results of the PT test can be expressed in rivaroxaban (µg/mL) rather than as PT ratio or international normalized ratio. A concentration-dependent prolongation of the clotting time was also observed with dPT, a test that may be clinically more significant than conventional PT because it is closer to *in vivo* conditions (i.e. it uses lower tissue factor concentrations). However, the increase in clotting time varied depending on the reagents used. Conventional methods for the HepTest and two-step PiCT (incubation times of 120 seconds and 180 seconds, respectively) resulted in a paradoxical response, with low concentrations of rivaroxaban reducing clotting times. This paradoxical response was not observed with shorter incubation times (0 or 30 seconds for one-step PiCT and 30 seconds for HepTest) or when antithrombin-immunodepleted plasma was used. Modification of these tests by shortening the incubation period is now recommended by the manufacturer. Rivaroxaban also increased the dRVVT in a concentration-dependent manner; however, this test is not specific. Rivaroxaban influenced the various parameters of the TGT in a concentration-dependent manner; peak effect was the most informative parameter. Rivaroxaban inhibited FXa activity in a concentration-dependent manner in the Staclot FXa coagulometric assay, with results expressed in clotting times; this assay, however, is not specific for FXa inhibitors. *Summary and Conclusions.* PT assays calibrated to rivaroxaban concentrations appear to be valuable assays for monitoring the pharmacodynamic effects of rivaroxaban. Encouraging results were also obtained with the Staclot FXa coagulometric assay, although further studies are required before this assay can be recommended for monitoring the pharmacodynamic effects of rivaroxaban.

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THE HEMOSTATIC PROFILE OF ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA (PV) PATIENTS IS INFLUENCED BY THE JAK2V617F ALLELE BURDEN

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Background. Thrombosis is a leading cause of morbidity and mortality in patients with Thrombocythemia (ET) and PV. Several mechanisms have been proposed to cause or to contribute to the acquired thrombophilic state of these patients. We recently described the occurrence of an acquired resistance to activated protein C (APC) in ET and PV patients, particularly in those carrying the somatic JAK2V617F mutation, due to a decrease in Protein S (PS) levels (Marchetti *et al.*, Blood 2008). *Aims*. This study investigated whether the JAK2V617F allele burden, quantified by real time PCR, may differently affect the APC resistance phenotype in a group of JAK2V617F-positive patients. *Methods.* APC resistance was evaluated in 27 ET and 26 PV patients by the endogenous thrombin potential (ETP) assay and expressed as normalized APC sensitivity ratio (nAPCsr). JAK2V617F allele burden was correlated to the plasma levels of procoagulant (FII, FV) and anticoagulant proteins (free PS, TFPI), known to be the major determinants of the nAPCsr, and to markers of neutrophil activation (plasma elastase, cell surface CD11b and CD14). *Results.* In our study subjects, the JAK2V617F allele burden ranged from 0.5-82% (mean: 25%) in ET and from 4.1-98.5% (mean: 51%) in PV patients. A significant positive correlation was found between the JAK2V617F allele burden and nAPCsr (R=0.359; p=0.009), plasma elastase (R=0.73, p<0.001), and surface expression of neutrophil CD14 (R=0.515; p=.005). An inverse and significant correlation was found between JAK2V617F allele burden and plasma levels of FII (R=-0.380; p=0.006), FV (R=-0.365; p=0.008) and free PS (R=-0.416; p=0.004). After correction for sex, age and hydroxyurea (HU) therapy, JAK2V617F allele burden was an independent predictor of the nAPCsr (B=0.396; p=0.004), while HU therapy was associated with a lower nAPCsr (B=-0.335; p=0.016). *Summary and Conclusions.* These data demonstrate that higher JAK2V617F mutational load is associated with a more profound increase in APCsr and neutrophil activation and dowregulation of anticoagulant PS, thus contributing to a prothrombotic phenotype of ET and PV patients. Further studies are warranted to clarify the molecular mechanisms underlying the relationship between this oncogenic mutation and the dysregulation of the hemostatic system in these diseases.

0441

SOLUBLE ENDOGLIN AND ITS INTERACTION WITH ADHESION MOLECULES IN PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Endoglin (CD105) is an accessory protein of the transforming growth factor-β receptor system expressed on vascular endothelial cells. Mutations on the endoglin gene are associated with hereditary hemorrhagic telangiectasias (Osler-Weber-Rendu syndrome) and, thus, endoglin has been extensively studied in the context of this disease. Endoglin is highly expressed on endothelial cells in healing wounds, developing embryos, inflammatory tissues and solid tumors. It is a marker of activated endothelium, while its vascular expression is limited to proliferating cells. Previous studies have shown that the endothelial function is impaired in patients with Thalassemia Intermedia (TI). Oxidative damage resulting from hemolysis and iron load, leads to increased expression of the intercellular and vascular adhesion molecules-1 (ICAM-1 and VCAM-1) and impaired nitric oxide (NO) bioavailability. *Aims.* As endoglin plays a critical role in angiogenesis and dysregulation of its expression and/or activity has been implicated in multiple vascular diseases, we aimed to investigate the expression of endoglin and its correlation with factors of endothelial dysfunction in patients with TI. *Methods.* Thirty adult patients with TI were included in the study, while 20 healthy individuals served as controls. Soluble endoglin, soluble forms of adhesion molecules ICAM-1, VCAM-1, E and P-Selectins, thrombomodulin, von Willebrand factor, as well as NO and vascular endothelial growth factor (VEGF), were measured in patients and controls, by immunoenzymatic methods. *Results.* The main results of the study are: a) levels of endoglin, E-selectin, thrombomodulin, VCAM-1, ICAM-1 and VEGF in patients with TI (5.5±0.4 ng/mL, 91.4±20.0 ng/mL, 48.8±16.5 ng/mL, 1413.7±176.1 ng/mL, 658.8±34.6 ng/mL and 619.1±227.8 pg/mL, respectively) were higher compared to controls (4.9±0.3 ng/mL, 10.3±2.2 ng/mL, 4.0±0.6 ng/mL, 328.3±43.8 ng/mL, 107.6±26.5 ng/mL, 81.3±58.1 pg/mL), (p<0.01), b) endoglin levels in patients with TI correlated positively with concentrations of ICAM-1 (r=0.760, p<0.003), VCAM-1 (r=0.520, p<0.05), E-Selectin (r=0.790, p<0.0020, P-Selectin (r=0.530, p<0.04), while these correlations were absent in normal individuals. *Conclusions.* Angiogenesis is a highly coordinated process in which VEGF, endoglin and hypoxia inducible factor 1 (HIF-1) play a pivotal role by coordinating interaction between endothelial cells, extracellular matrix and the surrounding cells. Taking into assumption that endoglin is a protective-repair tissue protein, our findings support the hypothesis that patients with TI exhibit increased degree of angiogenesis and endothelial regeneration, which are probably compensatory mechanisms in response to tissue hypoxia and damage.

0442

ORAL PREVENTION OF VENOUS THROMBOEMBOLISM AFTER TOTAL KNEE OR HIP REPLACEMENT SURGERY IN THE ELDERLY AND THOSE WITH MODERATE RENAL IMPAIRMENT: EFFICACY AND SAFETY PROFILE OF DABIGATRAN ETEXILATE

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Background. Oral dabigatran etexilate (Pradaxa®) is approved for preventing venous thromboembolism (VTE) after knee or hip replacement surgery in Europe, Canada and other countries. *Aims.* We aimed to determine the efficacy and safety of dabigatran etexilate in the combined sub-group of patients with moderate renal impairment and those older than 75 years (as renal function naturally declines with age) compared with subcutaneous (sc) enoxaparin. *Methods*. The RE-MODEL' and RE-NOVATE' trials studied the efficacy and safety of 220 mg and 150 mg dabigatran etexilate once daily (qd) compared with 40 mg sc enoxaparin qd and statistically demonstrated non-inferiority for the primary endpoint, total VTE and all-cause mortality. A post hoc pooled analysis was performed in patients older than 75 years or those with moderately reduced renal function (creatinine clearance 30-50 mL/min). The secondary, clinically relevant efficacy endpoint was major VTE and VTE-related mortality; the safety outcomes were major bleeding events (MBE; including peri-operative surgical-site bleeds) and major or clinically relevant bleeding events (CRBE/MBE). *Results*. Of 5371 treated patients, 841 (15.7%) were over 75 years and 337 (6.3%) had moderate renal impairment; the combined group comprised 953 patients (17.7%). Event rates and relative risks (RR; with 95% confidence intervals) compared with enoxaparin are shown in the Table. A RR <1 indicates a lower risk and a RR >1 a higher risk with dabigatran etexilate compared with enoxaparin. *Summary and Conclusions.* Dabigatran etexilate 150 mg qd showed less VTE and VTE-related mortality and bleeding compared with the enoxaparin regimen. The 220 mg dose showed a lower thromboembolic event rate with a higher bleeding rate. This analysis supports the use of the 150 mg dose of dabigatran etexilate in patients over 75 years or with moderate renal impairment.

Table. Comparison of dabigatran etexilate with enoxaparin.

Event	Dabigatran etexilate 220 mg qd	Dabigatran etexilate 150 mg qd	Enoxaparin 40 mg qd
Major VTE and VTE-related mortality	2.2% (5/230) RR: 0.34 (0.13–0.92)	4.3% (10/230) RR: 0.68 (0.31–1.48)	6.4% (15/235)
MBE	3.7% (12/321) RR: 1.13 (0.51–2.52)	1.3% (4/300) RR: 0.40 (0.13–1.25)	3.3% (11/332)
CRBE/MBE	10.6% (34/321) RR: 1.13 (0.71–1.80)	8.0% (24/300) RR: 0.86 (0.51–1.43)	9.3% (31/332)

0443

EFFICACY AND SAFETY PROFILE OF 220 MG DABIGATRAN ETEXILATE IN PATIENTS UNDERGOING TOTAL HIP OR KNEE REPLACEMENT SURGERY WITH A HISTORY OF STROKE, TRANSIENT ISCHAEMIC ATTACK OR VENOUS THROMBOEMBOLISM

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Background. The oral direct thrombin inhibitor dabigatran etexilate is approved for the prevention of venous thromboembolism (VTE) after knee or hip replacement surgery in multiple countries, including the European Union and Canada. *Aims.* In this post hoc analysis we investigated the efficacy and safety of dabigatran etexilate in the sub-group of patients with a history of VTE, non-haemorrhagic stroke or transient ischaemic attack (TIA). *Methods.* The RE-MODEL' and RE-MOBILIZE' trials studied patients undergoing knee replacement surgery and compared 220 mg and 150 mg dabigatran etexilate once daily (qd) with 40 mg subcutaneous (sc) enoxaparin qd (RE-MODEL') or 30 mg sc enoxaparin twice daily (bid; RE-MOBILIZE'). RE-NOVATE' compared 220 mg and 150 mg dabigatran etexilate qd with 40 mg sc enoxaparin qd after hip replacement. The endpoints studied in these pooled analyses were major VTE and VTE-related mortality and major bleeding events (MBE), including surgical-site bleeds, plus clinically relevant bleeding events (CRB). This analysis is focused on the group which received 220 mg qd dabigatran etexilate as this group is representative of a population without renal impairment. *Results.* A history of VTE, non-haemorrhagic stroke or TIA was present in nearly 4% of the total pooled population of 8135 patients. The incidence of major VTE and VTE-related mortality was 6.3% in the 220 mg dabigatran etexilate group and 6.7% in the

enoxaparin group, which translates into a risk ratio (RR) for the comparison of dabigatran etexilate 220 mg with enoxaparin of 0.95 (95% confidence interval [CI]: 0.29-3.15). The combination of MBE and/or CRB occurred in 8.8% and 7.9% in the two groups, respectively. The RR for the bleeding events was 1.11 (95% CI: 0.45-2.77). *Summary and Conclusions.* For the prevention of VTE after total knee or hip replacement surgery in patients at risk due to a history of VTE, stroke or TIA, the 220 mg dose of oral dabigatran etexilate showed good efficacy and a good safety profile compared with subcutaneously administered enoxaparin.

0444

LOW PLATELET GLYCOPROTEIN IB-POSITIVE MICROPARTICLES IN PATIENTS WITH CORONARY STENT THROMBOSIS

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Background and Aim. Heightened platelet reactivity and/or resistance to anti-platelet therapy may cause thrombosis in patients with coronary stents. We hypothesized that this event might involve adhesion mediated by glycoprotein Ib (GPIb)/von Willebrand factor, possibly resulting in the generation of GPIb-positive platelet-derived microparticles (GPIb+P-MP). Methods and Results. We measured by flow cytometry GPIb+P-MP in the blood of patients with coronary stents as potential markers of a prothrombotic tendency, and membrane expression of P-selectin along with binding of the antibody PAC-1 as markers of the inhibitory effect of anti-platelet drugs. We studied 51 patients with stable angina (SA) who had received coronary stents without complications; 16 patients with a history of stent thrombosis (S-TH); and 29 normal individuals. All patients were treated with aspirin and a thienopyridine at the time of study. The number of GPIb+P-MP was similar in normal individuals and SA patients, indicating that anti-platelet therapy has no effect on the generation of these microparticles, but was unexpectedly lower in S-TH patients. Thus, GPIb+P-MP levels allowed discriminating SA from S-TH patients with significant sensitivity and specificity. P-selectin expression and PAC-1 binding were lower in patients than normal subjects but with no difference between SA and S-TH patients, evidence that platelet inhibition was similar in the two groups. Conclusions. Enhanced GPIb/von Willebrand factor interactions may alter generation and/or adhesion of GPIb+P-MP at sites of vascular lesion through mechanisms not inhibited by current anti-platelet therapy. A decrease of GPIb+P-MP in blood may indicate a prothrombotic tendency in patients with coronary stents.

0445

YOUNG PATIENTS WITH STROKE, PFO (PATENT FORAMEN OVALE) AND THROMBOPHILIA

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In young stroke patients PFO is present in about 50%: but the risk for cryptogenic stroke in healthy people with PFO is only 0.1%. Unbalanced haemostasis (thrombophilia) could enhance the risk. *Aim of the study.* To evaluate the prevalence of trombophilia in patients with PFO (PFO^+) and cryptogenic stroke ($stroke^+$). *Materials and Methods.* In 330 consecutive subjects with cryptogenic stroke 149 (45.15%) were PFO^+ and 181 (54.9%) were PFO^-. We performed neurological examination, brain CT and /or MRI scan, extracranial Doppler ultrasonography, ECG, trans thoracic and/or transesophageal echocardiography, standard blood tests and PT, aPTT, fibrinogen, protein C, protein S, antithrombin, APC resistance, homocysteine, LAC, anticardiolipin antibodies, F VIII , test for thrombofilia mutations G1691A Factor V, G20210A Factor II, C677T MTHFR; 433 healthy subjects,matched for age and gender,were control population: 98 were investigated for PFO (43 PFO^+, 55 PFO^-). *Results.* Stroke is more prevalent in women than in men (F 54.3%, M 42.1%, p=0.002).Fibrinogen (14.8 vs. 8.9%, p=0.023), homocysteine (fasting:15.5% vs. 6.0%, p<0.001, post load: 25.7vs. 5.6%, p< 0.001), factor VIII (57.0 vs. 43.0%, p=0.002), LAC (2.7 vs. 0.0%, p=0.002)and anticardiolipin AbIgG (5.6 vs. 0.0%, p<0.001) were higher in patients $stroke^+$ vs $stroke^-$. The multivariate analysis confirm female gender (OR: 2.38, 95%IC:1.53-3.68, p<0.001), homocysteine (OR 3.49, 95%IC:1.92-6.34), FVIII (OR 1.93, 95%IC:14-3.26) as risk factors.In patients $stroke^+$ and PFO^+, APC resistance, homocysteine, MTHFR mutation and LAC have p<0.05. PFO seems to be a risk factor for stroke when right-to-left shunt is >10 microbubbles (34 vs 45%, p=0.071) In multivariate analysis PFO, age, female gender and APC resistance are significant. *Conclusions.* An hypercoagulable state in association with concomitant risk factors as PFO could contribute to provoke an intracardiac thrombosis with subsequent embolism.

0446

HIGHER CIRCULATING ACTIVATED PROTEIN C /PC AND APC/FACTOR II RATIOS DURING ORAL ANTICOAGULANT THERAPY. INFLUENCE OF THE HAPLOTYPE 1 OF THE ENDOTHELIAL PROTEIN C RECEPTOR GENE

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Background. Oral anticoagulation (OAC) therapy reduces levels of activated protein C (APC) remarkably less than levels of protein C (PC) and factor II (F II) in patients with systemic lupus erythematosus and in cardiac patients (Simmerlink MJA *et al.*, Blood 2002; 100: 4232). In addition, we have reported that carriers of the H1 haplotype in the endothelial PC receptor (EPCR) gene have higher APC levels than non carriers (Medina P *et al.*, Thromb Haemost 2004; 91: 905). *Aims.*To assess whether patients with venous thromboembolism under OAC therapy also show less decrease in APC levels, and whether this effect is associated with the presence of EPCR H1 haplotype. *Methods.* Blood was collected from 470 VTE patients, 143 (mean 44 years) with and 327 (mean 42 years) without OAC therapy. Levels of APC (España *et al.*, Thromb Haemost 2001; 86: 1368), PC (chromogenic) and F II (coagulometric) are expressed as the percentage of the level in pooled normal plasma from 30 healthy subjects. *Results.* The mean values for APC, PC and F II in patients under OAC therapy were 66%, 51% and 37%, whereas the values for patients without OAC therapy were 84%, 106% and 101%, respectively. For patients under OAC therapy the F II/PC ratio did not significantly vary for all PC values observed, whereas APC/F II and APC/PC ratios significantly increased as the F II and PC levels decreased, but only in patients under OAC therapy, showing a strong and inverse correlation between both ratios and its zymogen, reaching ratio values up to 3.5 for PC (Figure) and 5.0 for F II levels below 30%. Because the H1 haplotype in the EPCR gene is associated with higher plasma APC levels, we studied the influence of this haplotype on the analyzed ratios. Of the 18 patients under OAC therapy carrying the H1H1genotype, 8 (44%) showed APC/PC and 10 (56%) APC/F II ratios higher than 2, whereas of the 41 patients under OAC therapy not carrying the H1 haplotype only 5 (12%) and 4 (10%), respectively, had ratios higher than 2.0 (p<0.001). *Conclusions.* Our data confirm previous results showing that there is a disproportionately higher APC level in patients under OAC therapy and show that this effect is much more pronounced in homozygous carriers of the EPCR H1 haplotype. (Conselleria de Sanitat AP-151/08; RECAVA RD06/0014/004).

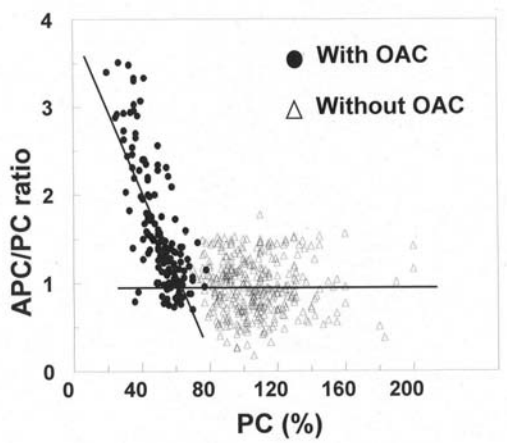

Figure.

0447

LAC TESTING IN PATIENTS WITH INFLAMMATORY STATUS

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Introduction. The presence of lupus anticoagulant (LAC) is one of the laboratory criteria for diagnosis of the antiphospholipid syndrome (APS). To exclude the transient nature of antiphospholipid antibodies, the International Society on Thrombosis and Haemostasis (ISTH) guidelines require a positive laboratory test on at least 2 occasions at least 12 weeks apart. In daily practice we observe transient LAC frequently in the course of infectious diseases. On the other hand we also observe unexplained prolonged aPTT in preoperative screening and intensive care patients. C-reactive protein (CRP) is an acute phase protein with a known affinity for phospholipids (PL). Hence, we investigated whether CRP interferes with LAC test results. *Materials and Methods.* CRP was measured in both the LAC positive and LAC negative sample of patients with transient presence of LAC. Patient plasma was qualified as LAC positive, by considering all ISTH criteria using a three step procedure in two test systems: an activated partial thromboplastin time (aPTT) (PTT-LA and Staclot-LA, Diagnostica Stago) and a diluted Russell's viper venom test (dRVVT) (LA-Screen and LA-confirm, Life Diagnostics). CRP was measured through a particle-enhanced immunoturbidimetric assay (CRPLX, Roche Diagnostica). Normal pooled plasma (NPP) was spiked with human CRP (Sigma-Aldrich) in a concentration range of 0.1 to 28.8 mg/dL to measure the effect on aPTT and dRVVT. A thrombin generation (TG) assay, measured via calibrated automated thrombinography (CAT), was performed with different types of PL at 1 µM and 5 pM tissue factor (TF). *Results.* Testing of 39 patients with transient LAC showed that CRP was significantly higher on the first occasion of LAC testing compared to the parallel testing on the second occasion (>12 weeks later), p=0.0001 (Wilcoxon test for paired samples). The aPTT test system was more sensitive to interference of CRP on LAC testing than the dRVVT test system. PTT-LA of CRP-spiked NPP showed an elevated normalised ratio from 2.4 mg/dL CRP onwards. Staclot-LA was strongly positive starting from CRP 5.3 mg/dL. dRVVT showed no elevated normalised ratio at any CRP concentration. TG using Staclot-LA hexagonal PL showed a decreased normalised peak/lagtime ratio starting from 2.4 mg/dL CRP. All other PL tested showed no decrease in normalised peak/lagtime ratio at any CRP concentration. *Conclusions.* Binding of CRP to negatively charged PL influences coagulation test results for LAC. The interaction strongly depends on the type of PL used in the reagent mixture. LAC testing should be interpreted with care in patients with inflammatory reactions.

0448

SILENT BRAIN ABNORMALITIES IN THALASSEMIA INTERMEDIA: DIAGNOSTIC CONTRIBUTION OF POSITRON EMISSION TOMOGRAPHY

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Background. Asymptomatic brain pathology has been described as a highly common phenomenon in patients with thalassemia intermedia (TI) and sickle cell disease (SCD). Current data in TI patients relied on the use of magnetic resonance imaging (MRI). However, in patients with SCD, the addition of position emission tomography (PET) to MRI identified a much greater proportion of patients with neuroimaging abnormalities, particularly in those who had no history of overt neurologic events. The utility of PET in detection of silent brain abnormalities in TI patients has not been evaluated. *Aims.* The present study examines the extent to which adding PET to MRI can improve the detection of silent brain abnormalities in adult patients with TI. *Methods.* Thirty patients (13 males and 17 females; with a mean age of 31.9 ± 11 years) carrying the diagnosis of TI - attending to the chronic Care Center, Hazmieh, Lebanon - were included in this study. Patients were randomly selected from a high-risk population of splenectomized, non-regularly transfused, non-anticoagulated TI patients. Patients were initially screened to confirm absence of neurological signs or symptoms and other associated stroke related disease. All patients underwent both MRI and 18F-FDG PET scan in the same day, after signing the informed consent. The criterion for abnormality on PET was the decrease in neuronal function (glucose utilization). MRIs were performed using T1 and T2 weighted spin-echo techniques. Abnormality was defined as an area of abnormally increased signal intensity on the T2 weighted pulse sequences. *Results.* Among the entire group of 30 subjects, 18 (60%) had abnormal MRI findings, 19 (63.3%) had abnormal PET findings, and 26 (86.7%) had either an abnormal MRI or abnormal PET or both. In patients with abnormality, 14 (53.8%) had multiple lesions on MRI, 14 (53.8%) had multiple findings on PET, and 19 (73.1%) had multiple findings on MRI, PET or both. Moreover, 13 (50%) had bilateral lesions on MRI, 3 (11.5%) had bilateral findings on PET, and 14 (53.8%) had bilateral findings on MRI, PET or both. Frontal lobe abnormalities were evident in 17 (65.4%), 3 (11.5%), and 18 (69.2%) patients by means of MRI, PET, or either; respectively. Parietal lobe abnormalities were evident in 9 (34.6%), 16 (61.5%), and 20 (76.9%) patients by means of MRI, PET, or either; respectively. Temporal lobe abnormalities were evident in 1 (3.8%), 13 (50%), and 14 (53.8%) patients by means of MRI, PET, or either; respectively. Occipital lobe abnormalities were evident in 3 (11.5%), 1 (3.8%), and 4 (15.3%) patients by means of MRI, PET, or either; respectively. Hence, concordance rates were 36.7% for detection of abnormality; 30.8% for detection of multiple lesions; 7.7% for detection of bilateral lesions; 7%, 19.2%, 0% and 0% for detection of frontal, parietal, temporal and occipital lesions, respectively. *Summary and Conclusions.* The addition of PET to MRI identified a much greater proportion of high-risk TI patients with silent neuroimaging abnormalities. PET lesions are equally extensive, less bihemispheric, and predominated in parietal and temporal lobes as compared to frontal lobes as with MRI.

0449

REVERSAL OF THE ANTIHAEMOSTATIC EFFECTS OF RIVAROXABAN BY ACTIVATED FACTOR VII AND ACTIVATED PROTHROMBIN COMPLEX IN PRIMATES

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Background. Rivaroxaban is a novel, oral, direct factor Xa inhibitor approved in the EU and several other countries for the prevention of venous thromboembolism in adult patients undergoing elective hip or knee replacement surgery. The drug is in advanced clinical development for other thromboembolic disorders. *Aims.* We determined whether recombinant activated factor VII (rFVIIa; NovoSeven®) and activated prothrombin complex concentrate (APCC; FEIBA®) can reduce the antihaemostatic effect of high-dose rivaroxaban in baboons. *Methods.* In baboons, levels of rivaroxaban higher than therapeutic concentrations were achieved by administering an intravenous (i.v.) bolus (0.6 mg/kg) followed by continuous infusion (0.6 mg/kg/h) for 60 minutes. This caused progressive pharmacological impairment of haemostasis, resulting in a three- to fourfold increase in prothrombin time (PT) from baseline and at least a twofold increase in template bleeding time (BT). Thirty minutes after the bolus injection, one group of baboons (n=7) received an infusion of APCC (50 U/kg over 25 minutes) and another group (n=7) received an i.v. bolus of rFVIIa (210 µg/kg). BTs and clotting times were measured to assess the APCC- and rFVIIa-mediated reversal of the antihaemostatic effect of high-dose rivaroxaban.

Table. The effect of activated prothrombin complex (APCC) and recombinant activated factor VII (rFVIIa) on markers of haemostasis impairment in baboons anticoagulated with high-dose rivaroxaban.

Time	BT (x-fold change from baseline) n=7	PT (x-fold change from baseline) n=7	TAT concentration (µg/l) n=7
APCC			
Baseline	1.00	1.00	3.51±0.08
30 minutes after rivaroxaban	2.02±0.56	3.04±0.43	3.01±1.37
At end of APCC infusion	1.02±0.33	2.20±0.29	10.35±1.41
20 minutes after end of APCC infusion	1.65±0.94	2.28±0.29	–
rFVIIa			
Baseline	1.00	1.00	7.35±4.17
30 minutes after rivaroxaban	2.54±0.79	3.17±0.42	2.95±0.79
5 minutes after rFVIIa	1.68±0.80	2.38±0.41	2.58±0.52
30 minutes after rFVIIa	1.96±1.26	2.48±0.49	4.00±1.12

Values are given as mean ± standard deviation.
BT, bleeding time; PT, prothrombin time; TAT, thrombin–antithrombin complex.

Results. In APCC-treated baboons, high-dose rivaroxaban prolonged

BT to 202±21% (mean ± standard deviation) of baseline, and PT was prolonged to 304±43%, 30 minutes after dosing. After the completion of APCC infusion, PT was reduced by 28±29% and BT returned to baseline. In rFVIIa-treated baboons, rivaroxaban prolonged BT to 254±79% and PT to 317±42% of baseline, 30 minutes after dosing. rFVIIa infusion decreased BT by 34±80% and shortened PT by 25±41%, 30 minutes after dosing. Rivaroxaban infusion decreased circulating thrombin-antithrombin complex (TAT) levels; this decreased level did not change significantly after administration of the rFVIIa bolus. However, APCC increased plasma TAT above baseline, suggesting systemic hypercoagulation (Table). *Summary and Conclusions.* We conclude that, in baboons, APCC or rFVIIa administration can rapidly attenuate haemostasis impairment caused by levels of rivaroxaban at higher than therapeutic concentrations, potentially providing antidotes for use during emergencies.

0450

THE RISK OF RECURRENT VENOUS THROMBOEMBOLISM IN PATIENTS WITH MULTIPLE GENETIC THROMBOPHILIC DEFECTS: A COMPARATIVE STUDY

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Thrombophilias are genetic defects that have been associated with an increased risk of venous thromboembolisms (VTE) occurrence and recurrence. The objective of the study was to compare the risk of VTE recurrence in patients with multiple thrombophilias (including homozygous mutations) to patients that only have one. This was a retrospective, population-based, comparative study that involved 824 patients, out of which 292 had thrombophilia. 32 of the thrombophilia affected patients had multiple genetic defects and were compared to a randomly selected control group of 50 patients with single thrombophilia. These two groups were compared with regards to various aspects such as VTE occurrence, VTE recurrence, type of anticoagulant (IV Heparin, LMW Heparin, Warfarin), anticoagulant therapy duration (short-term <=6mo, long-term >6mo), acquired risks (e.g. malignancy, surgery or trauma, oral contraceptive pill or hormone replacement therapy, pregnancy, immobilization, hypertension, morbid obesity), family history of VTE, and type of thrombophilia (Antithrombin, Factor V Leiden, Homocysteine, Lupus Anticoagulant, Protein C Deficiency, Protein S Deficiency, Prothrombin). Overall, it was found that there were more patients with multiple thrombophilic defects than patients with a single defect among the aforementioned categories. The difference was particularly significant between the two patient groups in the rate of recurrence ($p<0.01$), the rate of having associated acquired risk(s) ($p<0.005$), and the rate of receiving anticoagulation therapy for initial thrombotic event ($p<0.05$). There was no statistically significant difference in the duration of anticoagulation therapy for both initial thrombotic event and recurrent episodes. This study provided additional evidence for the increase in risk of VTE recurrence in patients with thrombophilic defects, and an even greater risk in patients with multiple defects. Further study is required in order to appropriately assess the duration of anticoagulant therapy for thrombophilic patients.

Table. Comparison of patients: multiple vs single defects.

Percentage of Patients	Patients with Multiple Thrombophilia (n=32)	Patients with Single Thrombophlia (n=50)
VTE Occurrence	100%	92%
VTE Recurrence	59.4%	32%
Anticoagulation for 1st Occurrence	96.9%	82%
Anticoagulation for Recurrence	53.1%	42%
Acquired Risks	96.9%	74%
Family History	75%	70%

0451

ADAMTS13 LEVELS IN HIV INFECTED PATIENTS WITH AND WITHOUT THROMBOTIC THROMBOCYTOPENIC PURPURA

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Background. Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease characterised by microvascular platelet deposition and thrombus formation in selected organs with resulting microangiopathic haemolytic anaemia, thrombocytopenia, neurological symptoms, and renal failure. Typically a very rare disorder, TTP is being seen with increased frequency in patients infected with the human immunodeficiency virus (HIV). Deficiency of the Von Willebrand factor cleavage protease, also known as ADAMTS13, has been implicated as a major aetiological factor in TTP. However, before studying the role of ADAMTS13 in HIV related TTP, it is necessary to know the normal values of ADAMTS13 levels in the population group in question because lower ADAMTS13 levels had been reported in some populations such as the Chinese. It is also necessary to know whether HIV infection in the absence of TTP has any effect on ADAMTS13 levels. *Aim.* The aim of the study was threefold: a) to compare the ADAMTS13 levels in the local Caucasian and African populations; b) to study the effect of HIV infection on ADAMTS13 levels by correlating CD4 counts and viral loads with ADAMTS13 levels in HIV positive patients without TTP; and c) to measure ADAMTS13 levels in HIV infected patients with TTP. *Methods.* Ethics approval was obtained from the Ethics Committee of the Faculty of Health Sciences, University of the Free State. An ELISA for ADAMTS13-antigen levels was standardised and optimised for routine use in our laboratory. ADAMTS13 levels (mean ± 1SD) were measured in both local Caucasian (n=19) and African (n=21) populations. Thirty six HIV-positive patients without TTP and with CD4 counts varying from 4 to 681 and viral loads varying from 3 400 to 700 000, were tested. The CD4 counts were determined with a Beckman Coulter flow cytometer (Epics XL.MCL) and viral loads with a Roche Amplicor HIV-1 (RT-PCR) kit according to the manufacturer's instructions. ADAMTS13 levels were also measured in 20 patients with HIV associated TTP. *Results.* No statistically difference in ADAMTS13 antigen levels could be found in the local Caucasian and African populations (739±77 ng/mL vs. 714±47 ng/ml; $p<0.5$). The ADAMTS13 levels were normal in the HIV patients without TTP (750±89 ng/mL) and the severity of HIV infection as reflected by either CD4 count or viral load had no effect on the levels. The ADAMTS13 levels in all 20 HIV associated TTP patients were low (244±121 ng/mL). *Conclusion*. Neither population group nor severity of HIV infection as reflected by CD4 count and viral load had any effect on ADAMTS13-antigen levels, making it a useful diagnostic tool in the setting of HIV positive patients with TTP.

0452

HIGH AVIDITY ANTICARDIOLIPIN ANTIBODIES ASSOCIATE WITH THROMBOSIS IN PATIENTS WITH ANTHIPHOSPHOLIPID SYNDROME

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Background. Anticardiolipin antibodies (ACA) are immunoglobulins that occur in the Antiphospholipid Syndrome (APS) and in a variety of disorders, including connective tissue diseases (secondary APS), syphilis and other infectious diseases. APS is an important cause of acquired thrombophilia and recurrent miscarriage; however not all ACA are associated with thrombotic events or pregnancy failure. Affinity/avidity of these autoantibodies and their association with thrombosis has not been sufficiently studied. *Aims.* The aims of our study were to evaluate if avidity of IgG ACA in sera from patients with primary or secondary APS or infectious disorders is associated with thrombosis or pregnancy loss. *Methods.* We studied sera from 60 patients: 22 with primary APS, 18 with APS secondary to SLE and 20 with syphilis. Avidity of IgG ACA was determined by enzyme-linked immunosorbent assay and modified buffer solution containing K-thiocyanate and Triton X-100 as dissociating agents. *Results.* High avidity IgG ACA (more than 70% of the initial binding) were detected in 18 primary and 10 secondary APS and were associated with thrombosis and/or pregnancy loss in 15 and 7 patients, respectively. In the other patients with APS and in those with syphilis ACA avidity was heterogeneous-low (<25%) and associated with venous thrombosis in 2 patients with SLE ($p<0.01$). *Summary and Conclusions.* IgG ACA with higher avidity appear to be significantly associ-

ated with thrombosis and/or obstetric complication in APS. Avidity of ACA may be a better predictor of predisposition to thrombosis and pregnancy loss than ACA levels , which may fluctuate over time owing to several factors. Thus, implementation of strategies for the characterization of ACA's avidity may have relevant clinical utility in the management of antiphospholipid syndrome.

0453

THE USE OF INFERIOR VENA CAVAL FILTERS AT A DISTRICT GENERAL HOSPITAL IN THE UK

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Background. Venous thromboemoblism (VTE) is a significant cause of morbidity and mortality worldwide. Anticoagulation remains the preferred treatment for deep vein thrombosis (DVT) and pulmonary embolism (PE), however, in recent years the use of inferior vena cava (IVC) filters has increased significantly as a preventative measure again PE. This has raised concerns about the indications for IVC filter placement and the management of IVC filters. In 2006 the British Committee for Standards in Haematology (BCSH) produced guidelines on the use of IVC filters. *Aims*. The aims were to establish which patients in the Royal Cornwall hospital were having IVC filters placed and if our practice was consistent with the current BCSH guidelines. *Methods*. Patients who had IVC filter inserted over a four year period (2004-2008) were identified using the Picture Archiving and Communication System. An audit proforma was designed to address each aspect of the current BCSH guideline. Clinical information was obtained retrospectively from the hospital notes. *Results*. 60 patients were identified over the four year period. 49 case notes were retrieved of which 23 were males and 26 females- 75% of the patients were aged above 60 years of age. All patients had IVC filters placed for appropriate reasons; 27 had a contraindication to anticoagulation, 14 were pre-operative patients with recent PE (within one month), 7 had VTE despite appropriate anticoagulation and 1 patient had VTE in pregnancy shortly before delivery (<2 weeks). Removal of IVC filter was delayed (>4 weeks) in 73% patients. A low rate of complications was observed after filter insertion with only 2 patients having IVC thrombosis, both of whom did not receive long-term anticoagulation and 1 patient with recurrent DVTs despite receiving anticoagulation. All 3 patients had underlying malignancy. There was no routine follow up in 76% of the cases. Survival was poor with death in 34/49 patients, of which 76% were secondary to malignancy. *Conclusions*. In our patient population IVC filters have been inserted for guideline appropriate indications. They appear to have had a useful role in the management of patients with VTE, in particular those who have contraindication to anticoagulation. In the majority of patients they were safe to use with low complication rates even without long-term anticoagulation and with prolonged placement. Routine radiological follow up may not be necessary in all patients as many have limited life expectancy due to underlying malignancy.

0454

IDENTIFICATION OF MUTATIONS IN THE PROTEIN C GENE ASSOCIATED WITH THE RISK OF VENOUS TROMBOSIS

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Background. During the diagnosis of protein C (PC) deficiencies in families with a history of venous thromboembolism (VTE), frequently occurs that some relatives of the investigated families show a PC level near the lower normal limit (60-70%), which makes clinicians difficult to reach a definitive diagnosis. *Aims.* To identify mutations in the PC gene responsible for the PC deficiency in 9 proposita with VTE and in their relatives. *Methods.* We sequenced the 9 exons (E) and their flanking intron (I) regions of the PC gene in the proposita and in two of their relatives, one with reduced and the other with normal plasma PC levels. Following identification of the mutation associated with the plasma PC deficiency, we genotyped the mutation in the remaining relatives by direct sequencing of the corresponding PC DNA fragment. *Results.* In total, we found 7 PC mutations, 2 of them in two different families. Three were mutations already described in the literature: Pro168Leu, Val297Met and Thr298Met. The first two mutations have been reported to induce an abnormal folding or thermodynamic instability in the PC molecule and the third would destabilize the structure of the hydrophobic pocket, blocking its function. We found 4 novel PC mutations. The Gly179Arg mutation, identified in two families, would induce an abnormal folding of the protein. The G3222T mutation modifies the consensus sequence of the E5-I5 splicing site, whereas the A3318G mutation modifies the consensus sequence of the I5-E6 splicing site, which could alter the sequence or the PC level. Finally, the Glu16Lys modifies the Glu16 residue in the Gla domain of the PC molecule, which is essential for the correct folding of the domain and for the PC anticoagulant function. *Conclusions.* Our study allowed us to identify the mutations responsible for the PC deficiency in all 9 families studied, and to provide genetic counselling to all family members (Conselleria de Sanitat AP-151/08; RECAVA RD06/0014/004).

0455

ARE THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR POLYMORPHISMS DETERMINANTS OF THE TYPE OF ACUTE CORONARY SYNDROME?

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Background. In patients with coronary disease at risk of acute coronary events it is unclear which biological factors could predict the type of acute coronary syndrome (ACS) clinical presentation. *Aim.* The aim of the study was to investigate the role of genetic polymorphisms in key proteins in fibrinolysis in the type of ACS. *Methods.* 248 patients with ACS (unstable angina or myocardial infarction) (77% male, mean age 60.75 SD 13.30 years) were prospectively recruited. PAI-1 (type-1 plasminogen activator inhibitor) 4G/5G and thrombin-activatable fibrinolysis inhibitor (TAFI) Ala147Thr, C+1542G, and Thr325Ile polymorphisms were determined by PCR. *Results.* 147 (59.3%) patients presented with ST-segment elevation (STE) ACS (all Q-wave myocardial infarction), and 101 (40.7%) with non-STE ACS (52 non-Q wave myocardial infarction, and 49 unstable angina). Homozygous TAFI +1542G and TAFI 325Ile genotypes were less prevalent in patients with STE ACS (p<0.001, OR: 0.22, 95% CI 0.10-0.50 and p<0.001, OR: 0.25, 95% CI 0.11-0.55, respectively). There were no differences in TAFI Ala147Thr or PAI genotype distribution between STE and non-STE ACS. In the multivariate analysis including clinical variables, the best model for STE ACS included TAFI +1542GG (p<0.001, OR: 0.17, 95% CI 0.07-0.30), age (in years, p<0.005, OR: 0.97, 95% CI 0.94-0.98) and dyslipidemia (p<0.005, OR: 2.33, 95% CI 1.42-3.80). *Conclusions.* TAFI polymorphism C+1542G and Thr325/Ile are related to the type of ACS. Patients with coronary disease would benefit from individualized cardiovascular prophylaxis based on genetic risk. Supported in part by grant FIS 02/0711 and FIS 05/0204 from the Fondo de Investigaciones Sanitarias, and HERACLES network RD06/0009, Ministerio de Ciencia e Innovacion, Instituto de Salud Carlos III, Spain.

Infectious diseases, supportive care I

0456

ORAL VALGANCICLOVIR AS EARLY PRE-EMPTIVE TREATMENT FOR CMV REACTIVATION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A SINGLE CENTER EXPERIENCE IN 45 PATIENTS

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Background. Cytomegalovirus (CMV) reactivation remains an important complication after allogeneic stem cell transplantation (allo-SCT), accounting for significant morbidity and mortality, despite recent advances in prevention and treatment. Preventive measures are both primary prophylaxis and preemptive therapy. According to the EMBT Infectious Disease Working Party and the European Conference on Infections in Leukemia both intravenous ganciclovir or foscarnet can be used as first line preemptive therapy; oral valganciclovir (VGCV) still remain an investigational option. *Aims.* We report the retrospective analysis of CMV treatment with valganciclovir and outcome in patients (pts) experiencing CMV reactivation after allo-SCT at our Institution in the last 4 years. *Materials and methods.* Between November-2004 and January-2009, 117 pts were evaluated for CMV reactivation within the first 3 months after allo-SCT. Pts were monitored for CMV quantitative pp65 antigenemia and quantitative DNA PCR twice weekly; response to treatment was defined as negativization of pp65 antigenemia for at least 2 consecutive determination under treatment. Preemptive therapies were: ganciclovir, foscarnet and VGCV. Toxicities were defined according to the Common Terminology Criteria for Adverse Events v3.0. *Results*. One-hundred-seventeen pts (median age 48 years, range 19-67; 71 males) received transplant for high risk haematological malignancies; 6/117 received a second transplant. Totally 123 allo-SCT were performed: 44 from HLA identical sibling, 36 from unrelated volunteer donor, 42 from family haploidentical donor (17 with *ex vivo* T-cell depletion, 25 without), 1 cord blood. Host/donor serostatus was: -/-7, +/+68, -/+6, +/-42. One-hundred-eleven pts received acyclovir as viral prophylaxis. Sixty-two pts out of 117 experienced CMV reactivation. Median time of CMV reactivation was 43 days (range 7-168). Median number of CMV Ag nuclei was 3 (range 1-35), median CMV DNA PCR was 2090 (range 131-66000). Forty-five out of 117 received oral VCGV as first line preemptive therapy; median time of treatment was 16 days (range 6-44). Thirty-one out of 45 (69%) completely resolved CMV reactivation, while 14 required drug crossover to ganciclovir or foscarnet iv for persistence of CMV positivity after a median time of 16 treatment-days. Thirty-one out of 45 patients (68%) developed haematological toxicities. Globally we observed: 16 grade IV neutropenia and 1 grade IV thrombocytopenia; 13 grade III neutropenia, 7 grade III anemia and 13 grade III thrombocytopenia. Six out of 29 pts with grade III-IV neutropenia experienced severe infectious adverse events (SAE), requiring hospitalisation. Twenty-one pts, out of the 31 who completely resolved CMV reactivation with VGCV did not present further reactivation. In 9/10 pts the recurrence of CMV reactivation within the first 3 months after transplant was successfully treated with VGCV. *Conclusions.* Oral valganciclovir treatment is effective as first line therapy early after allo-SCT. A prospective randomised study comparing oral VGCV and gancyclovit i.v. is planned in our Institution to assess the oral VGCV efficacy as standard treatment in allo-SCT.

0457

INCIDENCE AND RISK FACTORS OF PROVEN OR PROBABLE INVASIVE FUNGAL INFECTIONS IN 286 PATIENTS UNDERGOING RELATED OR UNRELATED ALLOGENEIC BONE MARROW TRANSPLANTATION

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Introduction. Allogeneic bone marrow transplantation (BMT) is increasingly used to treat hematologic diseases. Invasive fungal infections (IFI) remain an important cause of morbidity and mortality in this setting. Recent reports have indicate that the incidence of IFI (expecially aspergillosis) has increased in BMT recipients, particulary after engraftment. *Patients and Results.* To evaluate the epidemiology, outcome and risk factors of proven or probable IFI in allogeneic BMT recipients, we retrospectively reviewed the medical records of 286 consecutive adult patients (pts) who underwent allogeneic BMT (170 from related and 116 from unrelated donor) at our Department between 1992 and 2005; 213/286 (74%) pts received a myeloablative conditioning regimen and 73/286 (26%) a nonmyeloablative one. The median age of patients was 43 years (range 19-68). We identified 36 cases of proven or probable IFI (Aspergillus sp. 25, Candida sp. 5, Fusarium 2, Mucor 5) with an overall incidence of 13%. The incidence after related BMT (RD-BMT) was 9% while it was 16% after unrelated BMT (UD-BMT) (p<0,05). The incidence was the same in the myeloablative and non myeloablative setting. IFI occurred after a median of 38 days from BMT (range 5-1440); 16/36 (44%) cases occurred during pre-engraftment phase while 20/36 (56%) occurred after engraftment (with 12/20 cases after day 100). The sites of infection were: lung only 22/36 (61%), CNS 6/36 (17%), multiple sites 8/36 (22%). Advanced hematologic disease (relapsed or refractory) at time of transplant, history of pre-transplant IFI, presence of acute or chronic graft-versus-host-disease (GVHD), but not neutropenia, were significant risk factors (p<0.05). In the UD-BMT setting the incidence of IFI was significantly higher in patients who received a combination of immunosuppressive agents in the conditioning regimens (ATG +/- Fludarabine +/- Campath). Overall Survival after 100 days from diagnosis of IFI was only 20% and in these cases 64% of deaths were directly IFI related. *Conclusions.* 1) IFI is a significant cause of non-relapse mortality following RD and UD- BMT. 2) Aspergillus sp. remain the most important aetiologic agent. 3) Incidence of IFI in UD-BMT is significantly higher than in RD-BMT probably as a result of more intensive immunosuppressive conditioning regimen in this setting. 4) IFI can develop late after engraftment (after day 100 from transplant) and without neutropenia. 5) Status of hematologic disease (relapsed or refractory) at transplant, history of pre-transplant IFI and presence of GVHD, are important predisposing factors. 6) Retrospective studies, like this one, can be useful in order to identify high-risk BMT patients for which targeted and more effective diagnostic and therapeutic strategies should be used to prevent and treat IFI.

0458

ONCE-WEEKLY LIPOSOMAL AMPHOTERICIN B FOR PROPHYLAXIS OF INVASIVE FUNGAL INFECTION AFTER GRAFT VERSUS HOST DISEASE ì IN ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION (ALLO-SCT): A COMPARATIVE RETROSPECTIVE MONOCENTER STUDY

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Invasive pulmonary aspergillosis (IPA) is a significant risk in patients with graft versus host disease (GvHD) after allo-transplantation. A comparative retrospective single centre study was conducted to investigate the efficacy and safety of liposomal amphotericin B (L-AmB, Ambisome®) prophylaxis of fungal infections in allo-transplanted patients with GvHD. A total of 125 patients receiving high-dose prednisone (2 mg/kg/day) therapy for acute and/or chronic GvHD after allo-SCT were identified. 118 pateints (94%) had a RIC regimen and 7 (6%) a myeloablative conditioning; 95 patients (76%) had a family donor and 30 patients (24%) had an unrelated donor. Median age at transplant was 48 years (range 18-70). Forty-two patients received once-weekly high-dose (7.5 mg/kg/week) L-AmB as prophylactic treatment. Eighty-three patients of the control group received possibly other prophylactic antifungal drugs such as fluconazole, voriconazole, itraconazole, posaconazole. The prophylactic median dose of L-AmB was 500 mg/week (range 300-650) and the median duration of treatment was 7 weeks (range 2-15). The incidence of IPA disease was 8% in the prophylactic group at both one year and two years vs 36% and 44%, respectively, in the control group (p=0.008). No fungal infection-related deaths were observed post-transplantation in the prophylactic group vs 12 (14%) at one year, 14 (17%) at two years and 16 (19%) at three years post-transplantation in the control group (p=0.005). The OS rate at one year was 69% for the prophylactic group vs 75% for the control group; at two years the OS was 55% in the prophylactic group vs. 64% in the control group (p= 0.60). There were no differences in the TRM rates between the two groups at two years (18% vs. 19%) and at three years (18% vs. 21%) (p=0.99). Prophylactic treatment with L-AmB was well tolerated. Renal toxicity leading to treatment discontinuation was observed in only five patients (12%) who were concomitantly treated with other nephrotoxic drugs; nephrotoxicity was reversible in all five patients. These data suggest L-AmB prophylaxis is an effective and well tolerated treatment for the prevention of invasive fungal infection and can reduce the fungal infection related mortality in RIC allo-SCT patients presenting a severe

GvHD; further prospective clinical studies are required to confirm these single center data.

0459

INVASIVE ASPERGILLOSIS IN ACUTE MYELOID LEUKEMIA: REPORT OF SEIFEM-2008 MULTICENTER SURVEY

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Background and Aims. To evaluate epidemiological characteristics, and outcome of invasive aspergillosis (IA) in acute myeloid leukemia patients (AMLs), and to analyse efficacy of different therapeutic approaches. *Methods.* A multicenter survey conducted over 2004-2007 in 21 Italian Hematology Divisions. All proven/probable IA were reported. AMLs submitted to transplant were excluded. Principal demographic and clinical data, as well as antifungal therapies and outcome were analyzed. All available variables were investigated as predictors of death. Follow-up was assessed at 90th day from IA diagnosis. Response rate to 1st line antifungal therapy were thus analyzed. Our attention mainly focused on the three most frequently emploied drugs: voriconazole, liposomal amphotericin B (L-AmB), caspofungin.

Table. Therapuetic approaches employed in 140 patients and results of univariate analysis.

	N° cases (%)	N° exitus (AMR %)	*p-value*
Antifungal prophylaxis	20 (14%)	4 (20%)	
➢Topical	20 (14%)	6 (30%)	
➢Topical + systemic	81 (58%)	24 (30%)	0.75
➢Systemic	19 (14%)	4 (21%)	
➢Not performed			
Prophylactic sistemic drug		23 (34%)	
➢Itraconazole	68 (67%)	7 (21%)	0.19
➢Fluconazole	33 (33%)		
Empirical versus pre-emptive therapy	87 (68%)	25 (28%)	
➢Empirical	41 (32%)	10 (24%)	0.61
➢Pre-emptive			
Empirical/pre-emptive drug	27 (21%)	8 (30%)	
➢Caspofungin	54 (42%)	12 (22%)	
➢L-AmB	25 (20%)	6 (24%)	0.61
➢Voriconazole	14 (11%)	6 (43%)	
➢d-AmB	8 (6%)	2 (25%)	
➢Other [2]			
1° line therapy	84 (67%)	19 (23%)	0.46
➢Similar to empirical	42 (33%)	12 (29%)	
➢Different from empirical			
Drug in 1° line target therapy	*6* (5%)	1 (17%)	
➢d-AmB	37 (27%)	9 (24%)	
➢L-AmB	28 (21%)	9 (32%)	
➢Caspofungin	38 (28%)	7 (18%)	
➢Voriconazole	2 (1%)	0	
➢Posaconazole	3 (2%)	3 (100%)	0.79
➢Other [5]	22 (16%)	*5* (23%)	
➢Combined	*8*	*1 (12.5%)*	
▪*L-AmB + caspofungin*	*8*	*2 (25%)*	
▪*L-AmB + voriconazole*	*6*	*2 (33%)*	
▪*Caspofungin + voriconazole*			
G-CSF	93 (66%)	24 (26%)	
➢Yes	47 (34%)	14 (30%)	0.62
➢No			

AMR: aspergillosis-attributable mortality rate; d-AmB: deoxycolate amphotericin B; L-AmB: liposomal amphotericin B; G-CSF: granulocyte colony stimulating factor, AF: antifungal

Results. 140 cases were collected, most of which were probable IA (66%). Infection mostly occurred after the 1st course of chemotherapy (61%). The majority of AMLs experienced a deep neutropenia before the onset of symptoms (90%). Overall attributable mortality rate (AMR) was 27% (38/140), and it remained stable over years. Outcome was significantly influenced by AML phase ($p<0.001$), duration of ($p=0.05$) and recovery from neutropenia ($p<0.001$). Role of neutropenia duration was confirmed at multivariate analysis ($p<0.005$). Antifungal approaches are reported in Table 1. No differences emerged between empirical and pre-emptive therapy and none of the drugs employed resulted to significantly influence outcome. In 66% of pts initial empirical/pre-emptive drug remained unchanged after IA diagnosis, while in 16% clinicians shifted to a combined treatment. When we compared the 3 most frequently emplojed drugs as 1st line target therapy (Table), the overall response rate was 71%, ranging from 61% with caspofungin to 84% with voriconazole. Ninety-three pts received subsequent oral antifungal therapy, and voriconazole was the most frequently employed drug. *Summary and Conclusions.* Because of availability of complete information about pts, this study allows as to analyzed multiple factors, as potentially influencing outcome. Frontline therapy choice is a crucial point, influencing patients' outcome. Our series confirmed the good result obtained in other recent series and perception that the application of a correct and timely diagnostic work-up and the availability of more efficacious and less toxic antifungal drugs (i.e. voriconazole, liposomal amphotericin B [L-AmB], caspofungin) have modified the course of IA. However all newer drugs resulted comparable in terms of survival and response rate. Even combined antifungal treatment did not confer any advantage in survival analysis. Despite of recent progresses, management of hematological malignancy and recovery from neutropenia continue to be the most crucial prognostic factors.

0460

RAPID DETECTION AND IDENTIFICATION OF PATHOGENS IN HEMATOONCOLOGICAL PATIENTS USING MOLECULAR TECHNIQUES

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Background. Infectious complications in immunocompromised hosts (hematooncological patients) represent a serious clinical issue. In these individuals, not only common nosocomial agents, but also very unusual pathogens, zoonoses, pathogens with specific cultivation demands or fastidious pathogens can be identified. Moreover, in these patients, the disease course might be rapid, complicated and serious. *Aims.* The goal of the work was to improve the speed and precision of the laboratory diagnostics of infectious agents, as a supplement to the standard microbiological techniques. *Methods.* We have developed a system employing pathogen-specific probes and Real-Time PCR technology to detect 25 most frequent human pathogens causing severe nosocomial infections and 5 fastidious pathogens causing pulmonary infections. Each sample has also been tested using pan bacterial and pan fungal broad-range 16S and 18S rDNA PCR system coupled with direct sequencing of PCR products for precise identification of the causative agents. *Results.* We have investigated 1,248 clinical samples including peripheral blood, BAL, cerebrospinal fluid, sputum, aspirates from thorax cavity, drainage fluids, abscesses, tissue biopsies, urine, and stool. In addition to common pathogens we have identified a set of unusual and fastidious pathogens: Chlamydiophilla pneumoniae (BAL), Mycoplasma pneumoniae (BAL), Legionella pneumoniae (BAL, peripheral blood), Bordetella parapertussis (BAL), Peptostreptococcus micros (thorax cavity), Fusobacterium nucleatum, Listeria monocytogenes and Porphyromonas endodontalis (cerebrospinal fluid), Moraxella nonliquefaciens (urine), Mycobacterium tuberculosis (tissue, cerebrospinal fluid), Aspergillus flavus (tissue, sputum), Malassezia pachydermatis (tissue), Cryptococcus carnescens (BAL). Moreover, for the first time, we have identified a zoonosis caused by the tick-borne agent Candidatus Neoehrlichia mikurensis (CNM), in peripheral blood of a patient with relapsing mantle cell lymphoma. The CNM finding was corroborated by 16S rDNA sequencing and electron microscopy. *Conclusions.* Pathogen-specific Real-Time PCR technique coupled with direct pan bacterial and pan fungal sequencing represent a very fast and useful tool to accelerate and refine the diagnostics of infections in immunocompromised and critically ill patients. Timely information on the causative agents is highly important for rapid and accurate clinical decision making and targeted pharmacotherapy.

0461

COMBINED QUANTITATIVE CMV PCR MONITORING WITH PRE-EMPTIVE ORAL VALGANCLOVIR IS EFFECTIVE IN PREVENTING SYMPTOMATIC CMV INFECTION IN ALLOGENEIC HAEMOPOIETIC TRANSPLANT RECIPIENTS

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Background. Human cytomegalovirus (CMV) infection is a major cause of morbidity and mortality in haemopoietic transplant recipients prompting universal prophylaxis or treatment during the asymptomatic phase (pre-emptive treatment). Monitoring for CMV reactivation can be done by viral isolation, quantification of pp65 antigen or quantification of viral genome in the peripheral blood. Quantification of viral genome by a PCR based method is highly sensitive and allows rapid diagnosis and treatment. However the threshold for initiating (pre-emptive) treatment and the optimal therapeutic approach is not well defined. *Aims.* The aim of our study was to evaluate the use of a quantitative PCR CMV genome screening approach combined with standardised criteria for initiation of treatment with oral Valganciclovir in the control of the development of CMV disease in an allogeneic haematopoietic transplant (BMT) population. *Methods*. The study cohort included all allogeneic BMT patients treated in a single centre from January 2006 to December 2008. Peripheral blood samples were analysed for CMV reactivation at least weekly from day 0 of transplant until immunosuppressive drugs had been discontinued using a real time PCR method (Qiagen, Hamburg). The results were reported according to the manufacturers' instructions: copy number <650 was reported as not detected; copy number > 650 but <10000 was reported as detected; copy number > 10000 was reported as a numerical positive result. Patients were initiated on treatment, firstly, if they had CMV related symptoms and a positive result of any copy number and, secondly, if they were asymptomatic but either had two consecutive readings of a copy number of more than 10000 or two consecutive detectable readings with a rising titre. Treatment was discontinued when there were two negative results. Clinical data was collected by prospective follow up from case notes. *Results.* A total of 77 transplants (27 sibling and 50 unrelated donor) were performed. Thirty-five patients (45%) showed CMV reactivation amounting to 88 episodes. Twenty-three episodes (26%) did not fulfil the criteria for treatment initiation and resolved spontaneously. Fifty episodes (57%) were successfully treated with oral Valganciclovir and did not need hospitalisation. Twelve episodes were treated with Valganciclovir and then moved to IV Ganciclovir due to clinical deterioration / treatment failure or started on IV Ganciclovir initially and therapy continued on oral Valganciclovir. The remaining 3 episodes were treated with Ganciclovir as it was felt IV is preferable to oral therapy. There were two incidences of CMV retinitis while the peripheral blood CMV PCR was reported as not detectable and both these patients have previous incidence of positive CMV PCR that had required treatment. *Summary and Conclusions.* RT-PCR based method is a sensitive way of quantification of CMV viral genome. Oral Valganciclovir is effective in controlling CMV reactivation with minimal toxicity and avoiding patient hospitalisation. Two episodes of CMV retinitis while having low level of copy numbers (reported as not detectable by the present reporting method) raise the value for regular ophthalmology review for high risk patients.

0462

CANDIDEMIA IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES: THE ROLE OF PROPHYLAXIS AND THE IMPORTANCE OF LOCAL EPIDEMIOLOGY IN TREATMENT

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Background. Candidemia is a serious condition with a high mortality rate in patients with hematologic malignancies. It is thus important to understand the associated risk factors, as well as the need to establish adequate prophylaxis and early, effective therapy. *Aims.*To determine the incidence of candidemia in hospital patients with hematologic malignancies; to describe its clinical features and the risk factors associated with infection and with a poor outcome. *Methods.* An electronic database was used to identify cases between January 2000 to March 2008, of a positive blood culture for Candida spp in hemato-oncologic patients admitted to the Hematology Ward of Hospital Universitario La Paz. Clinical history of each identified case was reviewed. *Results.* 47 patients were identified, with an annual incidence of 1%. Species identified were: Candida parapsilopsis in 46% of cases (n=22) and Candida albicans in 21.3% (n=10); the remainder was distributed amongst C. guillermondi, C. tropicalis and C. krusei. Underlying hemalogic malignancies were: non-Hodgkin lymphoma (34%, n=16), multiple myeloma (19%, n=9) and acute myeloid leukemia (17%, n=8). 48.9% of patients underwent stem cell transplantation (45.3% allogeneic and 54.7% autologous). No significant association was found between the underlying hematologic malignancy and the species of Candida that was isolated. The antifungals used in treatment were: liposomal amphotericin 48.9%, fluconazole 12.7%, caspofungin 4.2%, voriconazole 4.2% and combined therapy in 30% of patients. MIC50 and MIC90 for fluconazole against C. parapsilopsis were 4 and 32, respectively, and 0.03 and 8, respectively against C. albicans. MIC90 against the other species was 0.03. MIC50 and MIC90 for amphotericin were 0.03 and 1, respectively, against C. albicans, C. parapsilopsis and C. krusei. Voriconazole, itraconazole and caspofungin were found to have an MIC90 of 0.03 against all species of Candida. 37.8% of patients were already receiving antifungal prophylaxis at the time of diagnosis of candidemia, although 90% of cases of C. albicans candidemia were not on prophylaxis (p<0.05). In terms of risk factors, 76.6% of patients had a central venous catheter, 78.8% were undergoing chemotherapy, 95.6% were receiving concomitant, broad-spectrum antibiotics, 21.7% were diabetic, 46.8% were receiving parenteral nutrition of which more than half (59.1%) were associated with C. parapsilopsis, 26.7% had a serious associated mucositis, 60.5% had less than 0.2×10^9/L neutrophils, 37% had kidney failure. Eight patients (17%) died as a result of candidemia: 4 from C. albicans, 2 from C. parapsilopsis, 1 from C. glabrata and 1 from C. krusei. Of the patients with C. albicans, 33% died, compared to 11.8% of those with other species of Candida (p<0.05). *Conclusions.* Candida parapsilopsis was found to be the the main causative species of candedemia in our centre, with a markedly high MIC50 and MIC90 for fluconazole, probably related to fluconazole prophylaxis. These findings highlight the importance of understanding the epidemiology of each centre when planning treatment and establishing an effective scheme of prophylaxis in high-risk patients to avoid the mortality associated with this type of infectious complication.

0463

MANAGEMENT OF FEBRILE NEUTROPENIA EPISODES IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES BY USING THE MULTINATIONAL ASSOCIATION FOR SUPPORTIVE CARE IN CANCER RISK INDEX

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Background. Febrile neutropenia (FN) is a common and potentially life threatening problem for patients with hematological malignancies. That is why it is very important to define which of these patients could be considered as low or high risk, in order to manage the episodes of FN by the best way possible. *Aims.* In this study, we try to determine whether the Multinational Association for Supportive Care in Cancer (MASCC) Risk Index is able to predict the outcome of the FN episodes in patients with hematologial malignancies, by separating them in risk groups. *Methods.* This is a retrospective study of 100 episodes of FN in patients with hematological malignancies, which were hospitalized in our Clinic from January 2007 to May 2008. MASCC Risk Index Score was calculated for every FN episode. This Score is based on seven independent factors present at the onset of the FN. These factors are: 1) burden of illness: absent or mild symptoms (5 points), moderate symptoms (3 points), 2) absence of hypotention (5 points), 3) absence of chronic obstructive pulmonary disease (4 points), 4) presence of solid tumor or absence of previous fungal infection (4 points), 5) outpatient status (3 points), 6) absence of dehydration (3 points), 7) age <60 years (2 points). MASCC Risk Index- Score ≥21 indicates low-risk patients. MASCC Risk Index-Score <21 indicates high-risk patients which were candidates for developing serious medical complications. In our study, MASCC Risk Index- Score was compared to the outcome of each episode of FN. The results were statistically analysed with Chi-Square, Fisher Exact Test and Logistic retrogression. *Results.* 46/100 (46%) episodes of FN were scored with MASCC Risk Index- Score ≥21. 40 of them (40/46, 86.95%), had a favourable outcome with immediate response to their therapy. 6/46 (13.04%), had unfavourable outcome, such as no response to the initial therapy, worsening of the patient's status and death. 54/100 (54%) episodes of FN were scored with MASCC Risk Index- Score <21. 4 of

them (4/54, 7.4%), had a favourable outcome. 50/54 (92.59%) had unfavourable outcome. Conclusively, MASCC Risk Index- Score could predict the outcome of the FN episodes, with worthy predictive value (87%), sensitivity (90.9%) and specificity (89.3%). *Summary and Conclusions.* MASCC Risk Index- Score is both safe and feasible way to predict the outcome of the FN episodes in patients with hematological malignancies, as it accurately identifies patients at low or high risk for medical complications. This Index could possibly be used for evaluation of the patients at the time of their admission to the Hospital. Low risk patients could be managed safely with early hospital discharge and oral antibiotics, always under the recommendations of the attendant physician.

0464

SERUM N-TERMINAL PRO-BRAIN NATRIURETIC PEPTIDE IN HAEMATOLOGICAL PATIENTS WITH NEUTROPENIC FEVER: A PROSPECTIVE COMPARISON WITH C-REACTIVE PROTEIN

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Serum amino-terminal pro-brain natriuretic peptide (NT-proBNP) is considered to be of importance in patients with severe sepsis. However, no data is available on NT-proBNP kinetics in haematological patients with neutropenic fever and severe sepsis. Seventy haematological patients were included into this prospective study. The median age was 56 years (range 18-70). Ten patients (14%) had previous cardiovascular disease. Nineteen patients received intensive chemotherapy for acute myeloid leukaemia and fifty-one patients were autologous stem cell transplant recipients. Laboratory samples for the determination of NT-proBNP and C-reactive protein (CRP) were collected at the beginning of the neutropenic fever (d0) and then daily up to 3-4 days. There were altogether 94 periods of neutropenic fever, of which 13 (14%) were complicated by severe sepsis. Two patients (15% of those with severe sepsis) died from septic shock. The median time after the onset of fever to the point when the criteria for severe sepsis were fulfilled was 1 day (range 1-7). The median pro-BNP (interquartile ranges) increased from 127 ng/l (57-393) on d0 to 542 ng/L (194-385) on d4. In patients with previous cardiovascular disease the NT-proBNP values were significantly higher than in patients without cardiac diseases throughout the whole study period. The median CRP increased from 35 mg/L on d0 to 109 mg/L on d2. Medians for NT-pro-BNP or CRP did not differ between periods with severe sepsis than those without on days 0-2. There was no correlation between CRP and pro-BNP measured on the same day. Neither serial NT-proBNP nor CRP showed any early predictive value in periods with severe sepsis. We conclude that NT-pro-BNP may not reflect inflammatory response in this patient group, rather it seems to be a marker of previous cardiovascular disease and a marker of cardiovascular stress during sepsis in these patients. It may thus serve as a useful adjunct to optimize management of patients with previous cardiovascular disease during sepsis.

0465

PALONOSETRON (ALOXI): A SINGLE-CENTER EXPERIENCE IN THE PREVENTION OF EMESIS IN PATIENTS AFFECTED BY HAEMATOLOGICAL MALIGNANCIES

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Chemotherapy (CT)-induced nausea and vomiting (CINV) still represents a concern for cancer patients care. Aloxi is the newest 5-HT3 receptor antagonist, with a higher receptor-binding affinity and a longer half-life (40 hours). It has demonstrated an improved antiemetic activity for delayed (>24 hours after the initial dose of CT) CINV. Limited data are available regarding its activity in a high-dose (HD) setting, in multiple-day CTs and in an allogeneic setting for haematological malignancies. Since January 2008, we are conducting a prospective monocenter study evaluating efficacy of Aloxi after administration of multiple-day and/or high-dose chemotherapy regimens and/or myeloablative conditioning treosulfan-based regimens for allogeneic bone marrow transplantation for different hematological malignancies. 100 consecutive patients received a double intravenous (IV) administration of Aloxi 0.25 mg over 30 seconds, approximately 30 minutes before the administration of chemotherapy on day 1 and on day 4 of each cycle plus dexamethasone 4 mg IV twice a day. No dosage adjustment has been performed in elderly patients (>65 years) and in patients with renal or hepatic impairment. No relevant adverse reactions occurred. Diagnosis was acute myeloid leukemia (65 patients), acute lymphoblastic leukemia (11 pts), non-Hodgkin's and Hodgkin's lymphomas (12 pts/4 pts), multiple myeloma (4 pts), chronic myelod and lymphocytic leukemia (1 pt/1 pt), severe aplastic anaemia (2 pts). 51 patients received an allogeneic myeloablative conditioning treatment with a treosulfan-based regimen; 12 patients received an autologous bone marrow transplantation after a myeloablative regimen with HD-melphalan alone or plus mitoxantrone; 24 patients received HD-aracytin alone or plus HD-methotrexate and 13 patients received multiple-day standard-dose treatments, as induction or consolidation therapies for acute leukemias. Major endpoints included complete response (CR) established as the proportion of patients with no nausea and/or emesis and no rescue medication during acute phase (<24 hours after initial dose of chemotherapy) and during delayed phase (>24 hours but <120 hours after initial dose of chemotherapy). Each patient was investigated by the assistant nurse with a specific tool of registration, in order to estimate the real CINV, otherwise just self-reported by the patients. As to acute-CINV evaluation, almost all 100 patients (93%) achieved a CR (no emesis, no need for rescue therapy) with only 7 patients (7%) experiencing nausea, followed by a minimum of one to a maximum of five episodes of emesis, treated with other 5-HT3RAs or metoclopramide as need. As to delayed-CINV evaluation, 13 patients (13%) suffered nausea, with up to 4 episodes of emesis, with a CR rate of 87%. Rescue therapies have been performed as above. The total amount of emesis events that have been registered during the whole observation time (0-120 hours) has been 32 events, corresponding to an overall response rate of 68%. Our experience with the combination of Aloxi (double injection) plus corticosteroid seems to be an effective therapy in the prevention of both acute and delayed CINV, confirming also its rational use in an allogeneic setting for patients affected by haematological malingnancies.

0466

PROLONGED FOLLOW-UP OF PATIENTS WITH HEMATOLOGICAL MALIGNANCIES INFECTED BY HBV AND HCV

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Introduction. Patients with hematological malignancies are the group of high risk of infection by HBV and HCV due to large transfusion load and large number of invasive procedures. The aim of the study was to evaluate the presentation rate of clinical hepatitis, patient's survival and how the fact of hepatitis infection influenced on their survival. *Results.* The studied group comprised all pts of a hematology department admitted since February 2004 till June 2006. The pts were monitored till June 2008. All pts were monitored by testing of HBsAg, aHCV, DNA-HBV, RNA-HCV, HBeAg, aHBs, aHBc, aHbe in scheduled timing by near 3 weeks. Liver biopsy was held for 64 pts, for 23 pts - with HBV and HCV immunohistology. Total 7800 biological samples were collected for HBV and HCV testing, 4000 of them was tested by PSR methods. Acute leukemias (AL) and aplastic anemias (AA) constituted 77% (205) of 265 investigated pts. Median age was 38 years (ranged from 15 to 79), male - 47% (125), female - 53% (140). Median transfusion load (the number of donors per 1 pt for investigation period) was 45 (ranged from 0 to 418). At first admittance to hematological department viral markers were found: for HBV in 15% (39/265) pts and for HCV in 7% (19/265) pts. It was postulated that the rate of infection process is extremely high in the hematology clinic. After total staying in the clinic 51% (135/265) pts had HBV markers, 19% (51/265) pts had HCV markers, 14% (37/265) pts had markers of both types (HBV+HCV). We have shown that up to 95% of HCV-positive pts and up to 60% of HBV-positive pts developed clinical-biochemical symptoms of viral hepatitis at the end of 3 years observation from the time of the first appearance of HBV and HCV markers. 154 (58%) pts survived, 111 (42%) pts died, death reason in 2 pts of them was fulminate liver dysfunction due to severe hepatitis B. The analysis of survival risk factors demonstrates that expected life duration decreases statistically significant after HBV infection. For AL pts HR=1.8 (p=0.034), for AA pts HR=4,3 (p=0.022). Statistical significant association the expected life duration and HCV infection was not proved. Proportional hazard regression model with time dependent covariates (PHREG SAS) was used for the analysis. *Conclusions.* The majority of pts infected by HBV and HCV developed the clinical recognizable viral hepatitis within 3 year from first appearance of viral markers. Pts with severe

immunosupression often do not demonstrate evident features of acute viral hepatitis. So pts with hematological malignances should be monitored regularly (at least once in a month) and carefully on HBV and HCV markers during all period of treatment and after treatment cessation for 1-2 years. Viral hepatitis B is proved to be survival risk factor for pts with AL and AA.

0467

COMPARISON BETWEEN FILGASTRIM AND PEG-FILGASTRIM IN REDUCING FEBRILE NEUTROPENIA AND MOBILIZING PBSC IN LYMPHOMA PATIENTS TREATED WITH DHAP OR R-DHAP

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Background. DHAP regimen is effective in relapsed/refractory Hodgkin and non Hodgkin lymphoma. This scheme is also effective in peripheral blood stem cell (PBSC) mobilization. In pre-growth factor era, after DHAP administration, 48% severe neutropenia and 28% documented sepsis were observed (Velasquez, Blood 1988). *Aims.* To compare usual employment of filgastrim (G-CSF) with PEG-filagastrim (PEG-G) in terms of 1) preventing febrile neutropenia, 2) mobilizing PBSC in patients undergone DHAP or R-DHAP schedules. *Methods.* Data regarding 69 DHAP or R-DHAP administrations in 67 patients (female 35, male 32) were retrospectively collected. In every case G-CSF or PEG-G were administered and PBSC harvest was planned. Incidence of neutropenia and infective complications were recorded. Data regarding efficiency of PBSC harvest were also collected. *Results.* Patients' median age was 41 years (range17-67). Forty-eight patients were treated with PEG-G on the second day (median, range 1-4) after the end of chemotherapy cycle. G-CSF was employed in 21 cases; a mean of 7.4 vials were administered for each cycle starting on day 8 (median, range 5-10) from the end of chemotherapy. Grade II neutropenia had a median duration of 2 days th with G-CSF (range 0-7) and with PEG-G (range 0-8). Grade IV neutropenia was observed for 1 day (median) both with G-CSF (range 0-5) and with PEG-G (range 0-6). Grade IV neutropenia lasting for at least 3 days was seen in 16 (23%) cases (4 with G-CSF, 12 with PEG-G) and lasting for at least 5 days only in 3 (4%) cases (all with PEG-G). Febrile events were observed in 7 (10%) cases (3 with G-GSF; 4 with PEG-G): 5 cases of fever of unknown origin, 1 case of pharyngitis and 1 case of bacterial pneumonia. No mortality due to infection was seen. PBSC mobilization was effective in 67/69 cases (both failures, i.e. harvest < 2 $\times 10^6$/kg with PEG-G, in previously over-treated patients). Mean harvest was 17.26×10^6/CD34/kg with G-CSF and 13.33×10^6/CD34/kg with PEG-G. In only 8 cases harvest was suboptimal (<5×10^6/kg): 4 after G-CSF and 4 after PEG-G. Cases where just a single harvest procedure was enough to reach PBSC target were 12/19 (63%) after G-CSF and 34/44 (77%) after PEG-G. This difference was not statistically significant (Mann - Whitney test V; p=0.3744). Median number of days to harvest from end of chemotherapy was 12 with G-CSF (range 7-15, mean 12, standard deviation 2.0) and 11 after PEG-G (range 9-16, mean 11.3, standard deviation 1.2). Variance is significantly different (test F; p=0.001 after logarithmic conversion), showing a better predictability of timing of harvest in PEG-G treated patients. *Conclusions.* Also in our experience the prophylactic use of neutrophil growth factors was able to reduce impact of infections in DHAP treated patients up to 10%. PEG-G seems more effective in reaching the PBSC target (>5×10^6/kg) with a single leukapheresis than G-CSF (77% vs. 63%), but this difference was not significant in this series. A higher predictability of harvest timing was shown in PEG-G treated patients.

0468

NATURAL HISTORY OF HEPATITIS E DURING THE COURSE OF HAEMATOLOGICAL MALIGNANCIES

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Background. Hepatitis E virus (HEV) is an RNA virus, endemic in developing countries and an emerging disease in industrialized countries. It was considered as an agent only responsible for acuting resolving hepatitis. However chronic hepatitis complicated by severe cirrhosis have recently been described in European solid organ transplantation recipients. Natural history of hepatitis E in haematological malignancies-related immunosuppressed patients remains largely unknown. *Aims.* To describe the clinical course of hepatitis E in patients with haematological malignancies. *Methods.* We retrospectively reviewed the clinical charts of patients followed in the hematology unit of the university hospital of Toulouse (France). Diagnosis of hepatitis E was based on abnormal liver tests of unknown origin and at least one concomitant positive blood PCR. In all patients, autoimmune hepatitis or viral hepatitis of other cause (CMV, HAV, HBV, HCV) were ruled out. *Results.* From 11/2003 to 11/2008, hepatitis E was diagnosed in six patients (5 males, 1 female; median age 46 years, range 32-65) followed for acute myeloid leukemia (N=2), mantle cell lymphoma (N=2), multiple myeloma (MM, N=1) and anaplastic large cell lymphoma (ALCL) (N=1). Delay between onset of hematological diagnosis and HEV diagnosis was 6 months (0-72). All patients but one were under specific treatment at the time of HEV diagnosis. Four patients were in complete response and 1 relapsing from AML. At the onset of HEV, AST and ALT were increased to 3-70N and 5-80N, respectively whereas prothrombine time was normal in all patients. Positive HEV viremia was detected for less than 6 months (acute hepatitis) in 4 patients and more than 6 months (chronic hepatitis) in 2. After HEV diagnosis, allogeneic (n=1), syngenic (n=1) and autologous (n=1) stem cell transplantation were succesfully performed. In one patient who had a syngenic allograft, transient recurrence (one month) of HEV viremia was observed. In one patient with MM-related hypogammaglobulinemia, chronic hepatitis occured despite IgIV supplementation and led to severe fibrosis (metavir score A2F3) at one year of follow-up. Chronic hepatitis (nine months) was also observed in a patient who received 4 cycles of ACBVD chemotherapy regimen for ALCL followed by autologous-SCT. Syngenic SCT graft was delayed in one patient and auto-SCT was challenged in one patient because of concomitant acute HEV. There was no evidence of liver failure in any patients regardless of HEV course and specific therapies. Of note, molecular evidence of patient-to-patient transmission of HEV in the hematology unit was demonstrated in 2 patients. Individual case reports, sequencing of HEV isolates and phylogenetic analysis will be presented at the meeting. *Conclusion*. Both acute and chronic hepatitis E may complicate the clinical course of patients with haematological malignancies. Evolution to cirrhosis seems improbable but patient to patient transmission is a concern. Whether management of malignancy, especially SCT, should be adapted to the HEV status remains to be determined.

0469

FILGRASTIM DOSE HAS AN IMPACT ON INFECTIOUS COMPLICATIONS IN PATIENTS WITH LYMPHOMA FOLLOWING AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Infections represent the leading cause of morbidity and mortality in patients with lymphoma treated with autologous stem cell transplantation. Duration and depth of neutropenia following transplantation correlates with incidence and severity of infections in the post-transplant period. Filgrastim shortens the duration of neutropenia but its impact on the course and outcome of infections as well as the preferred dose remain somewhat controversial. *Aims.*to evaluate infectious complications in patients with lymphoma treated with autologous peripheral blood stem cell transplantation (PBSCT) and investigate possible influence of different filgrastim doses on incidence, severity and outcome of these infections. Patients and *Methods.* 120 consecutive patients (median age 42, range 19-71 yrs, 63M/57F) with relapsed or refractory Non-Hodgkin's Lymphoma (NHL, n=92) and Hodgkin's Disease (HD, n=28) treated with PBSCT in a single center have been evaluated for infectious complications following transplantation. In the post-transplant period, all patients received filgrastim subcutaneously, starting from the day of WBC<1×10^6/L until the second consecutive day of WBC>1×10^6/L. Two different dose levels of filgrastim were used, *Low*: 300 mcg/day, administered in 58 patients; and *Standard*: median dose 600 mcg (SD 94, average 628 mcg), administered in 61 patients. *Results.* Febrile neutropenia occurred in 79 (65.8%) patients at a mean of 6 days after transplantation (range 1-9, SD 1.65), more often in patients with longer duration of neutropenia post transplant (p=0.0003). In these patients, microbiological work-up was done and empirical antibiotic therapy was initiated; piperacillin-tazobactam was administered to all patients not having a history of penicillin allergy. Empirical therapy was modified according to recommended guidelines in 19 (24.1%) patients:

vancomycin was added in 32 patients (40.5%), a systemic antifungal in 10 (12.7%) and both in 6 (7.6%) patients. Twenty-eight patients (35.4%) had proven bacteremias while 29 (36.7%) had other microbiologically documented infections (MDIs). Gram-positive microorganisms were responsible for the majority (64.3%) of all bacteremias. Patients receiving standard doses of filgrastim had significantly shorter duration of neutropenia (average of 9.06 vs. 9.82 days, p=0.02) and developed febrile neutropenia less often than the group receiving lower doses of filgrastim (57.4% vs. 75.9%, p=0.03). They also responded better to empirical therapy - addition of vancomycin was not needed as often as in the group receiving lower filgrastim doses (28.6% vs. 50%, p=0.05) and had shorter duration of antimicrobial treatment (average of 9.71 vs. 10.9 days, p=0.11). *Conclusions.* Infections are serious but manageable complications of PBSCT. Gram-positive microorganisms remain the major cause of documented infections. In our patients treated with two dose levels of filgrastim, standard filgrastim doses were more efficacious in both shortening the duration of neutropenia and reducing the incidence of fever during neutropenia. Also, compared to the lower filgrastim doses, patients receiving standard filgrastim doses needed shorter antimicrobial treatment and fewer modifications of empirical antimicrobial regimen.

0470

THE CONTRIBUTION OF NUTRITIONAL STATUS OF CHILDREN WITH MALIGNANCY TO MORBIDITY, MORTALITY AND THERAPY OUTCOMES

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Background. Children with malignancy frequently have associated cachexia with significant weight loss and malnutrition. Malnutrition has a deleterious effect on the results of therapy for malignant diseases in childhood. The impact of cytotoxic drugs on nutritional status is controversial. *Aims.*We aimed to evaluate the nutritional status of children with malignancy to determine whether the type of the tumor and aggressiveness of chemotherapy have any influence on nutritional status and to assess its relationship with survival and other outcomes of therapy.*Methods.* The study was carried out on 40 newly diagnosed cases of pediatric malignancies (23 males+17 females) at Pediatric Hematology and Oncology Unit in Zagazig University Hospital from December 2006 to January 2008. All patients were subjected to full medical history and examination with special emphasis on anthropometric parameters (WFA, HFA, WFH, BMI, MUAC, TSFT and MAMC). Laboratory investigations including hematological parameters [Hemoglobin level (Hb) and serum iron(S.Fe)] and biochemical parameters [Total protein (TP) and serum albumin (S.alb)] were done. The anthropometric, hematological, and biochemical parameters were measured at time of diagnosis and repeated after 1, 3, 5 and 7 months from start of chemotherapy.

Table. Comparison in outcomes of treatment of children with malignancy between well nourished and malnourished groups according to anthropometric parameters at diagnosis.

Parameter	Group	No of patients	Remission	Delay in therapy	Mortality	X^2			P value		
						A	B	C	A	B	C
WFA	wn	15	10(66.6%)	4(26.6%)	1(6.6%)	1.32	4.06	6.31	0.25	0.04*	0.011*
	mn	25	12(48%)	12(48.0%)	12(48.0%)						
HFA	wn	18	11(61%)	5(27.7%)	4(22.2%)	0.49	0.75	1.58	0.48	0.38	0.2
	mn	22	11(50%)	9(40.9%)	9(40.9%)						
WFH	wn	22	13(59.1%)	6(27%)	7(31.8%)	0.05	1.88	2.11	0.82	0.16	0.14
	mn	18	9(50.0%)	8(44.4%)	6(33.3%)						
BMI	wn	31	21(67.7%)	10(32.3%)	10(32.3%)	4.82	0.84	1.04	0.04*	0.35	017
	mn	9	1(11.1%)	5(55.5%)	3(33.3%)						
MUAC	wn	30	19(63.3%)	11(36.6%)	7(33.3%)	2.15	0.0	4.6	0.14	1.0	0.03*
	mn	10	3(30%)	3(30%)	6(60%)						
TSFT	wn	31	20(64.5%)	12(38.7%)	10(32.3%)	4.28	0.06	2.01	0.03*	0.8	0.13
	mn	9	2(22.2%)	3(33.3%)	4(44.4%)						
MAMC	wn	39	21(53.8%)	14(36%)	13(33.3%)	0.01	0.1	0.14	0.91	0.75	0.7
	mn	1	1(100%)	0(0.0%)	0(0.0%)						

wn= well nourished mn= malnourished A= Remission B =Delay in therapy C = Mortality * =significant relation

Results. Our study showed that the prevalence of malnutrition (based on WFA, HFA, and Hb level) was high among the studied cases at the time of diagnosis (>50%). As regard the effect of chemotherapy on different parameters along the whole period of the study, there was a highly significant increase in TSFT (p<0.001), significant increase in HFA and WFH (p<0.002) and a non significant increase in Hb level and WFA. Also there was a highly significant decrease in TP (p<0.001) and non significant decrease in BMI, MUAC, MAMC, S.alb and S.Fe. Except for malnutrition based on arm anthropometry (MUAC and TSFT) which was obviously higher (but non significant) among cases with solid tumors than in hematological tumors; there was no significant difference of prevalence of malnutrition based on other parameters between different types of tumors (p>0.05). Also, there was a significant increase of prevalence of malnutrition among non survivors than survivors of children with malignancy based on WFA, MUAC, TSFT, and S.alb.(p<0.05). As regard the outcome of therapy , there was a highly significant increase in remission response rates in well nourished children than in malnourished children (based on TP and S.alb) (p<0.001) ,meanwhile there was a significant increase in mortality rate in malnourished children than in well nourished children (based on the same two biochemical parameters) (p<0.003 and<0.04). *Summary and Conclusions.* Malnutrition is highly prevalent in children with malignancy at diagnosis. Malnourished children with malignancy have lower rate of achievement of remission, higher rate of delay in treatment and higher prevalence of mortality rates. Visceral proteins measurements (TP and S.alb), height and arm anthropometry are more reliable, easy, and economic parameters in diagnosis of malnutrition among children with malignancy than weight anthropometry especially for those with solid tumors.

SIMULTANEOUS SESSION I

Myeloma and other monoclonal gammopathies - Clinical

0471

BORTEZOMIB (VELCADE)-MELPHALAN-PREDNISONE (VMP) VERSUS VELCADE-THALIDOMIDE-PREDNISONE (VTP) IN ELDERLY UNTREATED MULTIPLE MYELOMA (MM) PATIENTS

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Introduction. MP has been the standard of care for elderly MM pts in the past 40 years. However, bortezomib and thalidomide-based combinations have demonstrated to improve the efficacy in terms of response rate and time to events as compared to MP. VMP has demonstrated to be highly effective in the VISTA trial in elderly untreated MM patients. *Aims*. Spanish Myeloma Group (PETHEMA/GEM) wanted to try to optimize the treatment for this pts population and to answer the question which agent is the optimal partner for bortezomib: an alkylating or an immunomodulatory drug. *Methods*. In order to answer this question, PETHEMA/GEM activated a phase III trial comparing VMP versus VTP in elderly untreated MM pts. Patients in VMP received bortezomib 1.3 mg/m^2 twice weekly (days 1, 4, 8, 11; 22, 25, 29 and 32) for one 6-week cycle, followed by once weekly (days 1, 8, 15 y 22) for five 5-week cycles plus melphalan 9 mg/m^2 and prednisone 60 mg/m^2 on days 1-4. Patients in VTP received the same bortezomib and prednisone regimen, but instead of melphalan they received continuous thalidomide at dose of 100 mg daily. The primary endpoint was overall response rate (ORR) after induction therapy and secondary endpoints included time to events, time to and duration of response, and safety. *Results*. As of October 30, 2008, 260 planned patients have been included in the study and 205 are evaluable for response to induction therapy. 98 patients were randomly assigned to receive VMP and 107 to VTP. Regarding baseline characteristics, both arms were well balanced. No significant differences were observed in response rate: ≥ PR in 81% of patients in both groups, with a CR rate of 22% vs 27% (p=NS) and CR/nCR of 41% vs 37% (P= NS). Median time to first response was similar in both arms (1,6 months) and there were not differences in the median time to achieve CR (4,4 vs 4,9 months). Both regimens were effective in patients with poor prognostic characteristics. Regarding haematological toxicity, VMP resulted in higher incidence of ≥G3 neutropenia (37% vs 21%; p=0,003) resulting in a higher incidence of ≥G3 infections (7% vs <1%; p=0,01); by contrast, VTP resulted in a higher frequency of ≥G3 cardiac events (8,5% vs 0%; p=0,001). The incidence of ≥G3 thromboembolic events was 4% in VTP arm and <1% in VMP; finally, 9% of patients in VTP developed ≥G3 peripheral neuropathy and 5% in VMP. 38% of patients in VTP arm developed related-serious adverse events and 15% in VMP (p<0,001). Six patients died in VMP, five of them because of severe infections; by contrast, six patient also died in VTP and five because of cardiologic events. *Summary*. This analysis indicates there are no significant differences in terms of efficacy between VMP and VTP, while the incidence of non-hematological AEs, especially cardiac events, was higher in VTP, resulting in more serious AEs. These data suggest that thalidomide may not be the partner of choice for combination with bortezomib and other IMIDs such as lenalidomide should be explored.

0472

BORTEZOMIB, MELPHALAN, PREDNISONE AND THALIDOMIDE (VMPT) VERSUS BORTEZOMIB, MELPHALAN AND PREDNISONE (VMP) IN ELDERLY NEWLY DIAGNOSED MYELOMA PATIENTS: A PROSPECTIVE, RANDOMIZED, PHASE III STUDY

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Background. In newly diagnosed myeloma patients the combination of bortezomib with melphalan-prednisone (VMP) was superior to MP. In relapsed-refractory patients the 4 drug combination of bortezomib-melphalan-prednisone-thalidomide (VMPT) induced a high proportion of complete responses (CR). *Aims*. In this prospective, randomized, phase III trial, we compared VMPT and VMP. The primary end point was progression-free survival (PFS). *Methods*. Patients (N=511) older than 65 years were randomly assigned to receive VMPT or VMP. Initially, patients were treated with nine 6-week cycles of VMPT (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 days 1-4; prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42, followed by bortezomib 1.3 mg/m^2 every 15 days and thalidomide 50 mg/day as maintenance) or VMP (bortezomib, melphalan and prednisone at the same doses and schedules previously described without maintenance). In March 2007, the protocol was amended: both VMPT and VMP schedules were changed to nine 5-week cycles and bortezomib schedule was modified to weekly administration (1.3 mg/m^2 days 1,8,15,22 in cycles 1-9). *Results*. Patient characteristics were similar in both groups, median age was 71 years. Patients who received at least 1 cycle were evaluated: 221 patients for VMPT and 229 patients for VMP. Data were analyzed in intention-to-treat. The very good partial response (VGPR) rate was higher in the VMPT group (51% versus 42%, p=0.06), including a CR rate of 35% in the VMPT group and 21% in the VMP group (p<0.0001).

Table 1. Outcome according to bortezomib schedule.

	VMPT group (n=221)		VMP group (n=229)	
	All patients (n=221)	Subgroup with bortezomib weekly infusion (n=150)	All patients (n=229)	Subgroup with bortezomib weekly infusion (n=165)
CR rate (%)	35	32	21	20
2-year PFS (%)	76	77	70	75
Grade 3-4 peripheral neuropathy (%)	12	5	10	4

In the subgroup treated with weekly infusion of bortezomib, VGPR was 50% for VMPT and 39% for VMP (p=0.06), including 32% CR for VMPT and 20% for VMP (p=0.01). Subgroup analyses did not show any statistical difference between responses and either ISS or chromosomal abnormalities (such as del13, t(4;14), t(14;16) and del17). After a median follow-up of 14.8 months, the 2-year PFS was 76.1% in the VMPT group and 70.0% in the VMP group (HR=0.70, 95% CI 0.44-1.11, p=0.13). In patients who received weekly infusion of bortezomib, the 2-year PFS was 76.8% in the VMPT group and 75.5% in the VMP group (HR=0.81, 95% CI 0.44-1.49, p=0.50). Factors predictive of longer PFS were age ≤ 75 years (p=0.006) in VMPT but not in VMP and the achievement of VGPR in both groups (p=0.02 and p=0.004). The 3-year overall survival (OS) was 89.6% in the VMPT group and 88.6% in the VMP group (HR=0.92, 95% CI 0.46-1.84, p=0.81). The incidence of grade 3-4 adverse events was similar in the VMPT group and in the VMP group: neutropenia (28% vs 28%), thrombocytopenia (19% vs 16%), peripheral neuropathy (12% vs 10%), infections (12% vs 7%), and gastrointestinal complications (5% vs 7%), respectively. The weekly infusion of bortezomib significantly decreased the incidence of grade 3-4 peripheral neuropathy to 5% and 4%. *Conclusions*. VMPT is superior to VMP in

terms of response rates. Longer follow-up is needed to assess their effects on PFS and OS. The weekly infusion of bortezomib significantly reduced the incidence of peripheral neuropathy without influencing outcome.

0473

FIRST ANALYSIS OF HOVON-65/GMMG-HD4 RANDOMIZED PHASE III TRIAL COMPARING BORTEZOMIB, ADRIAMYCINE, DEXAMETHASONE (PAD) VS VAD AS INDUCTION TREATMENT PRIOR TO HIGH DOSE MELPHALAN (HDM) IN PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA (MM)

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The randomized, open-label, phase III trial HOVON-65/GMMG-HD4 was designed to evaluate the efficacy of bortezomib prior to HDM for response and progression-free survival (PFS) in patients with newly diagnosed MM. The trial was performed in 75 referral centers in the Netherlands and Belgium (HOVON group) and Germany (GMMG group). Patients with Salmon & Durie (SD) stage II or III, age 18-65 years inclusive, were randomly assigned to 3 cycles of VAD (vincristine 0.4 mg, adriamycine 9 mg/m^2 days 1-4, dexamethasone 40 mg days 1-4, 9-12, and 17-20) or PAD (bortezomib 1.3 mg/m^2 days 1,4,8,11, adriamycine 9 mg/m^2 days 1-4, dexamethasone 40 mg days 1-4, 9-12, and 17-20). No thrombosis prophylaxis was given. Stem cells were mobilized using the CAD regimen, including cyclophosphamide 1000 mg/m^2 iv day 1, and G-CSF. After induction therapy, all patients were to receive 1 or 2 cycles of high-dose melphalan (HDM) 200 mg/m^2 with autologous stem cell rescue followed by maintenance with thalidomide 50 mg daily (VAD arm) or bortezomib, 1.3 mg/m^2 once every 2 weeks (PAD arm) for 2 years. Between May 4, 2005 and May 16, 2008, 833 patients were randomized. After the trial was closed, we here report the planned interim analysis data on response after induction and HDM-1 of the initial 300 (150 per arm) randomized patients. The 2 randomization arms were equal for SD stage of disease, ISS stage, and distribution of chromosomal abnormalities. 137 patients (91%) completed PAD or 136 (91%) completed VAD and 132 patients (88%) in each arm completed HDM-1. Full dose bortezomib could be administered in 95% (PAD1), 89% (PAD2) and 85% (PAD3) of patients. Successful stem cell apheresis was achieved in all 137 PAD treated patients who received CAD mobilization . Peripheral polyneuropathy CTC grade 3-4 during PAD vs VAD was 16% vs 6%, while DVT/pulmonary embolism was diagnosed in 3% during VAD and 4% during PAD. Responses were assessed according to EBMT criteria including VGPR and nCR after PAD/VAD, after HDM-1 and best response on protocol treatment. Complete Response (CR/nCR), Very Good Partial Response (VGPR) and Partial Response (PR) in both arms were compared by logistic regression (table 1). Deletion of chromosome 13q or presence of t(4;14) did not have a significant impact on VGPR or (n)CR. We conclude that PAD induces significantly more PR+VGPR+(n)CR as compared with VAD, and that this effect is sustained after HDM-1.

This trial was supported by the Dutch Cancer Foundation (EudraCT nr 2004-000944-26), the German Federal Ministry of Education and Research and a grant from Johnson and Johnson

Table 1.

Response ITT (%)	PAD	VAD	p	PAD+ HDM-1	VAD+ HDM-1	p	PAD HDM1/2 + Maint	VAD HDM1/2 + Maint.	p
CR/nCR	7	2	NS	26	14	0.01	43	26	0.003
≥VGPR	45	17	<0.001	31	44	0.003	73	60	0.02
≥PR	79	57	<0.001	91	79	0.003	94	84	<0.01

0474

SAFETY AND EFFICACY UPDATE OF PX-171-004, AN OPEN-LABEL PHASE II TRIAL OF CARFILZOMIB IN RELAPSED MULTIPLE MYELOMA

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Background. Carfilzomib (CFZ) is a selective proteasome inhibitor with single-agent activity in relapsed myeloma (MM). In preclinical studies, CFZ overcomes bortezomib (BTZ)-resistance (Kuhn, 2007) and lacks off-target activities associated with BTZ (Kapur, ASH 2008). *Aims*. PX-171-004 is an ongoing, multicenter Phase II trial to evaluate the safety and efficacy of CFZ in 155 patients (pts) with relapsed MM after 1-3 prior therapies. We previously reported an Overall Response Rate (ORR) of 35.5% for the first 31 pts (Vij, ASH 2008). Updated data are now available. *Methods*. The study enrolled two pt cohorts: BTZ-naïve and BTZ-exposed. CFZ 20 mg/m^2 was administered Days 1, 2, 8, 9, 15 and 16 in a 28-day cycle, for up to 12 cycles. Dexamethasone 4 mg po was administered prior to each dose in Cycle 1. The primary endpoint was ORR [Partial Response (PR) + Very Good Partial Response (VGPR) + Complete Response (CR)]. Secondary endpoints included Duration of Response (DOR), Time To Progression (TTP) and safety. *Results*. 31 pts were enrolled; 14 (45%) BTZ-naïve and 17 (55%) BTZ-exposed. The extent of BTZ exposure included single agent (2 pts), BTZ + chemotherapy (6 pts) and BTZ-transplant induction (9 pts). Additionally, 16 (94%) of BTZ-exposed pts had relapsed following > 1 IMID regimen. Overall, 23 (74%) pts had > 1 prior therapy and 27 (87%) relapsed after stem cell transplant. CFZ achieved an ORR of 57%, with a median DOR of 8.55 months (range >1.94 to >9.67 months), in BTZ-naïve pts. The median TTP has not yet been reached in this group. In BTZ-exposed pts, CFZ achieved an ORR of 18%. The median DOR has not yet been reached (>8.52 months) and the median TTP was 8.9 months. To date, 7 pts from each cohort (50% BTZ-naïve; 41% BTZ-exposed) are progression free. Overall, pts received an average of 6.6 treatment cycles and 9 pts (29%) completed 12 full cycles. 22 pts (71%) did not complete 12 cycles. Reasons for early discontinuation were progressive disease (11 pts), voluntary withdrawal (1 pt) and adverse events (AEs) (10 pts). AEs leading to study termination included rash (1 pt), multi-organ failure (1 pt), tumor lysis syndrome (1 pt), edema/dyspnea (1 pt), congestive heart failure (1 pt) and Grade 1 elevated creatinine/Grade 2 hypertension (1 pt). One pt was withdrawn due to hyperthyroidism, unrelated to CFZ. One pt with a history of thalidomide-associated Grade 1 peripheral neuropathy (PN), was discontinued for Grade 3 PN despite resolution to baseline prior to receiving the final CFZ dose. 2 pts discontinued due to AEs (abdominal pain and pneumonia) during Cycle 12; neither was considered unrelated to CFZ. *Conclusions*. These preliminary results demonstrate that CFZ monotherapy achieves durable responses in relapsed MM, even in patients previously treated with BTZ. A significant number of pts remain progression free after 12 cycles. These results are notable for an anthracycline and steroid-sparing regimen. CFZ is well tolerated for up to a year and the incidence of treatment-limiting PN is significantly lower than that reported for BTZ (Argyriou, 2008).

Table 1.

	ORR	CR	VGPR	PR	TTP	DOR
BTZ-Naïve (n=14)	8 (57%)	1 (7%)	2 (14%)	5 (36%)	Not Reached	8.55 months
BTZ-Exposed (n=17)	3 (18%)	0 (0%)	0 (0%)	3 (18%)	8.9 months	Not Reached

0475

LONG TERM FOLLOW UP OF A COMPARISON OF NONMYELOABLATIVE ALLOGRAFTING WITH AUTOGRAFTING FOR NEWLY DIAGNOSED MULTIPLE MYELOMA

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Background. We previously reported on a study where the treatment assignment of 162 newly diagnosed myeloma patients younger than 65 years was based only on the presence/absence of an HLA-identical sibling donor (Bruno et al, N Engl J Med, 2007). *Aims*. To report an update analysis. *Methods*. As mentioned in the original study, up-front treatment plans consisted of induction with VAD-based regimens, a cytoreductive autograft followed by a nonmyeloablative allograft (Auto-Allo) or a second melphalan-based autograft (Double-Auto). Primary endpoints were overall (OS) and event-free (EFS) survivals by intention-to-treat analysis. The 80 patients with a sibling donor were offered a Auto-Allo and the 82 without a Double-Auto after high (140-200 mg/m^2) or intermediate dose melphalan (100 mg/m^2). No maintenance and/or consolidation therapy was allowed by protocol after nonmyeloablative allografting. *Results*. After a median follow up of 6 years, OS and EFS were significantly longer in patients with donors: not reached versus 52 months (p=0.003) and 35 versus 29 months (p=0.008). Median OS was not reached in the 58 (out of 60 enrolled, 97%) patients who completed Auto-Allo and was 64 months in the 46 (out of 59 enrolled, 78%) who completed high-dose Double-Auto (p=0.03). Moreover, an extended experience consisting of 100 newly diagnosed myeloma patients treated with Auto-Allo in a prospective clinical trial by the Gruppo Italiano Trapianti di Midollo Osseo confirmed these encouraging findings. After a median follow up of 5 years, OS was not reached and EFS was 37 months. Incidences of acute and chronic GVHD were 38% and 50%, respectively. Complete remission (including molecular remission) was achieved in 53% of patients. Profound cytoreduction (at least very good partial remission) prior to allografting was associated with achievement of post-transplant remission (HR 2.20, p=0.03) and longer EFS (HR 0.33, p<0.01). Interestingly, development of chronic GVHD was not correlated with response duration. *Summary*. Overall, the Auto-Allo approach allows prolonged disease free survival especially in patients with reduced tumor burden at the time of allografting. We are currently investigating the role of "new drugs" in intensifying pre-transplant cytoreduction and post-transplant graft-vs.-myeloma effects to further improve clinical outcomes in patients with poor prognosis.

Chronic myeloid leukemia - Clinical I

0476

SUBCUTANEOUS OMACETAXINE MEPESUCCINATE IN IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS (PTS) WITH THE T315I MUTATION - DATA FROM AN ONGOING PHASE 2/3 TRIAL

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Background. Omacetaxine (formally homoharringtonine, HHT), a first-in-class cetaxine, shows clinical activity against Ph$^+$ Chronic myeloid leukemia (CML) with a mechanism independent of tyrosine kinase inhibition. Currently available tyrosine kinase inhibitors (TKIs) have no activity against T315I. *Aims*. To evaluate the safety and efficacy of omacetaxine in Ph$^+$ CML Pts with chronic (CP), accelerated (AP), or blast phase (BP) disease who have failed imatinib and have the T315I mutation. *Methods*. Adult CML Pts who have failed imatinib and harbor the T315I kinase domain mutation are enrolled. All patients receive informed consent and then omacetaxine induction at 1.25 mg/m^2 subcutaneous (SC) twice daily (BID) for 14 days every 28 days followed by maintenance at 1.25 mg/m^2 SC BID for 7 days every 28 days. Maintenance therapy begins after at least one induction cycle and achievement of initial haematologic response. *Results*. 69 Pts (40 CP, 16 AP and 13 BP) have been enrolled. All had failed prior imatinib and 80% failed ≥2 prior TKIs. Median age is 58 years. Median disease duration is 58 months. Omacetaxine is well tolerated with transient myelosuppression as the primary toxicity. Treatment-related grade 3/4 events occurring in ≥10% of patients are: thrombocytopenia (48%), anaemia (30%), neutropenia (28%), and neutropenic fever (12%). Non-haematologic grade 3/4 events are rare with diarrhea (2%) and fatigue (3%) as the most frequently reported. Injection site reactions are mild with only grade 1/2 events reported and erythema the only event reported in > 10% of Pts (13.1%). Efficacy data are available for 47 Pts (28 CP, 11 AP, and 8 BP). In CP Pts, the median number of cycles is 4 (1-25) with 39% having received ≥6 cycles of therapy. The overall CP Pt complete haematologic response (CHR) rate is 82% with a median duration of response of 8.7 months (2.1 to 28.7+) and an overall cytogenetic response (CyR) rate of 25% (4 complete, 1 partial, 2 minimal). Six CP patients entered the study in CHR and maintained their response for at least 8 weeks to be considered responders. In AP Pts, the overall haematologic response rate is 45% (2 CHR, 1 haematologic improvement, 1 return to chronic phase [RCP]) with a median duration of response of 9.7 months (8.3-10.9+) and an overall CyR rate of 9% (1 minimal response). In BP Pts, the overall haematologic response rate is 13% (1 RCP) with no cytogenetic responses observed. For progression free survival (PFS), progression is defined as the development of advanced disease or death and in CP Pts, the loss of CHR or major CyR. In CP Pts, PFS is 80% at 1 year and 70% at 2 years; in AP Pts, PFS is 25% at 1 year and 18 months; In BP Pts, PFS is 44% at 6 months. In 64% of Pts, the T315I clone is reduced to below detection limits. *Conclusions*. Omacetaxine in CML patients who have the T315I mutation results in de-selection of the T315I clone, induces durable haematologic and cytogenetic responses, and results in meaningful progression free survival.

0477

MULTICENTER OPEN LABEL STUDY OF SUBCUTANEOUS (SC) OMACETAXINE MEPESUCCINATE IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS THAT ARE RESISTANT OR INTOLERANT TO TWO OR MORE TYROSINE KINASE INHIBITORS (TKIS)

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Background. Omacetaxine (formally homoharringtonine, HHT), a first-

in-class cetaxine, shows clinical activity against Ph+ CML with a mechanism independent of tyrosine kinase inhibition. Omacetaxine has shown activity in multi drug-resistant CML patients. *Aims*. To evaluate the safety and efficacy of omacetaxine in Pts with imatinib-resistant CML with chronic (CP), accelerated (AP), or blast phase (BP) disease who have failed or are intolerant to at least 2 prior TKIs. *Methods*. Adult CML Pts who have failed multiple TKIs are enrolled. All patients receive informed consent and then omacetaxine induction at 1.25 mg/m^2 subcutaneous (SC) twice daily (BID) for 14 days every 28 days followed by maintenance therapy at 1.25 mg/m^2 SC BID for 7 days every 28 days. Maintenance therapy begins after at least one induction cycle and achievement of initial haematologic response. *Results*. 71 Pts have been enrolled (37 CP, 18 AP, and 16 BP). All have failed prior imatinib and at least one other TKI and 51% have failed ≥3 prior TKIs. Patients enrolled in India (N=8, recently enrolled and not included in efficacy evaluation) are accepted for enrollment after imatinib failure and at least one additional treatment. Median age is 58 yrs and 50% are male. Median disease duration is 74 months. At study entry, 39% of patients had Bcr-Abl mutations including 10% with compound mutations. Patients with the T315I mutation are enrolled into a separate clinical trial. In this study, the most frequently identified mutations are F317L (12%) and V299L (6%). Omacetaxine is well tolerated with transient myelosuppression as the primary toxicity. Treatment-related grade 3/4 adverse events occurring in ≥10% of patients are: thrombocytopenia (28%), neutropenia, (23%), and anaemia (13%). Non-haematologic grade 3/4 adverse events are rare, with fatigue (2%) as the most frequently observed. Injection site reactions are mild with only grade 1/2 events reported and erythema (8%) as the most common. Efficacy data are available for 30 Pts (12 CP, 9 AP, and 9 BP). The overall haematologic response (HR) rate in CP Pts is 75%, all of whom achieved complete haematologic response (CHR). Current median duration of response is 6.1 months. The overall HR rate in AP Pts is 44% with 22% achieving CHR with median duration of response of 3.5 months. In BP patients, the overall HR rate is 67% all of whom achieved return to chronic phase with a median duration of response of 5.0 months. Overall cytogenetic response (CyR) in CP patients is 17% (1 partial and 1 minor CyR). In AP patients, the overall CyR rate is 33% with 1 each, complete, partial and minimal CyR. No cytogenetic response is observed in BP patients. *Conclusions*. Omacetaxine therapy in heavily pre-treated CML Pts induces haematologic and cytogenetic responses and may provide an alternative therapy for patients resistant to multiple TKIs.

0478

RANDOMIZED CLINICAL TRIAL FOR THE OPTIMIZATION OF IMATINIB THERAPY BY COMBINATION, DOSE ESCALATION AND TRANSPLANTATION. DESIGNED FIRST INTERIM ANALYSIS OF THE GERMAN CML STUDY IV

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Background. In a substantial minority of CML patients imatinib fails or shows suboptimal responses. *Aims and Methods*. A 5-arm treatment optimization study was therefore designed to optimize responses and improve survival by randomized comparison of standard imatinib vs. imatinib + interferon alpha (IFN) vs. imatinib + low dose araC vs. imatinib after IFN failure (for low- and intermediate-risk patients) vs. imatinib 800 mg (for high-risk patients). Inclusion criteria were newly diagnosed BCR/ABL positive CML in chronic phase. In July 2005, randomization to the arms imatinib + araC and imatinib after IFN was discontinued and recruitment for imatinib 800 mg was expanded to low- and intermediate-risk patients. This arm therefore is not yet evaluable. Primary aims are: prolongation of survival and of duration of chronic phase, and determination of rates of hematologic, cytogenetic and molecular remissions, adverse events and role of allografting. Results. Since its activation in 7/2002, 1242 patients have been randomized. The current evaluation represents the first of three prospective, statistically adjusted interim analyses of 710 patients randomized by the end of 2005 to allow a follow-up of at least 3 years. Analysis was according to intention to treat. 687 patients (566 with primary imatinib, 121 with primary IFN) were evaluable for hematologic, 659 for cytogenetic, and 633 for molecular responses. Median age was 53 years, 60% were male, median values were for Hb 12.8 g/dL, WBC 71.2/nl and platelets 384/nl, 35% had low, 53% intermediate and 12% high risk (Euro score). Median observation time was 45 months. 5-year-survival probability of all patients currently is 92% (Figure 1). There are no significant survival differences recognizable yet. At 5 years, the rates of major cytogenetic remission (MCR) are 95%, of complete cytogenetic remission (CCR) 94% and of major molecular remission (MMR) 88% as determined by competing risk. There are no significant differences between the 4 evaluable treatment arms for CCR and MMR, except that the primary IFN group reaches CCR and MMR later. After five years, progression free survival (no death, patient still in first chronic phase) was 89%. 36 patients died, 56 patients were transplanted in first chronic phase, and 80 patients progressed, 43 of which were switched to alternative treatments (16 to new drugs, 18 to transplantation, 9 received both). Type and severity of adverse events (AE) did not significantly differ from those reported previously. Hematologic AEs (leukopenia, thrombocytopenia) were most frequent in the imatinib 800 mg arm. Nonhematologic AEs (gastrointestinal) were most frequent in the combination arms and with imatinib 800 mg. In no case recruitment had to be changed due to superiority or inferiority of any arm. Conclusion. This first interim analysis shows excellent survival and favorable long term response rates. Currently, survival in all treatment arms is better than in IRIS. Imatinib in combination with, or after, IFN or with low dose araC are feasible and safe treatment modalities. We expect that the final results of CML Study IV will optimize and improve therapy outcome in the future.

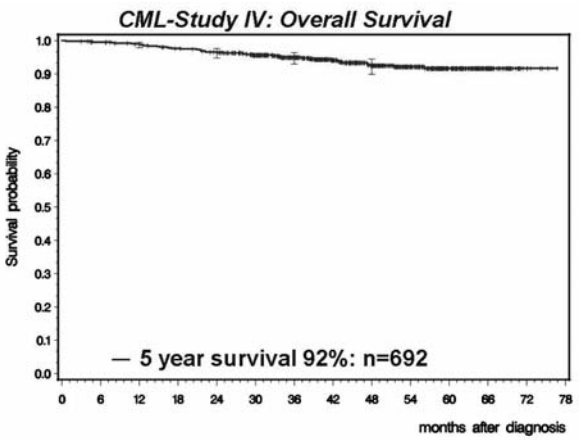

Figure 1.

0479

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) IN THE ERA OF TARGETED THERAPY: COMPARABLE SURVIVAL RATES FOLLOWING ALLOGENEIC HSCT AFTER IMATINIB AND IMATINIB THERAPY IN FIRST CHRONIC PHASE; RESULTS OF THE GERMAN CML STUDY IV

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Background. HSCT remains an important option for patients with chronic myeloid leukemia (CML) who failed imatinib. Studies published so far have focused on a potential unfavourable effect of imatinib on the outcome after HSCT. Further, there are hints that pre-transplantation imatinib therapy might even improve outcome in line with observations that reduction of leukemia load prior to transplantation is associated with better transplantation outcome. *Aim and Methods*. The German CML-Study IV (five-arm randomized controlled trial to optimize imatinib therapy by combination, dose-escalation and transplantation) is designed to determine the role of transplantation in the imatinib era. *Results*. By November 2008, 1242 pts were randomized, 84 (6.8%) pts transplanted. 55 pts were male (65%), median age at diagnosis was 37 years (yrs, range 16-62), median time to HSCT was 12.9 months (mo, range 3.5-55). Based on the indications for HSCT three groups were defined: i) elective HSCT (n= 20, 24%); ii) HSCT after imatinib failure or intolerance in first CP (n=36, 43%) and iii) HSCT in advanced disease (n=28, 33%) including 25 in blast crisis. All randomized therapies were evenly represented as treatments prior to HSCT.

Table 1.

		HSCT in 1. CP	HSCT in advanced phases
n		56	28
Euro score	high	15 (27%)	9 (32%)
	intermediate	17 (30%)	8 (29%)
	low	24 (43%)	11 (39%)
Male		33 (59%)	22 (79%)
Median age (range)		37(16-56)	38 (18-62)
Median time to HSCT (months) (range)		13,0 (4,8 – 32,3)	12,8 (3,5-55,1)
EBMT score	0-1	9 (16%)	0
	2	9 (16%)	1 (4%)
	3-4	36 (64%)	9 (32%)
	>=5	2 (4%)	18 (64%)
Best response prior to HSCT	CHR	41 (73%)	15 (54%)
	CyR	36/52 (69%)	10/24 (42%)
	minor CyR	30/52 (58%)	10/24 (42%)
	MCyR	25/52 (48%)	8/24 (33%)
	CCyR	14/52 (27%)	5/24 (21%)
	MMR	5/48 (10%)	2/19 (10%)
Response after HSCT	MMR	41/44 (93%)	14/15 (93%)
	CMR	38/44 (86%)	14/15 (93%)
Transplant source	Sibling	21 (38%)	9 (32%)
	Unrelated	35 (63%)	19 (68%)
	PB	41 (73%)	23 (82%)
	BM	14 (27%)	5 (18%)
Conditioning therapy	standard	39 (70%)	18 (64%)
	reduced	6 (11%)	3 (11%)
	Other/n.a.	7/3 (13%)	6/1 (25%)
Probability of survival at 2/3 years after HSCT		91,4%	58,8%

Median follow-up in the 56 CP patients was 30 mo. 3-year survival probability after HSCT in first CP was 91%. Incidence of transplant-related mortality was 7%. Median follow-up in 28 advanced disease pts was 24 mo (0-50). Survival probability at 2 years was 59%. To compare survival of transplanted with that of non-transplanted pts on imatinib therapy in first CP, a matched pair analysis was performed. For 53 transplanted pts two non-transplanted pts in first CP at the time of transplantation of the partner were identified. Survival estimates at five years (92% vs 93%) did not show any significant difference. After HSCT, molecular data for 59 of 70 living patients demonstrated major molecular remission (MMR) in 55 pts (93%) being complete (CMR) in 52 pts (88%) at most recent follow-up analysis. *Conclusions*. We conclude that reduction of tumor load by initial imatinib therapy and improvements in transplantation procedures translate into improved outcome of pts after HSCT. In view of the curative potential of transplantation and the survival results that are equally good as with drug treatment, we propose HSCT as preferred second line option after failure or intolerability of first line TKI-therapy for eligible pts.

0480

PHASE II MULTICENTRIC EXPLORATIVE STUDY OF INTERMITTENT IMATINIB (IM) TREATMENT (INTERIM) IN ELDERLY PATIENTS WITH PH+ CHRONIC MYELOID LEUKEMIA (CML) WHO ACHIEVED A STABLE COMPLETE CYTOGENETIC RESPONSE (CCGR) WITH STANDARD IM THERAPY

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Background. Standard therapy with Imatinib (IM) significantly prolongs the survival of Ph+ CML patients who obtain a complete cytogenetic response (CCgR). Elderly patients (i.e. > 65 yrs) have similar cytogenetic responses and survival, but they usually show a low compliance. *Aims*. The aim of the study is to investigate if CCgR that has been achieved with standard (daily administration) IM therapy can be maintained with the same dose of IM given intermittently (INTERIM). The study population is represented by elderly patients (at least 65 years) with Ph+ CML and with stable CCgR after at least 2 years of standard IM therapy (daily administration). *Methods*. IM is given at the same dose that was given at the time of enrollment by the following intermittent 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 will be evaluated at baseline (by conventional cytogenetics on bone marrow and FISH on peripheral-blood) and every 3 months during the study (only by FISH on peripheral-blood). Quantitative molecular assessment of BCR-ABL transcript by RQ-PCR on peripheral blood is due at baseline and every 3 months during the study and mutational analysis of ABL will be performed in case of loss of CCgR. If FISH documents a variation of the baseline value of more than 1% in two consecutive examinations, evaluation of marrow cells metaphases will be performed to confirm the loss of CCgR and to check for additional cytogenetic abnormalities. In case of loss of CCgR INTERIM will be stopped and standard therapy (daily administration) will be 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*. One-hundred and nine patients have been considered eligible, but 17 (16%) refused to enter into the protocol. Out of 92 enrolled patients, 61 started INTERIM, 4 patients (4%) went off the study for major protocol violation before the 3rd month and, at present, 57 patients are ongoing. Of these 57 patients, 25 and 10 completed the 3rd and 6th month, respectively. At the 3rd month, all the 25 evaluable patients maintained the CCgR. As detected by RQ-PCR, 19/25 (76%) maintained a major molecular response (MMR) and 6/25 (24%) showed at least 1 log increase of BCR-ABL ratio. At the 6th month, all the patients maintained the CCgR; 9/10 (90%) maintained a MMR and 1/10 (10%) showed a 1 log increase of BCR-ABL ratio. *Conclusions*. These preliminary data suggest that IM given intermittently can be sufficient to maintain the CCgR in those patients who have a stable CCgR, previously achieved with standard IM therapy.

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Acute lymphoblastic leukemia - Clinical I

0481

IMMUNOCHEMOTHERAPY WITH RITUXIMAB IN ADULT CD20+ B-PRECURSOR ALL IMPROVES MOLECULAR CR RATE AND OUTCOME IN STANDARD RISK (SR) AS WELL AS IN HIGH RISK (HR) PATIENTS WITH SCT

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Background. The effect of Rituximab with a chemo induction and consolidation therapy was studied in CD20+ and Ph/Bcr-abl negative B-precursor ALL (Pre-B/Common) ALL in GMALL Study 07/2003, since improvement of adult B-precursor ALL with chemotherapy was limited and also based on the encouraging results with combined intensive chemotherapy and Rituximab in adult Burkitt lymphoma / leukemia studies. *Methods*. Adult ALL patients (15 - 55 years) with standard risk B-precursor ALL being CD20 pos. (41% antigen expression of >20%) received Rituximab 375 mg/m^2 at day -1 before each induction course (phase I and II), the reinduction course and before each of the six consolidations for a total of 8 doses. Aim was to reduce MRD load and thereby the relapse rate. High Risk patients, candidates for a stem cell transplantation (SCT) in CR 1, received Rituximab three times (d -1 ind. I/II and Cons I) before SCT. *Results*. A total of 185 CD20 pos. patients were analyzed in the GMALL study 07/2003; 133 were SR and 52 HR patients. 117 received Rituximab (R+ arm) and were compared to 70 patients early recruited without Rituximab (R- arm), but identical chemo- and supportive therapy. In the SR there was no difference in the CR rate of 94% and 93% in the R+ vs. R- patients, neither in ED rate 5% vs. 4% or failure/PR 1% vs. 2%. However, MRD course differed substantially. Decrease in MRD load in the R+ vs. R- arm was faster with a Mol CR (MRD <10-4) at day 21 of 60% vs 19% and at wk 16 of 89% vs. 57%. Probability for continuous complete remission (CCR) at 3 years was 0.64 vs. 0.48 for R+ vs. R- pts. (p=0.009) and for overall survival (OS) 0.75 vs. 0.54. For HR pts OS at 3 yrs was 0.54 vs. 0.32 in the R+ vs. R- group. In the 66% HR pts receiving a SCT in CR1 OS was superior for the R+ vs. R- with 0.75 vs. 0.40, due to fewer relapses. OS was no excess toxicity in the R+ vs. R- patients, death in CR 1 was 4% in R+ vs. 3% in R. *Conclusions.* Intensive chemo- plus immunotherapy with Rituximab is feasible in adult SR and HR B-precursor ALL pts. There is a faster and higher Mol CR rate in the Rituximab arm with an improvement of CCR and OS. Thus, Rituximab + chemotherapy can improve outcome of adults with B-precursor ALL.

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0482

BLINATUMOMAB (ANTI-CD19 BITE?) FOR TARGETED THERAPY OF MINIMAL RESIDUAL DISEASE (MRD) IN PATIENTS WITH B PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): UPDATE OF AN ONGOING PHASE II STUDY

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Background. Blinatumomab (MT103/MEDI-538) is targeting the CD19 and CD3 antigens, and is a member of a novel class of bispecific BiTE antibodies that redirect T cells for lysis of target cells. In patients with B-precursor ALL, MRD positivity after induction therapy or at any time point later predicts a hematological relapse, despite undergoing further intense chemotherapy. A phase II study is being conducted by the German Multicenter Study Group on Adult Lymphoblastic Leukemia (GMALL) in patients with MRD-positive B precursor ALL. *Methods*. B-precursor ALL patients in complete hematological remission with molecular failure or molecular relapse (defined as MRD >10-4) starting at any time after consolidation I of front-line therapy were included. MRD is assessed by quantitative PCR of either individual rearrangements of immunoglobulin/TCR-genes or specific genetic aberrations such as bcr/abl or ALL1-AF4, defined as < 10-4. One treatment cycle of blinatumomab is a 4-week continuous i.v. infusion, which can be followed by allogeneic SCT or repeated cycles of blinatumomab with 2-week treatment-free intervals. The dose level is 15 microgram/m^2/24 hr. Based on clinical activity and lack of safety concerns, the dose may be increased in this trial. *Results*. At the date of submission, 10 patients have been enrolled and 7 patients are evaluable for response. Most common adverse events included pyrexia, chills, hypoimmunoglobulinemia, and lymphopenia. Except for 1 lymphopenia grade 4, 1 hypoimmunoglobulinaemia grade 3, and 1 port infection grade 3 (unrelated), these AEs had an intensity of grade 1 or 2. Most AEs resolved during treatment. Overall, treatment demonstrated no unexpected safety concerns and no permanent treatment discontinuation was required. Five of seven evaluable patients went into molecular remission (mCR) after one cycle of blinatumomab treatment. One of these responding patients became negative for both individual rearrangements and bcr/abl. Another patient with bcr-abl positive B-ALL, who has been treated with 4 cycles of blinatumomab (first three cycles in combination with dasatinib) remained at stable MRD level as did one patient with ALL1-AF4 after one cycle. Of the 5 responding patients one patient had an extramedullar testicular relapse followed by hematological relapse one month after discontinuation of treatment. *Conclusions*. Blinatumomab as a single agent has induced molecular remissions in 5 out of 7 patients with MRD-positive ALL. Treatment has exhibited a safety profile that supports further study and recruitment is ongoing.

0483

DASATINIB (SPRYCEL?) AND CHEMOTHERAPY FOR FIRST-LINE TREATMENT IN ELDERLY PATIENTS WITH DE NOVO PHILADELPHIA POSITIVE ALL (EWALL-PH-01): ANALYSIS OF RESPONSE AND RESISTANCE

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Background. Dasatinib (Sprycel®) is a potent multi-targeted kinase inhibitor (TKI) of BCR-ABL and SRC family kinases. Despite a 33% complete hematological response (CHR) rate in a Phase 2 study in patients with Philadelphia positive (Ph+) acute lymphoblastic leukaemia (ALL), the median duration of progression-free survival (PFS) was 3.3 months. A consensus has been reached by the EWALL (European Working group for adult ALL) to adopt a common first-line chemotherapeutic schedule for elderly ALL patients (aged 55 years or more). *Aims*. This schedule was adapted to test the addition of dasatinib in case of Ph+ ALL. *Methods*. After a prephase with dexamethasone 10 mg/m^2 d-7 to d-3, Dasatinib was administered at 140 mg QD (100 mg over 70y) during the induction period in combination with IV injections of vincristine 1 mg and dexamethasone 40 mg 2 days (20 mg over 70y) repeated weekly for 4 weeks. Consolidation cycles consisted in Dasatinib 100 mg/d sequentially with methotrexate 1000 mg/m^2 IV d1 (500 mg/m^2 over 70y) and asparaginase 10,000 UI/m^2 IM d2 (5,000 UI/m^2 over 70y) for cycles 1, 3 and 5 and cytarabine 1,000 mg/m^2/12h IV d1, d3, d5 (500 mg/m^2 over 70y) for cycles 2, 4 and 6. Maintenance phase consisted in Dasatinib sequentially with 6-MP and methotrexate orally 1 month every other

month and dexamethasone/vincristine in 2 months intervals up to 24 months of treatment. *Results*. A total of 30 patients were included from August 2007 to January 2009. Median age was 71 years (range, 61 to 83). The Ph+ chromosome was associated with complex abnormalities in 88% of cases. The CHR rate was 96.6% (29 out of 30 evaluable patients). The only case of induction failure was due to death during induction. The rate of complete molecular remission (CMR) after induction was 33% and MRD level continued to decrease with time. Eight relapses were observed after 2, 6, 6, 6 7, 9, 10 and 11 months of therapy. Seven relapses were associated with the detection of the T315I mutation and one with the F317L. We were able to describe the kinetic of the T315I mutation before haematological relapses using a specific T315I real time PCR. Four patients died while being on study, 1 during induction from invasive aspergillosis, 2 in CHR before consolidation from pulmonary embolism and physical deterioration and one during maintenance from pneumonia. Four other patients died from relapse. Events were defined as relapses, deaths, SAEs or study treatment discontinuations and median EFS was 212 days. However, median relapse free survival and overall survival were not reached with a median follow-up of 12.4 months. *Conclusions*. The EWALL-PH-01 protocol is highly effective with a 96.6% RCH rate in elderly patients with Ph-positive ALL. All relapsing patients except one harboured a T315I mutation and we were able to detect the kinetic of appearance of the mutation before haematological relapse. Of importance MRD level for responding patients was decreasing with time and median OS was not reached.

0484

IMATINIB AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (SCT) FOR PHILADELPHIA CHROMOSOME POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (PH+ALL): INTERIM ANALYSIS OF A RANDOMIZED PHASE III STUDY

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Background. Patients with Ph+ALL in whom bcr-abl transcripts reappear after allogeneic SCT are at high risk of relapse. In a previous study, approximately 50% of Ph+ALL patients with minimal residual disease (MRD) after SCT converted to PCR negativity in response to Imatinib (IM), started a median of 4.4 months after SCT. As failure to rapidly (within about 2 months) achieve PCR negativity was associated with relapse, we hypothesized that initiation of IM in the setting of a lower leukemic cell burden. i.e. earlier after SCT, may increase response rates and improve outcome. *Aims*. In this prospective, randomized multicenter clinical trial, we compared two strategies of intervention with respect to tolerability and duration of molecular and hematologic remission: the interventional administration of imatinib, triggered by PCR positivity after SCT, and the earliest possible initiation of IM after SCT, irrespectively of the patient´s MRD status. *Methods*. This report is a planned interim analysis of the first 40 enrolled patients, who within 6 weeks after SCT were randomly assigned to receive IM either up-front (cohort 1) (n=20) or subsequent to detection of MRD by real time quantitative and/or nested rtPCR (cohort 2)(n=20). Bcr-abl transcripts were assessed in peripheral blood and bone marrow samples every 3 and 6 weeks, respectively. Target dose of IM was 600 mg, 400 mg was permitted if deemed necessary. IM administration was scheduled for one year of PCR negativity. Informed written consent was obtained from all patients prior to enrolment. *Results*. SCT was performed in CR1 in 17 pts. of cohort 1 and in 18 pts. of cohort 2, 2 each were transplanted in CR2 and 1 with active disease (cohort 2). IM was started in 17/20 patients in the up-front IM and 10/20 in cohort 2. The majority of patients received 400 mg IM (13/17 pts. and 6/10 pts., respectively). Median time from SCT to start of IM was 45 days (cohort 1) and 89 days (cohort 2). Three patients (all in cohort 1) died in CR, only 1 one of whom had actually received IM. After a median follow-up of 438 days (131-1329d) and 577 d (196-1316d) in cohorts 1 and 2, respectively, none of the 35 pts. transplanted in CR 1 and 2 of 5 with SCT in CR2 or active disease have relapsed. IM was discontinued prematurely in 10/17 pts. in the IM up-front arm and 3/10 in the MRD-triggered IM cohort, due mostly to gastrointestinal toxicity (n=5) and GvHD (n=3). *Conclusions*. Administration of imatinib after SCT is associated with a low relapse rate and no evidence of increased non-relapse mortality both with the up-front and MRD-triggered schedules. Although imatinib appears to be less well tolerated when started very early after SCT, its routine use after allogeneic SCT is a promising strategy to improve outcome of patients with Ph+ALL.

0485

FLOW-CYTOMETRIC IMMUNOBEAD ASSAYS FOR THE DETECTION OF FUSION PROTEINS IN PRECURSOR B- ACUTE LYMPHOBLASTIC LEUKEMIA AND ACUTE MYELOID LEUKEMIA

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At present, the prognostic classification of acute leukemias is generally based on the presence or absence of specific genetic aberrations, particularly fusion genes, as detected by karyotyping, FISH or PCR. However, these techniques are time consuming and demand specialized laboratories. Therefore we are developing fast and easy flow cytometric bead assays (CBA's) for detection of fusion proteins in lysates of the leukemic cells that contain a well-defined fusion gene aberration. Previously, we developed CBA's for the diagnosis of BCR-ABL positive leukemias and PML-RAR· positive APL (AML-M3). Now we develop two multiplex CBA tubes: the precursor-B-ALL tube (detecting BCR-ABL, TEL-AML1, E2A-PBX1 and MLL-AF4 fusion proteins) and the AML tube (detecting PML-RAR·, AML1-ETO and CBF,-MYH11 fusion proteins). For each of the different fusion proteins two monoclonal antibodies were generated of which one is directed against the N-terminus and one against the C-terminus of the protein. The antibody binding sites on the various fusion proteins were carefully selected in order to recognize all variants of a fusion protein which result from translocations in the different breakpoint cluster regions. First "singleplex" CBA's were developed in which one antibody was coupled to BD CBA Flex beads to function as a catcher antibody and the other antibody was conjugated to biotin or a fluorochrome to serve as a detector antibody. A robust and specific signal in the flow cytometer was only detectable when the relevant fusion protein was present in the cell sample analyzed. When samples were analyzed that did not contain the fusion protein, no signal was obtained in the assay, since wild type proteins are not recognized by both antibodies of the couple. The assays are specific and sensitive and detect at least 10% (or less) of fusion protein positive leukemic cell lines diluted in a "background" of normal PB-MNCs or WBC's. Importantly, lysates of PB-MNC's or BM-MNC's of leukemia patients positive for the relevant fusion gene as assessed by PCR techniques, generated robust and specific signals in the assays. We conclude that the assays are sensitive, 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 new immunobead assays can be used for fast and easy diagnosis and classification of AML and precursor B-ALL patients expressing the various fusion proteins. This makes it possible to include these patients at an early stage in the right treatment protocols, much faster than by use of current techniques. At this moment, we are combining the singleplex assays into two multiplex tubes, thereby aiming at an easy flow cytometric approach for molecular diagnostics, which can be run in parallel to routine immunophenotyping, also applicable in countries that lack advanced molecular diagnostics.

Myelodysplastic syndromes

0486

ACQUIRED MUTATIONS IN TET2 ARE COMMON IN MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA

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Background. Myelodysplastic syndromes (MDS) represent a heterogeneous group of neoplastic hematopoietic disorders. They are characterized by dysplasia of the myeloid, megakaryocytic and/or erythroid lineages. Diagnosis of MDS is often difficult as many conditions (like infections, medication, toxic agents) may give rise to cytopenias and dysplasia without clonal disease. 30% of the patients eventually develop acute myeloid leukemia (AML). Several recurrent chromosomal aberrations have been associated with MDS, but the genes affected have remained largely unknown. The identification of genes that are mutated in MDS would facilitate a proper diagnosis of clonal disease, and could help to devise more targeted forms of therapy, comparable to the developments in chromic myeloid leukemia in the past decade. *Aims*. To identify relevant genetic lesions involved in the pathogenesis of MDS. *Methods*. We performed SNP-array-based genomic profiling of 102 MDS patients and subsequently genomic sequencing in a cohort of MDS and AML patients. *Results.* Six MDS patients showed aberrations on the long arm of chromosome 4, of which four patients showed uniparental disomy (UPD) of the 4q arm and two patients showed mono-allelic deletions. The smallest region of overlap contained two genes, TET2 and PPA2. Genomic sequencing showed homozygous mutations of TET2 in the patients with UPD of the 4q arm and hemizygous TET2 mutations in the patients with the deletions. Subsequently, missense and nonsense mutations in TET2 were found in 26% of our cohort of 102 MDS patients. Nonsense mutations were predominantly present in the N-terminal and central regions of the gene, while missense mutations were exclusively found in two evolutionary conserved C-terminal domains. Mutations of TET2 were also detected in a cohort of AML patients. Expression profiling of TET2 in healthy tissues showed highest TET2 levels in mature granulocytes compatible with a role for this gene in myelopoiesis. *Conclusions*. We conclude that TET2 is a novel gene important in the pathogenesis of MDS and AML.

0487

PREVALENCE AND PROGNOSTIC IMPACT OF TET2 MUTATIONS IN MDS

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Background. Genetic pathways involved in the development and progression of MDS remain poorly known. In addition, no gene mutation has demonstrated independent prognostic value in MDS. We recently showed TET2 gene, a member of Ten-eleven translocation family located at the 4q24 locus, to be frequently mutated in myeloid malignancies (F. Delhommeau, N. Engl. J. Med., in press) *Aims*. To establish the prevalence and prognostic impact of TET2 mutations in MDS. *Methods*. We retrospectively analyzed TET 2 mutations and their prognostic value, in 206 MDS and AML post MDS enrolled in GFM multicenter trials (41 RA/RCMD/MDS-U/5q-, 18 RCMD, 28 RARS/RCMD-RS/RARS-T, 43 RAEB 1, 32 RAEB 2, 44 AML post MDS). Univariate and multivariate survival analyses were conducted with Cox hazard proportional model. *Results*. We found 59 mutations of the TET2 gene by direct sequencing of exons 3 to 11 (27 frameshifts, 21 nonsense and 11 missense mutations in conserved domains) in 43/206 pts. The frequencies according to the WHO subtypes were 21.8% in RA, 5.2% in RCMD, 21.4% in RARS/RARS-T/RCMD-RS, 34.9% in RAEB 1, 15.6% in RAEB 2, 18.2% in AML post MDS. Other anomalies of the 4q24 region were found including a deletion in 1/46 pts analyzed by CGH and 3 LOH and 2 deletions in 5/23 pts analyzed SNP arrays. Thus, the overall prevalence of 4q24 anomalies was 22.3% pts (46/206). 20 pts had two anomalies of TET2 identified by direct sequencing (17 pts), or sequencing plus SNP array (3 pts), indicating that the two copies of the gene were targeted in 43.5% of mutated pts. Comparison between the 43 pts with TET2 coding sequence mutations and unmutated patients found no significant differences in initial characteristics for sex, age, previous exposure to chemo or radiotherapy, Hb level, WBC count, ANC, plt count,% bone marrow blasts, multilineage dysplasia, WHO and FAB subtypes, karyotype and IPSS. Five-year overall survival was 77.6% in mutated versus 46.4% in unmutated cases (p=0.0097). After excluding pts with AML post MDS, AML transformation at 5 years was 7.0% in mutated versus 49.7.% in unmutated pts (p=0.0018). In multivariate analysis (Cox hazard proportional model) adjusted for age, sex and IPSS, presence of TET2 mutation remained an independent favourable prognostic factor of overall survival [HR=3.3 (CI95%, 1.4-7.7) p=0.006]. *Conclusion*s. TET2 mutations are observed in about 20% of MDS, and are seen in all WHO or FAB subtypes. TET2 mutations constitute, to our knowledge, the first described independent molecular favourable prognostic factor of survival in MDS.

0488

GAIN-OF-FUNCTION MUTATIONS OF C-CBL TUMOR SUPPRESSOR IN MDS AND MDS/MPD ASSOCIATED WITH 11Q UPD

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Myelodysplastic syndromes (MDS) are heterogeneous neoplastic hematopoietic disorders, in which acquired uniparental disomy (aUPD) is a common genetic feature, as well as other allelic imbalances. However, the precise role of aUPD in the pathogenesis of MDS is not fully understood because of the scarcity of information about the gene targets of common aUPD. Through the genome-wide analysis of allelic imbalances in 171 MDS using high-density SNP arrays, we identify that a homozygous mutation of c-Cbl is the genetic target of the 11qUPD. aUPD is a common feature of cancer genomes, leading to loss of heterozygosity (LOH). aUPD is associated not only with loss-of-function mutations of tumor suppressor genes but also with gain-of-function mutations of proto-oncogenes in homozygous state. Here we show that homozygous gain-of-function mutations of c-Cbl tightly associated with aUPD in 11q have unique oncogenic properties, which are responsible for the pathogenesis of myeloid neoplasms. c-Cbl proto-oncogene, a cellular homologue of a viral oncogene, v-Cbl, encodes an E3 ubiquitin ligase, which negatively regulates signal transduction of tyrosine kinases. Homozygous c-Cbl mutations were found in most 11q aUPD-positive myeloid neoplasms, including MDS and MDS / MPD, as well as MDS-derived AML. We demonstrated that c-Cbl functionally and genetically acted as a tumor suppressor gene in hematopoietic cells as well as other cell types. c-Cbl mutants inhibited the E3 ligase activity of wild-type c-Cbl and lead to prolonged activation of tyrosine kinases in hematopoietic cells. When mutant c-Cbl was transduced, hematopoietic progenitors acquired increased sensitivity to a wide variety of cytokines, includ-

ing stem-cell factor, thrombopoietin, IL3, and Flt3 ligand with concomitant activation of downstream signaling, which was prominently enhanced in the c-Cbl'/' background. Our result clarified a unique relationship between a tumor suppressor proto-oncogene and its gain-of-function mutations. The phenotypes induced by mutant c-Cbl in c-Cbl'/' cells unequivocally indicated the presence of gain-of-function that are not ascribed to a simple loss-of-function or an inhibitory action against c-Cbl, which may explain the observation that c-Cbl mutations were associated with loss of wild-type c-Cbl alleles and the latter was predominantly caused by copy number (CN)-neutral, but not CN-reduced, LOH. Our findings provide a novel insight into the genetic role of aUPD associated with gain-of-function mutations of a tumor suppressor gene in the development of human neoplasm.

0489

IS ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION A REASONABLE THERAPEUTIC OPTION FOR ELDERLY (= 60 YEARS) PATIENTS WITH DE NOVO MYELODYSPLASTIC SYNDROMES ?

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With the rare exception of patients, who achieve long lasting remissions with chemotherapy, allogeneic hematopoietic cell transplantation (HCT) is currently the only modality with proven curative potential for MDS patients (pts). However, given the heterogeneity of MDS, and the potential complications associated with HCT, it is often difficult to decide when and in whom to perform an allogeneic HCT. A survival advantage with early transplantation in patients with MDS classified as intermediate-2 or high risk by IPSS criteria was demonstrated. However, this study was restricted to MDS patients below the age of 60 years. In fact, the results are therefore not a reflection of the "true" elderly MDS population who were, up to recently, not considered candidates for allogeneic HCT. Therefore, this study tried to investigate the outcome of 89 elderly (median age 64 years [range 60-71]) de novo MDS patients receiving allogeneic HCT between 2000 and 2008. MDS categories prior to HCT were either RA (n=3), RCMD (n=2), RAEB-1/CMML-1 (n=18), RAEB-2/CMML-2 (n=25) or secondary AML out of MDS (n=41). Almost all of the latter group had received at least one cycle of chemotherapy prior to HCT with 51% achieving a CR. Median time from MDS diagnosis to HCT was 10 months and HCT-comorbidity index (HCT-CI) ranged from 0 to 5 (median 1). The conditioning was of standard or reduced intensity in 30 and 59 pts, respectively and followed by donor cells from either related (n=29) or unrelated (n=60) donors. The graft versus host disease prophylaxis was cyclosporin A or tacrolimus based in all cases. With a median follow-up of 28 months the 3-year overall survival (OS) rate was 40% patients for all patients and was not different (p=0.5) between related and unrelated donor based HCT. OS differed according to marrow blasts (median 8%, range 0-80) prior to Tx (< 20%: 44% vs. ≥20%: 29%, p=0.04). In contrast, grouping pts according to IPSS cytogenetic risk group revealed no statistically significant differences (p=0.5). Of note was the observation that pts with HCT-CI of less or equal to 2 had a significant better OS compared to pts with more advanced comorbidities (50 vs. 18%, p=0.003). In a multivariate analysis only time from diagnosis to HCT (p=0.017), HCT-CI (p<0.001), and type of conditioning (p<0.001) influenced the overall outcome whereas age, donor type, cytogenetics and HLA-mismatch did not. These data demonstrate that allogeneic HCT is not only feasible but can also be associated with long-term disease control in elderly MDS pts. However, patient selection seems to be a definite prerequisite in order to prevent excess mortality. With the availability of new agents prospective randomized studies seem to be warranted in order to define the exact role and time point of allogeneic HCT in the treatment algorithm of advanced MDS.

0490

ANALYSIS OF THE MITOCHONDRIAL FERRITIN PHYSIOPATHOLOGICAL ROLE IN SIDEROBLASTIC ERYTHROPOIESIS

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Recently, in the erythroblasts of refractory anemia with ring sideroblasts (RARS), we identified a novel mitochondrial ferritin (FtMt) that may play an important role in regulating iron homeostasis and toxicity. FtMt may protect mitochondria from oxidative damage and increase cell resistance to the apoptotic signals. To clarify the possible role of FtMt in the ineffective erythropoiesis of RARS, we assessed its distribution in RARS erythroid progenitors and searched for possible correlations between FtMt expression and erythroid maturation, apoptosis, proliferation, clonogenic capacity. Moreover, we investigated the possible influence of experimentally induced FtMt overexpression on erythroid differentiation and its capacity to induce a sideroblastic phenotype in normal hematopoietic progenitors. CD34+ bone marrow cells from 24 patients with RARS, 20 patients with refractory anemia (RA) and 8 healthy donors were cultured for 14-21 days in a liquid medium according to a procedure which allows the expansion of high numbers of erythroid progenitors in the presence of Il-3, IL-6, stem cell factor and erythropoietin. At various days, cytospins were performed for FtMt, H-ferritin (HFt) and L-ferritin (LFt) immunocytochemical analysis, and samples of cultured cells were removed for other biological studies. Furthermore, we developed a lentiviral vector encoding FtMt under the control of the ubiquitous PGK promoter, which allowed the transduction of normal CD34+ bone marrow cells with an efficiency of some 30-40%, as evaluated by immunocytochemistry on day 7. FtMt transduced cells from 5 healthy donors were cultured by the same above procedure that allowed their differentiation to the erythroid lineage. RARS erythroid progenitors showed an early expression of FtMt and a continuous increase during the culture period (5-24%, day 4-14), with an inverse correlation between FtMt and HFt levels (p=0.04); studies of FtMt mRNA gene expression demonstrated the presence of the transcript even in cells lacking the protein. LFt levels were variable, while HFt expression was higher in RA (p<0.001). Cell growth was very low in all pathological samples. RARS and RA progenitors showed a tendentially higher apoptotic rate and an inverse correlation between apoptosis levels and BFU-E number. In RARS, apoptotic rate was higher in FtMt+ cells than in FtMt- cells (p<0.0001), whereas in RA a positive correlation between apoptotic rate and HFt expression was observed (p=0.005). Lentivirus-mediated FtMt transduction of normal CD34+ progenitors did not inhibit cell growth nor did it abrogate their ability to terminally differentiate but increased apoptosis of differentiated erythroid cells and reduced their HFt levels and heme content. In conclusion, we have demonstrated an abnormal phenotype characterized by mitochondrial or cytosolic iron overload in RARS and RA erythroid progenitors. The association of this phenotype with low proliferation and increased apoptosis suggests a close relationship between impaired iron metabolism and pathogenesis of myelodysplasia. Experimental overexpression of FtMt in normal erythroid progenitors may modify mitochondrial iron availability, impair heme synthesis and alter the balance between cell growth and death.

Chronic lymphocytic leukemia - Biology and Clinical

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INHIBITION OF LEF-1 EFFECTIVELY INDUCES APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA IN VITRO AND IN VIVO

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Background. A variety of aberrantly active signaling pathways has been associated with chronic lymphocytic leukemia (CLL), with one of them being the wnt/β-catenin/lef-1 cascade. Recent reports suggest autocrine activation of wnt signaling in CLL, with elevated levels of several wnts, fzd-3 and lef-1. Abnormal activation of this cascade induces expression various target genes involved in regulation of leukemic cell adhesion, B cell proliferation and survival. Hence, wnt/ β-catenin/lef-1 signaling is speculated to be involved in the prolonged survival of the CLL cells and therefore to be a potential target for CLL therapy. *Aims.* The aim was to investigate the role of lef-1-signaling in CLL cell survival. Furthermore we aimed on characterizing the effect of specific small molecules against β-catenin/lef-1 interaction on CLL cells *in vitro* and *in vivo*, to determine the suitability of β-catenin/lef-1 inhibition for CLL treatment. *Methods*. Enriched CLL-cells and healthy B-cells were used in this study. Protein levels of lef-1 target genes were detected by immunoblotting using specific antibodies. siRNA mediated knock down of lef-1 in primary CLL cells and JVM-3 cell lines was done using nucleofection, and viability was assayed by flow cytometry. *In vitro* cytotoxicity and LC50 of two small molecules inhibiting the β-catenin/lef-1 interaction was assessed using ATP based cell viability assay. Apoptotic response was investigated in time course experiments with different apoptotic markers. Specificity of the small molecules was demonstrated by co-immunoprecipitation experiments for the lef-1/β-catenin interaction. *In vivo* efficacy of the small molecules inhibitors were studied using a JVM-3 subcutaneous xenograft model in nu/nu mice. *Results*. We confirmed the overexpression of lef-1 in CLL at both, the mRNA and protein level. Knocking down of lef-1 led to increased apoptosis in CLL cells *in vitro* indicating that lef-1 has a vital role in extended survival of CLL cells. The viability was correlated to the lef-1 knock down by western blot. This observation was extended by using two small molecule inhibitors of β-catenin/lef-1 signaling (CGP049090 and PKF115-584) which induce apoptosis of CLL-like cell lines and primary CLL cells (LC50: ranging from 0.7μM to 0.9μM) Healthy B cells were not significantly affected, as ascertained by LC50 values of 8.5μM (CGP049090) and 5.7μM (PKF115-584). Co-immunoprecipitation proofed the selective disruption of β-catenin/lef-1 interaction. Further, a decrease of the lef-1 target genes c-myc, cyclin D1 and lef-1 could be demonstrated by immunoblotting upon incubation with inhibitors. We tested the *in vivo* efficacy of both inhibitors in a JVM-3 xenograft model. Mice treated with 25mg/kg for 14 days exhibited tumor inhibition of 69% with CGP049090 and 57% with PKF115-584 and the intervention was well tolerated. Kaplan-Meier survival analysis of this treatment significantly improved median survival by 12.5 days with CGP049090 and 15.5 days with PKF115-584 (p value <0.003). *Conclusions and Summary*. We showed lef-1 inhibition effectively induces apoptosis in CLL cells, via both, siRNA mediated knockdown, as well as inhibition of the active β-catein/lef-1 complex, which we additionally confirmed in an *in vivo* model. Therefore, targeting lef-1 signaling can be considered a potential attractive strategy for CLL treatment.

0492

TP53 MUTATION IN THE ABSENCE OF 17P DELETION DEFINES A GROUP OF PATIENTS WITH POOR OUTCOME IN CLL: ANALYSIS WITHIN THE CLL4 TRIAL (F VS. FC) OF THE GCLLSG

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Introduction. CLL patients with 17p deletion have a dismal prognosis after treatment. The exact prognostic role of TP53 mutations in the absence of 17p deletion and any differential impact of the mutation in cases with 17p deletion (vs. sole deletion) are currently unclear. *Methods*: We assessed the incidence, profile and prognostic impact of TP53 mutations in a well characterized patient population from a prospective trial (CLL4 trial GCLLSG: 1st line F vs. FC). We studied 340 of 375 CLL4 trial patients with available material. The population is well characterized and detailed genetic characteristics are available (genomic aberrations, VH-Status). We used DHPLC to detect TP53 mutations in the coding exons (2-11). Aberrant profiles were confirmed by sequencing including the use of fragment collection in cases with low grade mutations. *Results*. We found an overall incidence of TP53 mutations of 8.2% (28/340 patients). The mutations were exclusively located in the DNA binding domain. We observed 2 splice site mutations, 3 deletions, 2 insertions but the majority of mutations were missense mutations in exons 5-8. Two patients had 2 different mutations. Transitions were found in 11/24 missense mutations whereas transversions were seen in 13 of 24 mutations. Fourteen of 16 patients with 17p deletions had a TP53 mutation (87.5%). Interestingly, the 2 patients with a 17p deletion in whom no TP53 mutation was identified showed a low proportion of 17p- cells (19-21%) suggesting that detection limits of the technique might explain this finding. We found TP53 mutations in the absence of 17p deletions in 4.3% (14/326). Median progression-free survival and overall survival were significantly decreased in the group with TP53 mutation (PFS 23.4 vs. 61.8 months (p<0.001) and OS 29.2 vs. 84.6 months (p<0.001)). There were no significant differences in PFS or OS between the two treatment arms in the group with TP53 mutation. None of the patients with TP53 mutation showed a complete response (vs. 39/284 without TP53 mutation). Of the cases without response, 10/36 (28%) showed TP53 mutations. To assess the effect of the TP53 mutation in the absence of 17p deletion, we compared this group with the group with 17p deletion and found identical overall survival (Figure 1). These data suggest that the group of patients with TP53 mutation in the absence of 17p deletion forms a new prognostic subgroup with similar outcome as 17p deletion cases. Multivariate analysis of genomic aberrations, VH-Status, clinical parameters and serum markers revealed that TP53 mutation was the strongest negative prognostic marker regarding PFS (HR 25.6, p<0.001) and OS (HR 11.61, p<0.001). When comparing treatment results of the two arms among patients without TP53 mutation (n=312), there was a significant difference in OS with superior outcome for patients receiving FC (p=0.029).

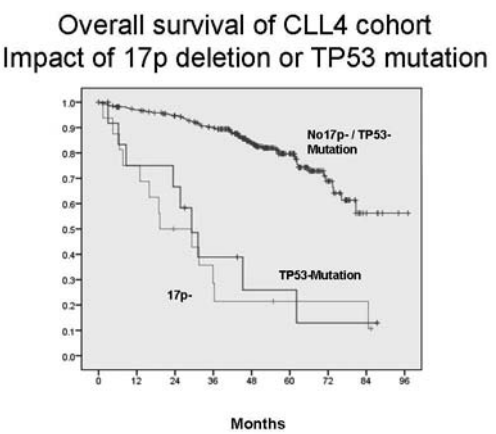

Figure 1. Survival by 17p-/TP53 mutation.

Conclusions. In this first line treatment trial population, TP53 mutations without 17p deletion occurred in 4.3% (14/326) of patients and the majority of cases with 17p deletion also have TP53 mutations. CLL with TP53 mutation carries a poor prognosis independent of the presence of the deletion 17p suggesting that alternative treatment approaches are needed for this group of patients. TP53 mutation emerged as the strongest prognostic factor in multivariate analysis in this trial population.

0493

DIFFERENTIAL GENOME-WIDE METHYLATION PROFILES IN PROGNOSTIC SUBSETS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous disease, characterized by accumulation of neoplastic B-cells due to deregulation of both apoptosis and proliferation. CLL can be classified into clinical subgroups depending on the presence or absence of somatic hypermutations in the immunoglobulin heavy-chain variable (IGHV) genes. Studies from previous investigations have shown that aberrant DNA methylation may play an important role in leukemogenesis. However, limited knowledge exists regarding the global methylation pattern in CLL. *Aims*. We here investigated genome-wide methylation using array-based technology in 27 CLL samples belonging to favourable (e.g. IGHV mutated CLL) and poor-prognostic subsets (e.g. IGHV unmutated and IGHV3-21 CLL). Methods: We applied a genome-wide methylation array from Illumina based on bisulfate conversion covering 28,000 CpG sites spanning 14,000 genes. The raw data was processed using Bead studio followed by bioinformatic analysis where the arcsin transformed data was used in a moderated t-test to find differentially methylated genes. Also, only genes with a large absolute difference between the groups were included. Methylation-specific PCR and realtime-PCR were also employed to verify the array data on selected genes. Furthermore, samples were treated with methyl-inhibitors to induce re-expression of methylated genes. *Results.* Overall, our results demonstrate a large number of differently methylated genes between the IGHV mutated and IGHV unmutated/IGHV3-21 groups. Specifically, we identified eight tumor suppressor genes (e.g. VHL, ABI3 and IGSF4) that were methylated in IGHV unmutated/IGHV3-21 CLL patients as well as ten genes involved in proliferation and tumor progression (e.g. ADORA3, LOC340061 and RASGRP3) which were overexpressed in these poor-prognostic groups. On the other hand, these latter genes were silenced by methylation in IGHV mutated CLL patients, whereas anti-apoptotic genes, such as BCL2 and PLD1, remained unmethylated in this subgroup. The methylation status was confirmed for four genes (BCL10, PRF1, ADORA3 and IGSF4) and the expression status of seven genes was verified using real-time RT-PCR (BCL10, PRF1, ADORA3, IGSF4, NGFR, ABI3 and VHL). We also re-expressed seven genes (e.g. NGFR, ABI3, VHL and IGSF4) by inhibiting DNA methylation using the methyl-inhibitor 5-aza-2'-deoxycytidine combined with and without the HDAC inhibitor trichostatin A. *Conclusion*. Our data reveals, for the first time, differences in global methylation profiles between favourable and poor-prognostic CLL subgroups, which appear to affect pathways involved in control of apoptosis and proliferation. Our novel data could possibly reveal new epigenetic-based silencing mechanisms of pathogenetic importance in these subsets.

0494

SINGLE-AGENT OFATUMUMAB, A NOVEL CD20 MONOCLONAL ANTIBODY, RESULTS IN HIGH RESPONSE RATES IN PATIENTS WITH FLUDARABINE-REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) ALSO REFRACTORY TO ALEMTUZUMAB OR WITH BULKY LYMPHADENOPATHY

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Background. Patients with fludarabine-refractory CLL also refractory to alemtuzumab (FA-ref) or ineligible for alemtuzumab due to bulky (>5 cm) lymphadenopathy (BF-ref) have poor outcomes with available salvage regimens. Ofatumumab, a unique human monoclonal antibody that targets a membrane-proximal small-loop epitope on the CD20 molecule, elicits potent *in vitro* complement-dependent cytotoxicity against malignant B cells with low CD20 expression, such as CLL cells. *Aims*. We evaluated the efficacy and safety of ofatumumab in patients with FA-ref and BF-ref CLL. Here we report updated results from the prospectively planned interim analysis of an international pivotal trial. *Methods*. Patients received 8 weekly infusions of ofatumumab followed by 4 monthly infusions (Dose 1, 300 mg; Doses 2-12, 2000 mg). The primary endpoint was overall response rate (ORR; 1996 NCI-WG criteria) over a 24-week period assessed by an Independent Review Committee (IRC). Secondary endpoints included duration of response, progression-free survival (PFS), overall survival (OS) and safety. *Results*. The interim analysis included 59 FA-ref and 79 BF-ref patients; 90% of patients received ≥8 infusions. Median age was 64 and 62 years, respectively, and median (range) number of prior regimens was 5 (1-14) and 4 (1-16), respectively. The ORR (99% confidence interval) was 58% (40, 74%) in the FA-ref and 47% (32, 62%) in the BF-ref groups based on IRC evaluation; all but one were partial remissions. The median time to onset of response was 1.8 months for both groups and the duration of response was 7.1 months in the FA-ref group and 5.6 months in the BF-ref group (response was sustained for approximately 2 months following cessation of treatment). Median lymph node, spleen and liver sizes decreased during the course of treatment. Median PFS and OS were 5.7 and 13.7 months, respectively, in the FA-ref group and 5.9 and 15.4 months, respectively, in the BF-ref group. A landmark analysis at Week 12 showed a significantly longer median OS (by ≥10 months) among responders (those responding by Week 12) versus non-responders; median OS had not yet been reached for responders in either FA-ref or BF-ref groups compared with 9.8 (p=0.04) and 10.2 months (p<0.0001), respectively, for non-responders. In addition to NCI-WG responses, improvements (maintained for ≥2 months) in important clinical parameters were observed in a large proportion of patients (Table). Resolution of B-symptoms, hepatomegaly, or reductions in lymphadenopathy, occurred in about 50% of patients. Median hemoglobin and platelet counts increased during the study; neutrophils remained relatively stable, with only 5 events of grade 4 neutropenia reported among 4 patients. Treatment was well tolerated, with transient grade 1 or 2 infusion-related reactions in approximately 60% of all patients. The most common grade 3 or 4 adverse events during the treatment period were infections (FA-ref, 24%; BF-ref, 23%). Early death (≤8 weeks from treatment initiation) occurred in 4 FA-ref and 2 BF-ref patients, none of which were considered related to ofatumumab. *Conclusions*. Ofatumumab monotherapy results in high ORR, improves disease symptoms and has a favorable safety profile in heavily pretreated patients with FA-ref and BF-ref CLL.

Table. Measures of clinical improvements with a minimum duration of 2 months.

	FA-ref		BF-ref	
Improvement in clinical parameter from baseline to Week 24, %	N*	n (%)†	N*	n (%)†
Complete resolution of B-symptoms	31	15 (48)	46	29 (63)
Complete resolution of lymphadenopathy (<1 cm nodes)	55	9 (16)	74	8 (11)
≥50% reduction in lymphadenopathy	55	34 (62)	74	36 (49)
Complete resolution of splenomegaly	30	14 (47)	46	16 (35)
Complete resolution of hepatomegaly	18	9 (50)	21	11 (52)
Neutrophil count from <1.5 × 10^9/L to ≥1.5 × 10^9/L	19	1 (5)	17	5 (29)
Hemoglobin from ≤11 g/dL to >11 g/dL	26	8 (31)	42	11 (26)
Improvement in platelet count from ≤100 × 10^9/L to 50% increase or >100 × 10^9/L	29	12 (41)	44	17 (39)

*Total number of patients with abnormal baseline parameters; †Number of patients with improvement (lasting for at least 2 months) from baseline to Week 24
FA-ref=fludarabine- and alemtuzumab-refractory; BF-ref=bulky fludarabine-refractory

0495

PHASE I STUDY OF RO5072759 (GA101) IN RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. RO5072759 (GA101) is the first humanized and glyco-engineered type II monoclonal anti-CD20 antibody in clinical trials. Glycoengineering results in a significant increased antibody-dependent cytotoxicity (ADCC) compared to rituximab. Additionally, high affinity to a type II epitope on CD20 is characterized by reduced complement-dependent cytotoxicity (CDC) and strongly enhanced direct cell death compared to type I antibodies. *Aims.* In this Phase 1/2a study, RO5072759 was administered as a single agent to patients with CD20+ malignant disease for whom no therapy of higher priority was available in order to determine the safety, tolerability and pharmacokinetics of RO5072759. After reporting an encouraging activity of RO5072759 in lymphoma patients (Salles et al., ASH 2008), we present here for the first time the data for B-CLL patients. *Methods.* Patients were treated with RO5072759 by i.v. infusion (premedication: acetaminophen, antihistaminics, allopurinol/adequate hydration and no steroids) administered as a flat dose on Days 1, 8 and 22 and every 3 weeks thereafter for a total of 9 infusions. The dose was escalated based on the safety in a 3+3 design. *Results*. Thirteen B-CLL patients received RO5072759 at doses from 400 mg up to and including 2000 mg. Median age was 64 years [46-81], hemoglobin at baseline 12.6 g/dL [9.4 -14.9], WBC 51.8x10^9/L [10 -124], platelets 191x10^9/L [48-404]. The median duration of CLL was 8 years (2.8-15.7), median prior regimens 3 [1-8]. All 13 patients had received prior fludarabine therapy and 8 (62%) rituximab-containing regimen. To-date 7 of 11 evaluable patients have responded to treatment with responses (assessed by IWCLL criteria) observed with doses given at 400-2000 mg (best response): 1 CR, 6 PR and 4 SD. All patients showed an almost total and sustained reduction of CD19+ blood cell count following the 1st infusion. RO5072759 was well tolerated with no DLTs and no dose reductions. Grade 1 or 2 (CTCAE V3.0) infusion related reactions were associated with the 1st infusion with Gr 3 in 2 pts. Patients responded well to the slowing or interruption (10 pts) and steroids (11 pts) during the 1st infusion (2 during any subsequent infusion). Grade 3/4 AEs were: transient neutropenia in 9 patients (recovering spontaneously or with G-CSF), and transient thrombocytopenia (3 pts). SAEs were reported in 3 patients (febrile neutropenia, thrombocytopenia, bronchitis, tooth infection, neutropenia and tumor lysis syndrome. Infections were Grade 1-3 in 10 patients. The pharmacokinetics of RO5072759 showed a dose dependent increase in exposure with significant inter- and intra-patient variability. Time-dependent clearance was noted, which is consistent with a reduction in target-mediated antibody clearance with increasing duration of treatment. *Conclusion*. These preliminary results indicate that RO5072759 has promising activity in B-CLL patients with a 64% response rate as single agent in pretreated patients and has a similar safety profile to that observed in NHL patients treated with RO5072759 with the observation of an increased incidence of neutropenia in B-CLL.

Stem cell transplantation - Experimental and Clinical

0496

AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) VS ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-SCT) AS FIRST TRANSPLANT PROCEDURE IN PATIENTS WITH WALDESTRÖM MACROGLOBULINEMIA. RESULTS OF A RETROSPECTIVE ANALYSIS OF THE LYMPHOMA WORKING PARTY OF THE EBMT.

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The role of both autologous (ASCT) and allogeneic stem cell transplantation (allo-SCT) in the treatment of patients with Waldeström macroglobulinemia (WM) is not clearly defined at the present time. Herein, we present the results of a retrospective analysis that compares the long-term outcome in a group of 230 patients (147 males and 83 females, median age at diagnosis 48 (20-67) years, median age at transplant 52 (23-71) years) that underwent an ASCT (n = 153) or an allo-SCT (n = 77) as first transplant between 1995 and 2005 and were reported to the EBMT Database. In the allogeneic group, 63 patients (82%) were allografted from a HLA identical sibling donors and the remaining 14 (18%) from matched unrelated donors. In 34 patients, a conventional conditioning regimen was used and in 43 (56%) patients, a reduced intensity procedure. Patients treated with an allogeneic procedure did show poorer prognostic features before transplantation than those treated with an autograft; time interval between diagnosis and transplant was significantly longer [31 (5-310) months vs 20 (3-244) months, p= 0.01) and the percentage of patients receiving ≥3 treatment lines before the transplant was significantly higher (67% vs 33%, p<0.001). In addition, patients treated with an allo-SCT more frequently showed chemorefractory disease at the time of transplantation (30% vs 7%, p<0.001). Patients treated with an ASCT were significantly older [median age at transplant 53 (25-70) years vs 49 (23 - 64) years, p=0.006). With a median follow up for the surviving patients of 53 (6-130) months (allo-SCT group) and 48 (6-155) months (ASCT group) (ns), 5-yr non-relapse mortality (NRM), relapse rate (RR), progression free survival (PFS) and overall survival (OS) were of 12.7 (95%CI 9 - 18), 42.1 (95%CI 35 - 50), 45.2 (95%CI 38 - 53) and 66.7 (95%CI 60 - 74), respectively for the whole series. In the multivariate analysis, allo-SCT was significantly associated with a higher NRM [relative risk 2.7 (95%CI 1.1 - 6.6), p= 0.03] as well as a poor performance status at the time of transplant [relative risk 4.2 (95%CI 1.5 - 11), p=0.006] and the use of bone marrow [relative risk 2.8 (95%CI 1.2 - 6.6), p=0.02]. On the contrary, ASCT was significantly associated with a higher RR [relative risk 4.1 (95%CI 2.0 - 8.5), p<0.001] together with refractory disease [relative risk 2.5 (95%CI 1.2 - 5), p= 0.01], ≥3 lines of therapy before transplant [relative risk 2 (95%CI 1.2-3.2), p=0.004] and poor performance status [relative risk 4 (95%CI 1.3-12), p=0.01]. The impact of the type of transplant on both PFS and OS was non-proportional over time. ASCT was an independent adverse prognostic factors for both outcomes in the second time period (≥24 months after transplantation) [relative risk 4.2 (95%CI 1.8 - 9.8), p= 0.003 and relative risk 5.1 (95%CI 1.8-14.6), p= 0.002, respectively]. Type of transplant was not a prognostic factor for both PFS and OS in the first time period. Other adverse independent prognostic factors for PFS were refractory disease [relative risk 2.0 (95%CI 1.2 - 3.5), p=0.01], ≥3 lines of therapy [relative risk 1.8 (95%CI 1.2 - 2.7), p= 0.007] and poor performance status [relative risk 3.6 (95%CI 1.7 - 7.6), p= 0.001] and for OS, ≥3 lines of therapy [relative risk 2.2 (95%CI 1.3-3.6), p=0.004] and poor performance status [relative risk 3.4 (95%CI 1.4 - 7.8), p=0.005]. In summary, both transplants procedures are feasible in a selected population of young patients with WM. Although NRM is

higher in the allogeneic setting, the over time decreased relapse rate due to the additional immune effects favors allo-SCT in the long run.

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BORTEZOMIB INDUCTION BEFORE AUTOLOGOUS TRANSPLANTATION, FOLLOWED BY LENALIDOMIDE CONSOLIDATION-MAINTENANCE IN UNTREATED ELDERLY MULTIPLE MYELOMA PATIENTS

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Background. New agents have been introduced as induction prior to autologous stem cell transplant (ASCT) and as consolidation/maintenance thereafter to improve complete response (CR) rates. *Aims*. In this phase II study we evaluate a sequential approach with bortezomib as induction prior to autologous transplantation followed by lenalidomide as consolidation-maintenance. Primary endpoints were safety (incidence of grade 3-4 adverse events) and efficacy (response rate). *Methods*. Newly diagnosed multiple myeloma (MM) patients, aged 65-75 years, were eligible. The induction included four 21-day cycles of bortezomib (1.3 mg/m^2 days 1,4,8,11), pegylated-liposomal-doxorubicin (30 mg/m^2 day 4) and dexamethasone (40 mg: days 1-4, 8-11, 15-18, cycle 1; days 1-4, cycles 2 to 4) (PAD). Autologous transplantation was tandem Melphalan 100 mg/m^2 and stem-cell support (MEL100). Consolidation included four 28-day cycles of lenalidomide (25 mg/day days 1-21) plus prednisone (50 mg every other day) (LP), followed by maintenance with lenalidomide (10 mg/day days 1-21 every 28 days) (L) until relapse. *Results*. One-hundred and two patients have been enrolled. In a per-protocol analysis, PAD induced: 58.5% at least very good partial response (VGPR), including 12.8% complete response (CR); MEL100 autologous transplantation: 82.0% at least VGPR and 38.6% CR; LP-L consolidation-maintenance: 86.0% at least VGPR and 66.0% CR. Eleven of fifty patients showed a further improvement in response rate during LP-L consolidation-maintenance. After a median follow-up of 20.3 months, the 3-year progression-free survival was 68.8%, the 3-year time to progression (TTP) was 74.7% and the 3-year overall survival was 86.3%. By exploratory subgroup analyses, patients with high risk cytogenetic profile - including del17 or t(4;14) or t(14;16) - and patients with standard cytogenetic profile had similar TTP (HR 0.49; 95% CI, 0.13-1.81; p=0.28). The results did not change with the inclusion of patients with del13q in the high risk group (HR 0.62; 95% CI, 0.20-1.97; p=0.42). During PAD induction, grade 3-4 adverse events included thrombocytopenia (16.7%), neutropenia (9.8%), peripheral neuropathy (15.7%), and pneumonia (9.8%). During LP-L consolidation-maintenance grade 3-4 adverse events were neutropenia (16.5%), thrombocytopenia (6.3%) and cutaneous rash (3.8%). *Conclusions*. Bortezomib as induction before transplantation, followed by lenalidomide as consolidation-maintenance induced very high response rate and prolonged 3-year progression-free survival.

0498

ALLOGENEIC STEM CELL TRANSPLANTATION IN ELDERLY PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN FIRST COMPLETE REMISSION: EXPERIENCE OF THE GMALL STUDIES

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In the German Multicenter ALL studies (GMALL) patients aged over 55 years with high risk (B lineage ALL with WBC at diagnosis >30000, late CR, t (4; 11), complex aberrant karyotype or prae T or mature T ALL) or very high risk (Ph+/BCR-ABL+) ALL in first complete remission are increasingly candidates for allogeneic stem cell transplantation (SCT) with a HLA identical sibling donor (MRD) or a matched unrelated donor (MUD). Here, we report on 27 elderly patients transplanted within the GMALL studies 06/99 and 07/03. Median age of the patients was 60 years (56 - 65). 19 patients belonged to the very high risk group, VHR (Ph+ ALL) and 8 patients to the high risk group, HR (prae T ALL n=5, mature T ALL n= 1, pro B ALL n=1, prae B ALL, Ph- n=1). 9/27 patients were transplanted from a HLA identical sibling donor and 18/27 from a matched unrelated donor. Conditioning regimens for MRD SCT were myeloablative (MAC) in 6 patients (TBI 12 Gy and chemotherapy n=2, radioimmunotherapy + chemotherapy n=2, chemotherapy only n=2) and 3 patients received reduced intensity conditioning (RIC).Conditioning regimens for MUD SCT changed over time with an increasing number of RIC in the study 07/03. In total, 7/18 patients received MAC (TBI 12 Gy and chemotherapy n=5, chemotherapy only n=2) and 11/18 patients received RIC. *Results*. After allogeneic SCT 14 out of 27 patients (52%) are alive in complete remission (CCR) with a median follow up of 1052 days (24 - 2321). 13/27 patients died (48%). Causes of death were transplant related mortality (TRM) in 9/13 patients (69%) in median on d+129 (18 - 736) and relapse in 4/13 patients (31%) between d+761 and d+1071 after SCT. Risk factors for TRM: In MRD transplantation 2/9 patients (22%) died due to TRM in contrast to 7/18 patients (39%) after MUD SCT, mainly due to infection and GvHD. With standard conditioning (MAC) 7/13 patients (54%) died due to TRM in contrast to 2/14 patients (14%) with RIC. All 7 patients receiving TBI 12 Gy and chemotherapy are dead, 6/7 patients due to TRM. Therefore, conditioning with TBI 12 Gy is omitted in further studies. In Ph+ ALL patients 8/19 died due to TRM vs 1/8 patients with Ph- ALL. Risk factors for relapse: In MRD transplantation 3/9 patients (33%) died due to relapse in contrast to 1/18 patients (6%) after MUD SCT. After allo SCT and MAC 2/13 patients (15%) died due to relapse and 2/14 patients (14%) after RIC. Due to the small number of patients, no difference between MAC and RIC regarding relapse after allo SCT could be found. In Ph+ ALL patients 1/19 died due to relapse vs 3/8 patients with Ph- ALL. *Conclusions*. Our data show that allo MRD but also MUD SCT is very effective in a selected population of elderly ALL patients. Since the survival of elderly patients with chemotherapy only at 3 years is about 20%, more patients should be encouraged to have an allogeneic SCT, even from an unrelated donor.

0499

AUTOLOGOUS STEM CELL TRANSPLANTATION FOR MULTIPLE SCLEROSIS PATIENTS: A 10-YEAR EXPERIENCE OF THE RUSSIAN-AMERICAN COOPERATION

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During the last decade high dose chemotherapy (HDCT) with autologous haematopoietic stem cell transplantation (AHSCT) has been used as a therapeutic option for multiple sclerosis (MS) patients. The goal of our research was to study safety and efficacy of HDCT+AHSCT in MS patients both with progressive and remitting disease course. 152 patients with MS (secondary progressive - 59 patients, primary progressive -26, progressive-relapsing - 10 and relapsing-remitting - 57) were included in this study (mean age - 32.0, range: 17-54; male/female - 64/88). BEAM or BEAM-modified conditioning was used. Median EDSS at base-line was 5.0 (range 1.5 - 8.5). The mean follow-up duration was 21 months (range 6 - 120 months). Neurological evaluation was performed at base-line, at discharge, at 3, 6, 9, 12 months, and every 6 months thereafter; MRI examinations - at baseline, at 6, 12 months, and at the end of follow-up. Transplantation procedure was well tolerated by the patients with no transplant-related deaths. The efficacy analysis was performed in 79 patients who had had follow-up for at least 6 months. At 6 months post transplant the following distribution of patients according to clinical response was observed: 42 patients (53%) achieved an objective improvement of neurological symptoms; 37 patients (47%) had disease stabilization. At long-term follow-up clinical response was classified as improvement in 40 patients (50.6%) and stabilization in 34 patients (43.1%). One patient relapsed 9 months post-transplant. Two patients deteriorated to a worse score after 18 months of stabilization; 2 other patients progressed after 12 and 30 months of improvement, respectively. All of the patients with clinical stabilization and improvement had negative MRI scans. All the patients without disease progression were off therapy throughout the post-transplant period. In conclusion, HDCT+AHSCT resulted in clinical improvement or stabilization in the vast majority of MS patients included in the analysis. The results obtained point that HDCT+AHSCT is effective both for progressive and for remitting disease course. Further studies should be done to establish the best timing for transplantation and to validate HDCT+AHSCT regimens.

0500

INTRA-BONE MARROW (IBM) TRANSPLANT OF CORD BLOOD CELLS IS ASSOCIATED WITH BETTER ENGRAFTMENT, FASTER PLATELET RECOVERY AND LESS SEVERE AGVHD AS COMPARED WITH INTRAVENOUS ADMINISTRATION. AN EUROCORD MATCHED PAIRED ANALYSIS

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Background. Intravenous infusion (IV) is the method conventionally used to transplant hematopoietic stem cells (HSC) despite the relatively low seeding efficiency. This problem is particularly important in cord-blood transplantation (CBT). CBT potentially represents an effective treatment for haematological diseases, but only a small proportion of adult patients can undergo this procedure because the combination of low number of nucleated cells contained in a cord-blood unit with HLA disparity is associated with delay or failure of neutrophil, and especially platelet, recovery. To solve this problem, after preclinical experiments in mice, a phase II trial in 32 patients with acute leukaemia has suggested that IBM transplant might be associated with better engraftment and faster platelet recovery. *Aims*. In order to validate this initial observation, we performed a matched pair analysis by selecting in the Eurocord data base, single CB unit injected IV (IVT) (patient 129) in patients with haematological malignancies and compared with 69 patients transplanted with CB cells injected IBM. *Methods*. The matching criteria were: patient age, number of cells collected, number of HLA disparities, conditioning regimen, status of the disease at transplant and previous autologous transplantation. IBM patients (n=69) were matched to 129 IVT recipients. The median follow-up was 13 vs 12 months in IVT and IBM groups. There were no statistical differences between the 2 groups according to diagnosis (60 and 59% had acute leukaemia), age (median 35 and 38 years), number of cells collected (median 2.8×10^7/kg), HLA disparities (5/6, n=39, 4/6 n=152 and 3/6 n=7), previous autograft (18%), status of the disease at transplant (60% were in advanced phase) and conditioning regimen (RIC: 13% in the IVT group vs 16% in the IBM group). However, IBM group patients were transplanted more recent (2003 vs 2007) ($p<0.001$). *Results*. The cumulative incidence (CI) of neutrophil recovery at day 60 was 74±5% in IVT group vs 80±6% in IBM group ($p=0.12$); the median day to achieve >500/mm3 was 26 and 23 days respectively. Patients receiving CB IBM had higher CI of platelets recovery at day 180 (72±6%) compared to IVT group (50±4%; $p<0.0001$); also time to platelet recovery was quicker in IBM 35 days (16-70) compared to 51 days (16-348) in IVT group. If we consider adult patients receiving myeloablative conditioning the results are: CI of neutrophil recovery was 75±5% vs 77±6% ($p=0.32$); CI for platelets recovery 48±5% vs 76±6% ($p=0.00001$) for IVT and IBM respectively. In the overall population, cumulative incidence of acute GVHD grade III-IV at d100 was 0% compared to 11±3%, ($p<0.001$) respectively. Overall survival at one year was 39±5% compared to 50±7% ($p=0.26$) for IVT and IBM respectively. *Conclusions*. The transplantation of CB cells via IBM is associated with better engraftment and faster platelet recovery. The reduced severity of acute GVHD observed in IBM patients is intriguing and promising.

Hodgkin - Clinical

0501

FINAL RESULTS OF THE HDR2 STUDY - A EUROPEAN MULTICENTER TRIAL IN PATIENTS WITH RELAPSED HODGKIN LYMPHOMA

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Background. In patients with relapsed Hodgkin lymphoma (HL), high dose chemotherapy (HDCT) followed by autologous stem cell transplantation is being regarded as standard of care. However, the optimal regimen and intensity of chemotherapy is unclear. Therefore, the European intergroup study (HDR2) performed by the GHSG, EORTC and EBMT evaluated the impact of additional sequential high dose chemotherapy (sHDCT) after 2 cycles of DHAP and before BEAM to improve the efficacy of the standard regimen without sHDCT. *Methods*. Patients with histologically confirmed relapsed HL in first relapse with CR > 3 months or second relapse without prior HDCT were included. Responding patients after two cycles of DHAP were randomized between BEAM or sequential high dose (CTX, MTX, VP-16) followed by BEAM. Freedom-from-treatment-failure (FFTF) was the primary end point and progression-free survival (PFS) and overall survival (OS) were secondary end points. Kaplan-Meier estimates were used for the evaluation of survival and follow-up time. A prognostic score based on stage of disease, relapse type and presence of anemia before treatment (Josting *et al.*, JCO 2002) was used to predict PFS. *Results*. A total of 284 patients were included in this trial. The median follow-up time was 42 months. 240 patients were randomized after DHAP and first restaging. There were no major differences in patient characteristics between the two arms with most of the patients in late first relapse (CR > 12 months). The intensified experimental arm showed significantly longer mean treatment duration, more frequent WHO-Grade IV toxicity before BEAM and more frequent protocol violations ($p<0.05$). Although there was slightly lower mortality in the intensified arm (16% vs. 20%), there were no differences in terms of FFTF, PFS and OS. The respective 3-year-rates for the standard arm vs. the intensified arm were: FFTF: 71% vs. 67%, PFS: 72% vs. 69%, and OS: 87% vs. 83%. Patients with Ann-Arbor stage IV, early or multiple relapse and anemia had a significantly higher risk of recurrence of HL (all single bivariate $p<0.05$, combined $p<0.001$). *Conclusions*. Both regimens tested showed equally favorable results in outcome and survival. Since further intensification did not improve results, 2 cycles of DHAP followed by BEAM are the standard of care for patients with relapsed HL in our hands.

0502

EARLY CHEMOTHERAPY INTENSIFICATION WITH BEACOPP IN HIGH-RISK, INTERIM-PET POSITIVE ADVANCED-STAGE HODGKIN LYMPHOMA, IMPROVES THE OVERALL TREATMENT OUTCOME OF ABVD: A GITIL MULTICENTER CLINICAL STUDY

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Background. Early interim-PET after two courses of chemotherapy (PET-2) is the most powerful tool to predict treatment outcome in advanced-stage Hodgkin Lymphoma (asHL) treated with ABVD. The 2-year (2-y) Progression Free Survival (PFS) of PET-2 positive (PET-2+) patients is only 12%, but the optimal treatment for this patient subset is still unknown. *Aims*. for this reason a treatment strategy was designed by GITIL (Gruppo Italiano Terapie Innovative nei Linfomi) to early intensify chemotherapy with BEACOPP (4 escalated + 4 baseline cycles) for all the HL patients with a PET-2+ after 2 ABVD courses consecutively admitted to GITIL centers from January 2006 till December 2007. *Patients and methods.* 164 patients, 120 in stage IIB-IVB and 44 (26%) in stage IIA with adverse prognostic factors (more than 3 nodal sites, ESR > 50 mm, bulky lesion), were treated with two ABVD courses and re-evaluated with PET-2. Four PET-2+ patients treated with other therapies for medical decision were excluded from the analysis. Twenty-four (15%) showed a PET-2+ (Arm A) and 136 a PET-2- (Arm B), respectively. The two cohorts of patients were well matched in terms of prognostic factors: age, male sex, stage III-IV, bulky, hemoglobin, leukocyte and lymphocyte count, albumin, and B-symptoms. IPS ≥3 was equally frequent in both arms (29% and 28%, $p=0.95$). The median interval between the end of 2nd ABVD course and PET-2 was 11 days (3-29). Interim-PET scans were interpreted with a five-point, semiquantitative score with the mediastinal vessels and liver as reference organs for FDG residual uptake. PET scans were centrally reviewed: the results of the review will be presented. *Results.* After a mean follow-up of 20 months (4-37), 142 patients remain in continuous CR (CCR) and 18 experienced treatment failure for disease progression (12) or relapse (6). Of the 24 arm A patients, 15 (62%) are in CCR after BEACOPP therapy and 9 progressed; the mean duration of CR for the responding patients was 18 months (11-37). In Arm B 127 patients (93.5%) are in CCR after standard ABVD therapy and 9 progressed/relapsed. The 2-y PFS of Arm A+B, Arm A and Arm B was 87%, 56% and 93%, respectively. The positive and negative predictive value of interim-PET on 2-y PFS were 37.5% and 93.5%, respectively. The 2-y overall survival was 96%: 3 patients died for progressing lymphoma (1 in Arm A and 2 in Arm B) and 3 for treatment related toxicity. In multivariate analysis the only factor turning out significant for PFS was PET-2+ (HR 6.33, 95% CI 2.4-16.9, $p=0.0002$). *Conclusions.* These data seem to suggest that: (1) in asHL a risk-adapted therapy based upon interim-PET results after 2 ABVD courses improves the overall efficacy of standard ABVD treatment; (2) The positive Predictive Value of PET-2 drops from 90% after ABVD to 37.5% after BEACOPP intensification; (3) for PET-2 negative patients the most appropriate treatment is standard ABVD chemotherapy (4) the 2-y PFS of PET-2+, ABVD-treated HL patients can be substantially improved (from 12% to 56%) by early switching to BEACOPP chemotherapy.

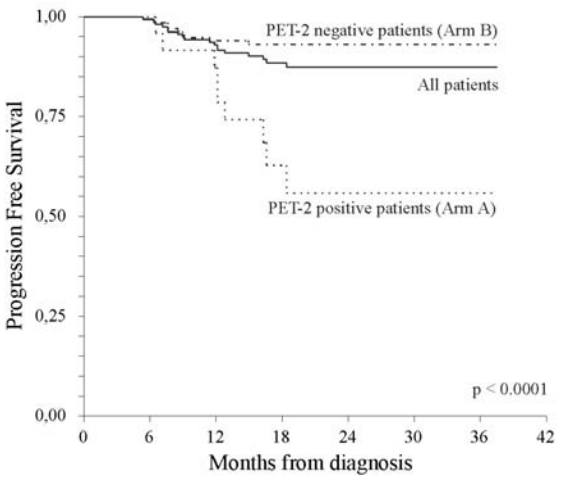

Figure 1.

0503

ROBUST ANTITUMOR ACTIVITY OF THE ANTIBODY-DRUG CONJUGATE SGN-35 WHEN ADMINISTERED EVERY 3 WEEKS TO PATIENTS WITH RELAPSED OR REFRACTORY CD30 POSITIVE HEMATOLOGIC MALIGNANCIES IN A PHASE 1 STUDY

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Background. SGN-35 is an antibody-drug conjugate (ADC) that is being developed for treatment of CD30-expressing malignancies such as Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (sALCL). SGN-35 consists of an anti-CD30 antibody conjugated to the antitubulin agent monomethyl auristatin E (MMAE). Upon binding to CD30 on the cell surface, SGN-35 is internalized and MMAE released. Subsequent binding of MMAE to tubulin disrupts the microtubule network, leading to cell cycle arrest and apoptosis. *Aims*. To characterize the safety and antitumor activity of SGN 35 in patients with relapsed or refractory CD30 positive hematologic malignancies. *Methods*. A Phase 1, multicenter, dose-escalation study was conducted in patients with relapsed or refractory CD30-positive lymphomas. Informed consent was obtained prior to initiating therapy. SGN 35 was administered every 3 weeks as a 2 hr outpatient IV infusion; dose levels between 0.1 and 3.6 mg/kg were evaluated. Patients who had stable disease or better after 2 doses were eligible to receive additional SGN-35 treatment. Investigator assessment of tumor response was done between Days 15-21 of the second cycle. CT scans were also retrospectively reviewed by an independent review facility (IRF) and the level of concordance between investigator and IRF assessment of tumor reduction was determined. *Results*. A total of 45 patients (HL: n=42, sALCL: n=2, angioimmunoblastic T cell lymphoma: n=1) were treated in this study. Median age was 36 years (range 20-87). Patients had received a median of 3 prior chemotherapy regimens (range 1-8) and 73% of patients had previously received an autologous stem cell transplant. Treatment-emergent adverse events occurring in ≥20% of patients were fatigue (42%), pyrexia (33%), peripheral neuropathy (31%), diarrhea (22%), nausea (22%), headache (20%), tachycardia (20%), and vomiting (20%); most events were Grade 1 or 2. Dose-related neutropenia was also observed. The maximum tolerated dose was identified as 1.8 mg/kg every 3 weeks. Reductions in target lesion size were seen in 86% of patients per investigator and IRF CT assessment. Of the 28 evaluable patients treated at doses of 1.2 mg/kg or higher, 26 patients (93%) had tumor reductions and 15 patients (54%) had objective responses (complete remission [CR] + partial remission [PR]); the CR rate was 36% (n=10). In addition, 2 patients, who were treated at 0.6 mg/kg, had PRs. The median progression free survival for patients treated at doses of 1.2 mg/kg or higher is 27 weeks (range 2-54+) vs. 9.7 weeks for patients treated at lower doses. *Summary and Conclusions*. SGN-35 was generally well tolerated at doses up to 1.8 mg/kg and induced durable objective responses in heavily pretreated patients with HL and sALCL. Strong concordance was observed between investigator and retrospective independent evaluation of tumor reduction. A pivotal trial of SGN-35 in Hodgkin lymphoma has been initiated.

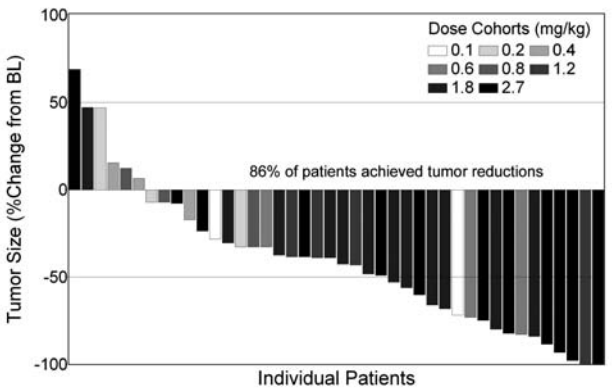

Figure 1.

0504

PET-CT ADAPTED THERAPY AFTER 3 CYCLES OF ABVD FOR ALL STAGES OF HODGKIN LYMPHOMA. RESULTS IN 118 PATIENTS

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Background. ABVD can be considered as first line treatment for Hodgkin Lymphoma (HL). Our cooperative group has an experience with 584 patients in early or advanced stage treated with 3 or 6 cycles of ABVD plus involved field radiotherapy (IFRT) with a complete remission (CR) of 91% and an event free survival and overall survival at 60 months of 79% and 95%. A negative PET-CT, either early during treatment of ABVD or after completion of it, has shown to be a powerful prognostic tool (Hutchings: Blood 2006; Gallamini: Haematologica 2006). *Aims*. Test the feasibility and efficacy of treatment to all stages of HL adjusted to PET-CT results after 3 cycles of ABVD. Evaluate the outcome of patients who have a negative PET-CT after 3 cycles of ABVD and receive no further treatment. Offer more intense therapy to patients who have persistent hypermetabolic lesions in PET-CT after 3 cycles of ABVD. *Method.* Since October 2005, 141 newly diagnosed patients with HL have been included in a prospective multicenter trial, 118 have already finished treatment. Patients with more than 50% of anatomic reduction of mases but persistent hypermetabolic lesions after 3 ABVD were considered in partial response (PR) and completed 6 cycles of ABVD and IFRT on PET-CT positive areas. Those patients with less than PR after 3 cycles received high doses of chemotherapy and an autologous stem cell transplant (ASCT). All patients were reevaluated at the end of treatment. The median age at diagnosis was 28 years. Ninety five (80%) had localized stage at diagnosis (I-II) and 23 (20%) presented with advanced stage (III-IV). Sixty (57%) patients had IPS 0-1, 38 (36%) had IPI 2-3 and 8 (7%) patients had IPI 4-5. Seventeen (14%) patients had bulky disease at diagnosis. *Results.* All patients completed treatment as planned. One hundred (85%) achieved CR with negative PET-CT after 3 cycles of ABVD. Eighteen were PET positive, two with PD who achieved CR after ESHAP and ASCT. The other 16 patients completed a total of 6 cycles of ABVD + RT in PET positive areas. Fourteen achieved CR and 2 persisted with hypermetabolic lesions. One died of progressive disease and the other one is in CR after third line treatment. Seven patients relapsed after achieving CR with ABVD x 3 and are now all in 2nd CR after salvage treatment. With a median follow up of 18 months, 107 (91%) are in first CR after 1st line treatment, 5 in CR after 2nd or 3rd line treatment, 2 relapsed after ASCT, 1 died of disease and 3 under treatment. The event free survival and overall survival at 18 months is 87% and 97% respectively. *Conclusion.* Treating patients with ABVD, evaluating response after 3 cycles with PET-CT, and adapting further therapy, leads to a higher rate of CR and reduces the number of cycles of ABVD and need for IFRT, avoiding long term toxicity. A larger number of patients and further follow-up is needed to confirm these preliminary results.

0505

PANOBINOSTAT HAS ACTIVITY IN TREATMENT-REFRACTORY HODGKIN LYMPHOMA

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Background. Panobinostat (LBH589) is a potent pan-deacetylase inhibitor (DACi) targeting epigenetic and non-epigenetic oncogenic pathways. Panobinostat is currently under clinical investigation in a variety of solid tumors and hematologic malignancies. This Phase IA/II trial evaluates oral panobinostat in patients with advanced hematologic malignancies. *Aims*. To determine the overall response rate (ORR) to panobinostat in a subset of patients with treatment-refractory Hodgkin lymphoma (HL), and to compare response assessments between the

study sites and a central reading facility. *Methods*. Patients with a range of hematologic malignancies are treated with two schedules of oral panobinostat: once-a-day on Monday/Wednesday/Friday (MWF) every week (qwk) or every other week (qowk). Each schedule is assessed in two groups of patients, which are defined by the underlying disease and the definition of dose-limiting hematologic toxicity: Group X - leukemias or high-risk myelodysplastic syndrome; Group Y - lymphoma or myeloma. PET and CT data are evaluated for best response by both the site investigators and a central reading facility. PET images are evaluated by institutional criteria for site assessment, and are classified as either progressive disease (PD; ≥25% sSUVmax ↑ or new lesions), partial response (PR; ≥25% sSUVmax, no new lesions), or stable disease (SD) by central assessment. CT scans are evaluated by Cheson criteria (1999 and 2007 for site and central assessments, respectively). *Results*. Patients in Group Y (n=49) have been treated with oral panobinostat (MWF): 20-60mg qwk or 30-60mg qowk. Safety analysis in Group Y (cut-off Oct 17, 2008) reveals that the most common Grade 3/4 AEs (≥5%) have been thrombocytopenia (71.4%), neutropenia (28.6%), fatigue (18.4%), and anemia (6.1%). The maximum tolerated dose for patients in Group Y is 40mg MWF qwk or 60mg MWF qowk. As of Nov 20, 2008, 31 patients with HL have been treated MWF, at doses ranging from 30 to 60mg qwk or 45 to 60mg qowk. Twenty-eight patients with HL are evaluable for assessment of response. Prior therapies (not available for one patient) included: surgery (19 patients), radiotherapy (24 patients), stem cell transplantation (SCT; 24 patients), and cytotoxic chemotherapy (all 27 patients). The median number of prior chemotherapeutic regimens was five (range 3-16). ORR by PET was 68% by the study sites (4% CR + 64% PR) and 64% by the central reading facility (5% CR + 59% PR). ORR by CT was 42% by the study sites (4% CR + 38% PR) and 35% by the central reading facility (35% PR). Of the 22 patients with HL who achieved CR or PR by PET or CT in either site or central reads, 15 received panobinostat MWF, qwk and 7 received panobinostat MWF, qowk. Of these 22 patients, 19 had prior SCT, and 13 had at least five chemotherapy regimens. *Summary and Conclusions*. Panobinostat shows promising clinical activity in heavily pretreated patients with HL. ORRs are similar between the study sites and the central reading facility. A global Phase II study of panobinostat at 40mg/day MWF, every week in patients with HL is currently underway.

Table 1.

Assessments		Responders (CR + PR), n (%)	Number of responders treated with the following number of chemotherapy regimens**:		
			3–4	5–6	7–16
PET	Site reads (N=25)	17* (68%)	5	8	3
	Central reads (N=22)	14* (63.6%)	3	8	2
CT	Site reads (N=26)	11 (42.3%)	4	5	2
	Central reads (N=20)	7* (35%)	2	2	2

*for one patient, prior medications are unknown; **patients were treated with a minimum of 8 to a maximum of 21 different drugs.

Myeloid Biology

0506

NIPA CHECKPOINT CONTROL IS ESSENTIAL FOR EFFICIENT ONCOGENIC TRANSFORMATION BY C-MYC

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The regulated oscillation of protein expression is an essential mechanism of cell cycle control. The SCF class of E3 ubiquitin ligases is involved in this process by targeting cell cycle regulatory proteins for degradation by the proteasome, with the F-Box subunit of the SCF specifically recruiting a given substrate to the SCF core. We previously reported the cloning of NIPA (Nuclear Interaction Partner of ALK) in complex with constitutively active oncogenic fusions of ALK, which contributes to the development of lymphomas and sarcomas. Subsequently we characterized NIPA as a F-Box protein that defines an oscillating ubiquitin E3 ligase targeting nuclear cyclin B1 in interphase thus contributing to the timing of mitotic entry. Using a conditional knockout strategy we inactivated the gene encoding NIPA. NIPA-deficient animals are viable, but sterile due to a block of spermatogenesis. Moreover, our studies demonstrate that loss of NIPA has no substantive effect on the physiological cell cycle progression of primary MEFs indicating that this cell cycle checkpoint is inactive under optimal proliferation conditions. Interestingly, NIPA checkpoint control can be unmasked by oncogenic transformation by c-Myc. Here we show that transformed focus formation assays revealed higly significant differences in c-Myc-induced transformation in NIPA-null and wild-type MEFs. c-Myc transduction caused a pronounced upregulation of cyclin-B in NIPA-null MEFs, which was completely reversible by ectopic NIPA expression. This increased cyclin-B1 expression after c-Myc transduction in the absence of NIPA has considerable functional consequences for the cells: Focus formation ability of c-Myc-infected Nipa-/- MEFs was greatly reduced in comparison to wild-type MEFs (24.6% vs. 100%). Moreover, c-Myc expression caused 12.8% apoptotic subG1 cells in wild-type MEFs, whereas Nipa-/- MEFs were more affected by c-Myc-induced apoptosis (22.45%). By contrast, transduction with other oncogenes like k-Ras in p53 knockdown Nipa-/- and Nipa+/+ MEFs showed no differences in various transformation and apoptosis assays pointing out the exclusive role of the G2/M checkpoint NIPA in c-Myc induced transformation. Furthermore, we investigated the impact of these findings for the pathogenesis of c-Myc induced tumorigenesis *in vivo*. Recipient mice transplanted with c-Myc transduced wild-type bone marrow rapidly developed an AML-like disease (median survival 33 days) characterized by bone marrow infiltration and expression of the myeloid lineage markers CD11b and Gr1. In contrast, animals transplanted with c-myc transduced NIPA knockout BM showed a substantially delayed onset of leukemia and survived significantly longer compared to the control group (median survival 52 days, $p<0.01$). Taken together, our data demonstrate that NIPA is required for efficient c-Myc transformation *in vitro* and and *in vivo* in a murine bone marrow transplantation model. Moreover, our results highlight the functional importance of NIPA in cell cycle regulation and suggest that deregulation of the protein provides a substantial contribution during the process of tumorigenesis.

0507

ABERRANT INTRACELLULAR RETENTION OF MUTATED RECEPTOR TYROSINE KINASE FLT3 IN HUMAN LEUKEMIC CELLS AS A POTENTIAL MECHANISM SUPPORTING THE PATHOGENESIS OF ACUTE MYELOID LEUKEMIA

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The exact regulation of FLT3 expression, a type III receptor tyrosine kinase (RTK), contributes to controlled proliferation and differentiation of blood cells at different stages during hematopoiesis. Activating mutations of FLT3 are found in AML, ALL and MDS and contribute to their pathogenesis, but it is unknown how mutant FLT3 deregulates hematopoiesis. It has been reported that murine mutant FLT3 signal transduction, namely with the most frequent Internal Tandem Duplication (ITD), differs from FLT3 wild type signalling. A key question for the

treatment of leukaemia is what are the mechanisms causing the aberrant constitutive signalling of FLT3-ITD which contribute to the pathogenesis of leukemia. Therefore, the aim of this study was to investigate if the intracellular trafficking of mutant FLT3 is altered compared to wild type (wt) FLT3 and if an altered localization might represent one possible mechanism for a different signalling quality leading to pathogenic effects observed in leukemia, like it has been shown for the other mutated type III RTKs PDGFR and KIT, which are aberrantly localized in human cancer. The localization of mutant FLT3-ITD as well as wild type FLT3 was investigated in retrovirally transduced COS7 cells, human leukemic primary cells and cell lines as well as in primary CD34$^+$ hematopoietic stem cells, enriched by MACS technique with an anti-CD34 antibody. Subcellular distribution of FLT3 was determined by confocal laser scanning microscopy. Internalization kinetics was studied by fluorescently labelling the FLT3 ligand (FL), time lapse imaging, and flow cytometry. The protein glycosylation status and effects on signal transduction were investigated by Western blot analysis of protein size and protein phosphorylation for FLT3 and downstream targets (ERK, STAT5, PIM1). FLT3wt localized to the plasma membrane, whereas mutant FLT3-ITD accumulated in the trans Golgi network (TGN). Consistently, FL was not internalized in FLT3-ITD expressing cells, while internalization in FLT3wt expressing cells started at around 4 min. The STAT5 pathway was FL-independently activated only by FLT3-ITD, but not by FLT3wt. The inhibition of the constitutive FLT3-ITD phosphorylation by the inhibitor PKC412 led to a shift of FLT3 accumulation out of the TGN towards the plasma membrane. At the same time the predominant high mannose form, typical for FLT3-ITD, shifted to complex glycosylation, characteristic for the FLT3wt protein. After PKC412 treatment, surface FLT3-ITD was internalised when stimulated with FL, similar to FLT3wt. Taken together, our data show that TGN accumulation of human FLT3-ITD and the lack of FL internalization might support FL-independent autonomous signalling from intracellular compartments similar to signalosomes. This TGN retention appeared to be induced predominantly by the constitutive phosphorylation of the FLT3-ITD protein instead of an aberrant protein folding or altered protein conformation. The TGN environment may provide interactions with additional/different substrate(s), altering subsequent signalling quality (as shown here for STAT5) and thereby favouring uncontrolled proliferation of undifferentiated cells. In conclusion, our data further unravel the mechanisms of the aberrant signalling of mutant FLT3-ITD and the effects of a specific inhibitor.

0508

CSF-1-INDUCED OSCILLATIONS IN PI3K/AKT ARE REQUIRED FOR CASPASE ACTIVATION IN MONOCYTES UNDERGOING DIFFERENTIATION INTO MACROPHAGES

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Background. The differentiation of human peripheral blood monocytes into resident macrophages is driven by CSF-1 (colony-stimulating factor-1) that, upon interaction with its specific receptor, CSF-1R, induces within minutes the phosphorylation of its cytoplasmic tyrosine residues and the activation of multiple signaling complexes. Caspase-8 and -3 are activated later, at day 2-3 after CSF-1 stimulation, and contribute to macrophage differentiation. The connection between activated CSF-1R and caspase activation remained unknown. *Aims*. To identify the signaling pathways connecting CSF-1R interaction with its ligand to downstream caspase activation. *Results*. Here, we demonstrate that the phosphatitidylinositol-3 kinase (PI3K) and the downstream serine/threonine kinase AKT connect CSF-1R activation to caspase-8 cleavage. Most importantly, we demonstrate that successive waves of AKT activation with increasing amplitude and duration are required to provoke the formation of the caspase-8 activating molecular platform. Inhibition of the PI3K/AKT pathway 24 hours after CSF-1/CSF-1R interaction still prevents caspase activation. Although CSF-1R is partly down-regulated, inhibition of its phosphorylation by an ATP-competitive inhibitor prevents further oscillation of the AKT pathway. The ERK1/2 pathway is activated with a coordinated oscillatory kinetics in a CSF-1R-dependent manner but plays an accessory role in caspase-8 and -3 cleavage and activation. *Conclusions*. Altogether, our results reveal that CSF-1 stimulation activates a molecular clock that involves PI3K and AKT to promote caspase activation. This oscillatory signaling pathway, which is coordinated with ERK1/2 oscillatory activation, may involve the CSF-1R and control the terminal differentiation of monocytes into macrophages.

0509

EVIDENCE FOR AN ANTI-CANCER BARRIER IN A MIXED LINEAGE LEUKEMIA MOUSE MODEL IN VIVO

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Mixed Lineage Leukemia (MLL) mutations identify a unique group of acute leukemias with distinct biological and clinical features. Although the role of MLL in leukemogenesis has been extensively studied, a precise mechanism of leukemia development is not known. We generated a switchable MLL-ENL-ERtm mouse model, in which the MLL-ENL oncogene has been introduced by homologous recombination and is controlled by the endogenous MLL promoter, thus, expressed at physiological levels. The activity of the MLL-ENL-ERtm protein is dependent on continuous provision of tamoxifen or 4-hydroxytamoxifen. The MLL-ENL-ERtm mice have developed a myeloproliferative disorder (MPD) characterized by persistent mature neutrophilia after 484,5±75,68 days of latency on a tamoxifen diet, in association with high white cell counts in peripheral blood, splenomegaly and occasionally with anemia. Blood smears showed large numbers of mature myeloid elements consisting of 40-80% neutrophils (band forms in abundance), admixed with immature myeloid elements, 3-11% monocytes and 2-6% myeloblasts. The phenotype of MPD also involved myelomonocytic proliferation with 35% immature monocytic cells in one animal and severe anemia with increased numbers of immature erythroid cells in peripheral blood in another animal. High penetrance and long latency of leukemia in our model permits the study of early leukemia development. In preleukemic animals (3-6 months after tamoxifen treatment) that represents the initial phase of MLL-ENL-driven cell transformation, bone marrow smears revealed expansion of band and segmented neutrophils indicating reactivation of self-renewal/proliferation properties in committed progenitors. We observed that a myeloid cell population mainly consisting of band neutrophil elements and showing granulocyte/ macrophage surface marker expression (CD34-CD43+Mac-1+Gr-1+CD16/32+) infiltrates the spleen at the very early stage of the disease. Infiltration of spleen correlates with increased proliferation rate measured by 5-bromo-2-deoxyuridine (BrDU) incorporation *in vivo* compared to wild-type matched control. Interestingly, the preleukemic bone marrow showed subsequent decline in BrDU incorporation associated with increased expression of p21CIP1 and p16INK4A and activation of senescence-associated ß-galactosidase activity typical for cellular senescence. Paralelly, we also detected apoptosis using the TUNEL assay in the preleukemic bone marrow. Our results suggest activation of a potential tumor suppressor mechanism in response to activated MLL-ENL - induced aberrant proliferation in early stages of cellular transformation. We are currently investigating potential tumor suppressor pathways that might provide a barrier to malignant progression in MLL.

0510

A HISTONE H3 LYSINE 9 TRIMETHYLATION SIGNATURE PREDICTS SURVIVAL IN AML PATIENTS

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Regulation of chromatin organization by histone modification is known to be crucial for fundamental regulatory processes. Its dysregulation is increasingly anticipated to play an essential role in leukemogenesis. However, little is known about histone modification patterns in acute myeloid leukemia or other cancers. Chromatin-Immunoprecipitation coupled with microarray hybridization (ChIP-Chip) was used to identify global histone modification patterns in acute leukemias and their association with survival. ChIP-Chip assays were performed for Histone H3 acetylation (H3Ac) (n=161) and lysine 9 tri-methylation (H3K9me3) (n=173) in acute myeloid and lymphoblastic leukemia, in $CD34^+$ hematopoietic progenitor cells, and in white blood cells. ChIP-Chip analyses revealed disease specific histone modification signatures in AML and ALL patients *in vivo*. Both histone modifications distinguished AML from ALL and from normal $CD34^+$ hematopoietic progenitor cells. Alterations in histone modification patterns were associated with transcription factor binding sites especially of ETS and CREB family members. A signature derived from the pattern of H3K9me3 modification predicted survival in AML patients significantly better than clinical parameters such as karyotype, age and NPM/FLT3 mutations alone. These data establish that gene-specific histone modification patterns in acute leukemias. These patterns are determined by the type of disease and the hematopoietic differentiation status. These signatures bear prognostic implications and might aid in establishing novel clinical and prognostic subgroups.

Developmental hematopoiesis, stem cells and microenvironment

0511

THE PERIPHERAL CANNABINOID RECEPTOR REGULATES HUMAN AND MOUSE HEMATOPOIESIS, BONE MARROW RECOVERY, AND HEMATOPOIETIC STEM AND PROGENITOR CELL MOBILIZATION

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Endocannabinoids, as well as exogenous cannabinoid ligands, bind to and activate the cannabinoid receptors CB1 and CB2. Both receptors are G protein-coupled seven transmembrane receptors (GPCRs), and are highly conserved during evolution. The CB1 receptor is one of the most abundant GPCRs expressed in brain, whereas the CB2 receptor is predominantly expressed in immune cells, such as B cells, natural killer cells, monocytes, neutrophils and T cells. In the hematopoietic system, GPCRs play an important role in regulating the spatial distribution of immature and mature hematopoietic cells, including their release into the circulation and their homing to hematopoietic compartments. The mobilization of hematopoietic stem and progenitor cells (HSPCs) from their main site of production in the bone marrow into circulation is a complex and incompletely understood process. While several studies have addressed the expression and function of cannabinoid receptors in mature hematopoietic cells, the effect of cannabinoids on hematopoiesis and mobilization of HSPCs has not been investigated in depth. Therefore, in this study we focus on analyzing the expression and function of cannabinoid receptors in human and murine HSPCs. We observe that both CB1 and CB2 receptors are expressed in human $CD34^+$ cells, murine side population and LSK (Lin^-, $sca1^+$, $ckit^+$) cells. Given the expression of cannabinoid receptors in immature cells, we investigate the effect of cannabinoid ligands in HSPC, and demonstrated that cannabinoids induce chemotaxis and colony formation of human $CD34^+$ cells and murine HSPC. Murine transplantation assays allowed us to study the effect of cannabinoids *in vivo*, and showed that Cb2 cannabinoid agonists induce mobilization of murine short-term and long-term HSC. In addition, we demonstrated that 20 weeks after transplantation the cannabinoid-mobilized cells contribute to the tri-lineage reconstitution (B-, T-, and myeloid cells) with similar efficiency than the G-CSF-mobilized cells. Finally, we provide evidence that Cb2 deficient mice present reduced numbers of hematopoietic stem cells, and impaired recovery following sublethal irradiation, while irradiated mice treated with Cb2 agonist show accelerated bone marrow recovery after damage. Altogether, these results demonstrate that the cannabinoid system regulates hematopoiesis and cell mobilization, and may be therapeutically applied in clinical conditions, such as bone marrow transplantation and irradiation induced bone marrow failure.

0512

THE NOVEL CDK-INHIBITOR P26-INCA1 CONTROLS THE RESPONSE OF HEMATOPOIETIC STEM CELLS TO AGING ANG TOXIC STRESS

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The hematopoietic stem cell (HSC) compartment is strictly regulated to sustain blood cell production but to avoid exhaustion. CDK inhibitors and their regulatory mechanisms are thought to play a crucial role in maintaining stem cell quiescence. A better understanding of stem cell regulatory mechanisms is essential for a wide range of therapeutic interventions in stem cell research, transplantation as well as cancer therapy. We identified p26INCA1 as a novel inhibitor of Cyclin - CDK2 complexes. Recombinant p26INCA1 inhibited Cyclin A-CDK2 complex activity in a dose dependent manner. CDK inhibition by p26INCA1 required a Cyclin interaction domain and was associated with cell cycle arrest and reduced growth of hematopoietic progenitors. Aging p26INCA1 knockout mouse harbored an increased number of HSC. Predominantly, long-

term HSC numbers were increased and Inca1-deficient bone marrow showed improved long term reconstitution properties. Exposing Inca1-/- animals to the myeloablative agent 5-FU resulted in premature death of Inca1-/-mice due to exhaustion of hematopoietic progenitors cells. Inca1-/- bone marrow cells exhibited increased proliferation upon competitive transplantation and a higher replating efficiency in colony formation assays. Further, INCA1 expression was induced by growth factor deprivation but suppressed by the mutant Flt3 tyrosine kinase. These findings demonstrate that p26INCA1 provides an additional layer of CDK-mediated stem cell regulation. Our results suggest a novel mechanism of growth factor regulated stem cell control. p26INCA1 could be an important target for HSC expansion and therapy of leukemic stem cells.

0513

STRUCTURE-FUNCTION ANALYSIS OF HUMAN HEMATOPOIETIC PBX INTERACTING PROTEIN (HPIP): A NOVEL HUMAN STEM CELL REGULATORY PROTEIN

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Human hematopoietic PBX interacting protein (HPIP) is a 731 amino acid protein, discovered as a novel interacting partner of the PBX homeodomain protein. HPIP inhibits the ability of PBX-HOX heterodimers to bind to target sequences and strongly inhibits the transactivation activity of E2A-PBX1 [t(1;19) translocation, which occurs in 25% of pediatric pre-B cell acute lymphocytic leukaemia] (Abramovich C. *et al.* JBC, 2000; Oncogene, 2002). HPIP cDNA was cloned in pMSCV-IRES-YFP cassette. Umbilical cord blood enriched with $CD34^+$ population of stem cells was obtained to perform *in vitro* and *in vivo* experiments. Mutants, with deletions of the microtubule binding region (ΔMBR-HPIP), and nuclear receptor and PBX1 interacting motif (ΔNRPID-HPIP) were generated and tested *in vitro* and *in vivo*. The constitutive expression of HPIP wt and ¢MBR-HPIP in human cord blood cells ($CD34^+$) enhanced erythroid colony formation in CFC assay (p=0.008, n=6) while the ΔNRPID-HPIP mutant nullified the effect. Both mutants of HPIP augmented significantly, the formation of primitive colonies (GEMM and GM) in methylcellulose assay ($p \leq 0.01$, n=6) as compared to YFP control and HPIP wt. In replating CFC assays ΔNRPID-HPIP showed an increased number of myeloid colonies ($p \leq 0.01$, n=6) and GM (p=ns) colonies but a decrease in granulocytic colonies ($p \leq 0.05$, n=6) compared to YFP control and HPIP wt. Long-term culture initiating cell assay (LTC-IC) demonstrated that HPIP protein enhanced the frequency of LTC-IC ($p \leq 0.1$, n=3) while mutants did not show any significant increase as compared to control YFP. HPIP wt and the mutants did not enhance the yield of LTC-IC derived CFC per million cells (p=ns, n=3). Infected cells were transplanted into NOD/SCID mice. HPIP induced, a significant increase in $CD34^+CD19^+$, $CD10^+$ and $CD117^+$ ($p \leq 0.05$ and $p \leq 0.1$) cells. Intriguingly, there was a significant increase in scid repopulating cell frequency as compared to control YFP in NOD/SCID mice.

0514

THE ROLE OF THE MADS TRANSCRIPTION FACTOR MEF2C IN REGULATING MLL-LEUKEMIC AND HEMATOPOIETIC STEM CELLS

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AML is maintained by a small population of leukemic stem cells (LSC), which share two main characteristics with hematopoietic stem cells (HSC): 1) self-renew ability, thereby creating daughter cells with the capacity to replicate almost indefinitely; and 2) differentiation capacity, by which progressively differentiating cells are generated. In addition, other common attributes have been described, including efficient engraftment, quiescence of cell cycling, cytoprotection, and surface marker expression. Fusion proteins generated from translocations involving the MLL gene on chromosome 11q23, which are associated with infant mixed-lineage leukemia (MLL) and adult AML with poor prognosis, have been shown to generate LSC from committed myeloid progenitors. MLL regulates gene transcription at the chromatin level and MLL leukemias are invariably associated with aberrant expression of selected HOX and TALE homeobox genes. More recently, the MEF2C transcription factor, encoded by a member of the (MCM1-agamous-deficiens-serum response factor) MADS family of homeotic genes, has also been implicated in the establishment of MLL-induced LSC. Taking advantage of a conditional Mef2c knock-out mouse strain, we have investigated the role of Mef2c in the MLL-induced LSC and in normal HSC. We demonstrated that Mef2c deficiency does not impair the establishment nor maintenance of LSC generated *in vitro* by MLL/ENL fusion proteins - however, its loss led to compromised homing and invasiveness of the tumor cells. Similarly, although the number of HSC, as defined by a lineage negative, Sca1+, Kit+ (LSK) phenotype, in Mef2c deficient mice was normal, competitive repopulation assays demonstrated a strikingly reduced ability of the Mef2c-/- cells to repopulate hematopoiesis in mice. This defect in the LSK compartment could be attributed to reduced homing to the bone marrow. Mef2c-dependent targets included several genes encoding matrix metalloproteinases and chemokine ligands and receptors, providing a mechanistic link to the homing defect shared by both HSC and LSC lacking Mef2c.

0515

STAT5 MEDIATES MASSIVE EXPANSION OF LEUKEMIA STEM CELLS

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Background. Leukemias are considered hierarchically organized being maintained by a leukemia stem cell (LSC). Whereas LSCs become the primary focus for targeted therapies, little is known about the pathways regulating LSC function. *Methods*. Using retroviral gene transfer of MN1, NUP98HOXD13 (ND13), or HOXA9 oncogenes and limiting-dilution transplantation before and after a 6-day culture period of leukemic cells we modelled murine leukemias with different LSC frequencies, characterized the LSC expansion potential, and validated critical signaling pathways mediating LSC expansion by loss-of-function analysis. *Results*. It was shown previously that constitutive expression of MN1 is sufficient to induce a rapidly lethal AML in mice. We found that in MN1 leukemias LSC numbers increased 68-fold over an *in vitro* culture period of 6 days, whereas in MN1 leukemias cotransduced with a second oncogene, the HOX fusion oncogene ND13 (MN1+ND13), LSC numbers increased 9000-fold as determined by the competitive repopulation unit (CRU) assay (132 fold difference comparing MN1 vs. MN1+ND13 leukemias). To screen for functional differences of the two models we screened for differential cytokine responses *in vitro*. Interestingly, MN1+ND13 expressing cells proliferated in response to GM-CSF, whereas MN1 cells or ND13 cells did not. This was confirmed as well for MN1+HOXA9 expressing cells compared to MN1+CTL or HOXA9+CTL expressing cells. We found that downstream members of the GM-CSFR pathway Stat1, Stat3, Stat5, and Erk1/2 were selectively phosphorylated upon cytokine stimulation in MN1+ND13 and MN1+HOXA9 cells compared to single-oncogene transduced cells. To characterize the role of Stat1 and Stat5b in LSC expansion, Stat1 -/- and Stat5b -/- cells were co-transduced with MN1 and HOXA9 and compared to wildtype cells *in vivo*. CRU assays with MN1+HOXA9-transduced Stat1 -/- and Stat5b -/- cells demonstrated that during a 6-day culture period *in vitro* LSC expansion was 6 and 77 fold lower, respectively, than in wildtype control mice. Thus, Stat5b is one of the key mediators of LSC expansion in MN1+HOXA9 leukemias. As MN1 and HOXA9 are upregulated in distinct subsets of normal karyotype AML we speculated that their combined overexpression may model subsets of complex karyotype AML. We performed gene set enrichment analysis on cytogenetic subsets of previously published gene expression data from 285 AML patients. 12 of 13 Stat-related pathways were enriched in complex karyotype patients compared to 4 and 8 of 13 Stat-related pathways in inv(16) and normal karyotype AML, respectively, thus supporting a critical role of Stat activation in LSCs of AML with multiple active pathways like complex karyotype AML. *Conclusions*. Here we establish the concept that LSCs have a massive but variable expansion potential depending on the number of activated oncogenes, and functionally prove that Stat5b mediates massive LSC expansion in MN1+ND13 induced leukemias. Stat5b may become an important therapeutic target in complex karyotype AML.

SIMULTANEOUS SESSION II

Acute lymphoblastic leukemia - Biology I

0516

ETV6/RUNX1 ABROGATES THE SPINDLE CHECKPOINT AND DIRECTLY TARGETS ITS KEY PLAYER MAD2L1

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ETV6/RUNX1 (E/R) is expressed in 25% of childhood acute lymphoblastic leukemia (ALL) with t(12;21) chromosomal translocation. The fusion gene encodes a protein that contains the N-terminal region of ETV6 fused to the almost entire RUNX1, retaining the runt domain (RD) required for DNA binding and heterodimerization of RUNX1. The fusion gene acts as an aberrant transcription factor and represses or disrupts the regulation of RUNX1 target genes. Recently, a near tri- or tetraploid karyotype has been strongly associated with the E/R fusion gene in B cell precursor ALL, accounting for overall 5% of cases in this genetic subgroup. Moreover, 15% of E/R positive leukemias have a somatically acquired trisomy 21. Assuming that these types of chromosomal imbalances are caused by a common mechanism, the chromosome mis-segregation during mitosis, this would imply that E/R generally interferes with the spindle assembly checkpoint (SAC), the respective surveillance mechanism. The aim of this study was to assess the function of SAC in E/R expressing model systems and leukemias and to explore the molecular mechanisms leading to the proposed attenuation of SAC. Here, we show that E/R expressing Ba/F3 and leukemic cells had significantly reduced proportions of cells arrested in mitosis (determined by DNA content analysis and phospho-histone H3 stain) in the presence of nocodazole, consistent with a weak SAC. To gain insight into the molecular mechanism of SAC attenuation, we focused on the mitotic arrest deficient 2 (Mad2) protein MAD2L1. MAD2L1 is an essential member of SAC and its deregulation has been shown to concur with aneuploidy. Furthermore, micro array data suggest a differential regulation of MAD2L1 in E/R positive leukemias. The reduction of MAD2L1 expression was confirmed at mRNA and protein levels in E/R expressing cell lines. Based on the presence of several consensus RUNX binding motifs in the promoter region we assumed that MAD2L1 is directly regulated by E/R. We thus tested the transcriptional activity of RUNX1 and E/R in luciferase-based reporter assays. Two constructs containing endogenous MAD2L1 promoter sequences with one or three perfect RUNX sites were used and revealed a consistent dose-dependent, up to two-fold reduction of RUNX1-induced activation by E/R. An E/R construct containing a point mutation in the DNA binding domain, previously reported to greatly reduce DNA binding, did not show these effects. To validate the direct interaction of E/R, the ability to bind to the endogenous promoter was assessed by chromatin immune precipitation. In fact, using myc-RUNX1 or myc-E/R stably expressing 293 cells we clearly demonstrate that both, RUNX1 and E/R, bind to the RUNX sites of MAD1L1. Together, these data provide first evidence for the direct regulation of MAD2L1 and attenuation of SAC by E/R and link the fusion gene to the mitotic checkpoint. renate.panzer@ccri.at Financial support: FWF P17551-B14 and GENAU-CHILD GZ200.136/1-VI/1/2005 to ERPG.

0517

LEUKEMIA-INITIATING CELLS ARE FREQUENT IN VERY HIGH RISK CHILDHOOD PRECURSOR B ACUTE LYMPHOBLASTIC LEUKEMIA

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Poor risk acute lymphoblastic leukemia (ALL) has been proposed to arise from a limited immature leukemia-initiating cell (LIC) compartment that may confer treatment resistance. Functional assessment of LIC frequency using syngeneic leukemia mouse models and recent data showing that different ALL subpopulations, sorted based on expression of CD19, CD34 and CD38, can reconstitute ALL in NOD/SCID mice challenge this view. We have established xenotransplantation of primary ALL cells in immunodeficient NOG mice (NOD/SCID strain with additional IL-2 receptor common gamma chain deletion), starting from carefully selected very high risk (VHR) and standard risk (SR) ALL patients classified by the ALL-BFM study group. Here we show that ALL cells from VHR and from SR patients display heterogeneous immunophenotypes, some having a large $CD34^+/CD19^+$ compartment while others have a large $CD34^-/CD19^+$ compartment. Moreover, analysis of immunophenotypes of VHR and SR-ALL cells reveals heterogenous staining patterns and does not identify subtype specific subpopulations. In addition, aldehyde dehydrogenase expression, a marker of hematopoietic stem cell and progenitor function, was also heterogeneous. Thus it appears unlikely that the LIC compartment can be identified in ALL with such markers. To functionally evaluate the number of unsorted cells required for reconstitution of VHR-ALL in immunodeficient mice, we performed orthotropic xenotransplantation of primary ALL cells using intrafemoral injection in NOG mice. One million of unsorted ALL cells generated leukemia in NOG mice in 5/5 cases, and 100 cells were sufficient for engraftment (in 3/5 cases) without conditioning, despite the xenograft barrier. The leukemia immunophenotype as well as the hepatosplenic and bone marrow involvement pattern was conserved, and secondary transplantations demonstrate conserved self-renewal properties. Based on these observations it is conceivable that most if not all ALL cells can engraft immunodeficient mice, recapitulate the whole leukemia phenotype and thus retain LIC properties.

0518

THE CALM INTERACTOR CATS REPRESSES THE TRANSACTIVATION CAPACITY OF THE LEUKEMOGENIC FUSION PROTEIN CALM/AF10 AND INTERACTS WITH KEY PROTEINS OF THE APOPTOTIC PATHWAY

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Background. CATS is 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. In T-ALL with T-cell receptor (TCR) gamma/delta rearrangement, the CALM/AF10 fusion is the most frequent rearrangement found in up to 30% of cases. CATS increases the nuclear and nucleolar localization of CALM/AF10. When fused to heterologous DNA-binding domain (GAL4-DBD), CATS functions as a repressor of transcription in reporter gene assays. *Aims*. We previously showed that CALM/AF10 is a nuclear-cytoplasmatic shuttling protein, which can be retained in the nucleus upon interaction with CATS. Therefore we investigated the potential transcriptional property of the CALM/AF10 fusion protein and whether CATS might influence this property of CALM/AF10. In addition, we analyzed CATS protein expression in resting and proliferating cells, and identified CATS interacting proteins. Methods. A GAL4-based reporter gene assay was used. Vectors expressing the GAL4-DBD-CALM/AF10 fusion protein, the CATS gene as well as a luciferase reporter gene were cotransfected into mammalian cells. We analyzed CATS expression in activated and resting lymphocytes by Western blotting. Primary human peripheral blood lymphocytes (PBLs) were stimulated for 3 days with phytohemagglutinin. For the initial identification of protein interactions we used the yeast two-hybrid system. Protein interactions identified were confirmed by coimmunoprecipitation. *Results*. GAL4-DBD-CALM/AF10 activates the reporter gene expression under the control of a GAL4-tk responsive promoter 4.2-fold compared to GAL4-DBD alone. Coexpression of CATS leads to a dose dependent up to 2.5 fold decrease of CALM/AF10-mediated transcriptional activation. Western blot analysis showed that the CATS protein is only detectable in activated PBLs, whereas no CATS protein was observed in the quiescent cells. Finally, our yeast two hybrid screen identified HAX-1 and SIVA-1 as CATS interacting partners. Both proteins are involved in the regulation of apoptosis. HAX1 has been found in anti-apoptotic signaling counteracting the pro-apoptotic effects of BAX. HAX1 was identified as a protein interacting partner of HS1 (hematopoietic lyn substrate 1), which is part of the B-cell and T-cell receptor signaling cascade. SIVA is a pro-apoptotic protein and important intracellular signaling molecule that transduces CD27-, GITR- and TCR-mediated apoptotic responses. *Summary and Conclusions*. This study demonstrates for the first time that CALM/AF10 has transcriptional activation potential. Moreover, we show that CATS antagonizes the transactivation capacity of CALM/AF10 in a dose-dependent manner. The absence of CATS in resting lymphocytes and its rapid appearance in activated lymphocytes, together with the fact that CATS interacts with HAX-1 and SIVA suggests that CATS might be

involved in regulating the apoptotic and proliferative response of lymphoid cells. Thus our results suggest a link between the leukemogenic fusion protein CALM/AF10 and T- and B-cell receptor signaling.

0519

IDENTIFICATION OF GENETIC MUTATIONS THAT COOPERATE WITH CONSTITUTIVELY ACTIVE FLT3 IN THE INDUCTION OF ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Acute lymphoblastic leukemia (ALL) is the most common form of childhood malignancy and is a heterogeneous disease with subtypes that differ markedly in their cellular and molecular characteristics, as well as their response to therapy and subsequent risk of relapse. The FMS-like receptor tyrosine kinase FLT3 contributes to normal differentiation, proliferation and apoptosis of primitive hematopoietic cells in both early myeloid and lymphoid lineages. Mutations constitutively activating the FLT3 receptor are commonly found in acute leukemias of both lineages, making FLT3 one of the most frequently mutated genes in hematological malignancies. The availability of FLT3 inhibitors provides an attractive therapeutic strategy for patients harboring these mutations. In ALL, FLT3 mutations are associated with either MLL or hyperdiploid subtypes, raising the question if specific genetic events synergize with FLT3 mutations in ALL induction and if inhibiting FLT3 activation alone will be effective in leukemia treatment. To better understand the role of FLT3 activating mutations in ALL, we have established an allograft mouse model, in which bone marrow (BM) progenitors were transduced with a retroviral vector expressing FLT3 with an internal tandem duplication (FLT3-ITD), followed by transplantation into syngenic recipients. Mice receiving murine FLT3-ITD bone marrow succumbed to aggressive leukemia with a mean latency of 45 days. Based on surface marker analysis and immunoglubulin (Ig) gene rearrangements, these tumors could be classified as pre-B-cell tumors. The disease was transplantable down to a cell number <200 and clonal (based on retroviral integration sites). Strikingly, the FLT3 inhibitor PKC412, as well inhibitors of the PI3K and MAPK pathways or dominant-negative forms of STAT5, profoundly inhibited proliferation of leukemic cells *in vitro* but did not induce apoptosis. However, anti-apoptotic stimulus provided in methylcellulose cultures was necessary to maintain leukemic cells in culture, which coincided with upregulation of surface expression of the Ig heavy chain of the preB-cell receptor (preBCR). These results suggest that the preBCR is active in these tumors but progression to the B-cell stage is blocked, either through FLT3 signaling and/or through independent lesions. Strikingly, each leukemia contained up to 6 to 8 independent retroviral integrations sites, a much higher number than would be expected given the circa 15-20% infection frequencies of BM cells before transplantation. The high number of integrations could reflect two events: 1) preferential infection of a subset of cells more permissive to infection; and/or 2) the preferential outgrowth of these infected cells due to the increased likelihood that cooperating genes were activated through the integration site. To determine if integration of the retroviral vector near putative oncogenes or tumor suppressors contributed to the induction of leukemia, retroviral integration sites were analyzed. Consistent with this hypothesis, integrations were found near genes that have been identified as 'common integration sites' (CIS) or loci that are targeted in independent tumors - an event highly unlikely to occur merely by chance. Based on the function of gene products deregulated by the retroviral integration, we predict that disruption of the preBCR signaling pathway is necessary for FLT3-induced preB-cell leukemia.

0520

PHOSPHO-PROFILING IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) REVEALS CONSTITUTIVE AND CYTOKINE INDUCED SPECIFIC SIGNATURES

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Oncogenesis and tumor progression are supported by alterations in cellular signaling. We used phospho-specific antibodies in flow cytometry to analyze specific signaling profiles of leukemia cells at a single cell level in 7 B cell precursor (BCP)- ALL leukemia cell lines and 12 primary pediatric BCP- ALL xenograft samples. Peripheral blood lymphocytes gated on CD19-positive B cells were used as normal nonmalignant controls. Cells were stimulated by different stimulants and cytokines and activation of various phosphoepitopes was analyzed and compared to the basal state of unstimulated samples. Signaling profiles of normal B-lymphocytes were compared to those of the BCP- ALL cell lines as well as to the BCP- ALL xenograft samples. In a second step signaling profiles of the primary xenograft samples were analyzed in respect to prognostic subgroups. Basal phosphorylation for all signaling molecules analyzed was significantly higher in the leukemia cell lines and leukemia xenografts than in normal B-lymphocytes. Interestingly, the BCP- ALL cell lines also had significantly higher basal phosphorylation levels than the primary BCP- ALL xenografts with the exception of Stat5, which was strongly phosphorylated in all leukemia samples. However, when comparing the amounts of phosphorylation before and after stimulation, normal B-cells displayed significantly higher profiles for most phosphoepitopes compared to the leukemia cell lines and xenografts. The leukemia cell lines and the primary xenograft samples both displayed high levels of constitutive phosphorylation in general, reducing their ability to react to a given stimulus compared to normal B-lymphocytes. With the most important exception of Stat5: we consistently found that Stat5 phosphorylation is increased in ALL cell lines and primary xenografts after stimulation with IL-7 compared to normal B-lymphocytes despite the already strong activation of this transcription factor in unstimulated samples. Stat5 is known to enhance proliferation and protect from apoptosis and has been shown to play an essential role in a variety of hematological malignancies, predominantly AML. Our data now strongly suggest that Stat5 and Stat5 dependent pathways are critically involved in leukemogenesis of pediatric BCP- ALL. Analyzing the xenografted primary patient samples according to prognostic subgroups, we identified a marked difference in p38 phosphorylation after treatment with anisomycin. The patient samples with favorable prognosis demonstrated significantly higher levels of pp38 after stimulation with anisomycin, although there were no differences in basal phosphorylation. Anisomycin is a potent inducer of apoptosis through p38-signaling and these results therefore suggest that leukemia samples of patients with good prognosis are more prone to undergo apoptosis after a given stimulus than the samples from patients with a poor prognosis, the weaker reaction suggesting a more apoptosis resistant phenotype. Since we could identify significant and specific phosphorylation signatures characteristic for BCP- ALL cells that also distinguish prognostic subgroups, this provides a strategy to define pathways important for continued survival, proliferation and resistance of leukemia and allows identification of therapeutic targets and novel biomarkers associated with clinical outcome.

Deep vein thrombosis

0521

FACTOR V LEIDEN MUTATION INCREASES THE RISK FOR VENOUS THROMBOEMBOLISM IN PATIENTS WITH MALIGNANCY

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Background. Patients with malignancy frequently develop venous thromboembolism (VTE). The most common genetic risk marker for VTE, the factor V Leiden (FVL), has not yet been prospectively evaluated in patients with malignancy. *Aims*. It was the aim to investigate in our prospective Vienna Cancer and Thrombosis Study (CATS), if carriers of FVL have an increased risk for VTE during their course of disease. *Methods*. CATS is an ongoing observational study initiated in 2003 in patients with newly diagnosed cancer or progression of disease after remission. FVL was determined by the allele specific, mutagenically separated PCR method, the investigators were blinded with regard to the thrombosis history of the patients during the study. Until 12/2008 we evaluated 954 patients (median age =62 years, 430 women, 520 men), of whom 122 had glioblastoma, 141 carcinoma of the breast, 137 of the lung, 156 of the gastrointestinal tract, 64 of the pancreas, 104 of the prostate, 140 had hematologic and 90 other malignancies. Patients were contacted in 3-4 months intervals during the study period to obtain information on new development of VTE. The study was approved by the Ethics Committee and all patients provided written informed consent. *Results*. Of these 954 consecutive patients evaluated in the study, 68 (7.1%) were carriers of FVL (66 heterozygous, 2 homozygous). This prevalence is consistent with the frequency in the Austrian population. In our cohort, 10/68 (15%) patients with and 63/886 (7%) without FVL developed objectively confirmed VTE during the observation period. Multivariable analysis showed that FVL carriers had a significantly increased risk for VTE after adjustment for age, sex, surgery, chemotherapy, and radiotherapy (hazard ratio 2.3, 95% confidence interval, 1.2-4.4, p=0.017). The cumulative probability of developing VTE after 6 months was 14% in patients with FVL and 5.7% in those without (p=0.012). *Conclusions*. We conclude that carriership of FVL is independently associated with a more than 2fold increased risk for VTE in patients with malignancy, which adds to the already high VTE risk in this group. FVL is rather easy to determine, and results are independent of clinical circumstances. Determination of FVL could help to individually assess the risk of thrombosis in cancer patients and to improve prophylactic strategies in these high risk patients.

0522

A POOLED ANALYSIS OF FOUR PIVOTAL STUDIES OF RIVAROXABAN FOR THE PREVENTION OF VENOUS THROMBOEMBOLISM AFTER ORTHOPAEDIC SURGERY: EFFECT ON SYMPTOMATIC VENOUS THROMBOEMBOLISM AND DEATH, AND BLEEDING

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Background. Rivaroxaban is an oral, direct Factor Xa inhibitor approved in the EU and several other countries for the prevention of venous thromboembolism (VTE) after total hip or knee replacement (THR/TKR). Four randomized, double-blind, phase III studies (RECORD1, 2, 3 and 4) investigated rivaroxaban for the prevention of VTE after THR or TKR. A total of 12,729 patients received either oral rivaroxaban 10 mg once daily (od), starting 6-8 hours after surgery, or subcutaneous enoxaparin 40 mg od starting the evening before surgery (RECORD1-3) or 30 mg twice daily starting 12-24 hours after wound closure or adequate haemostasis (RECORD4). In RECORD1 and 2, patients undergoing THR received rivaroxaban for 31-39 days. Enoxaparin was given for 31-39 days in RECORD1 or 10-14 days followed by placebo up to day 31-39 in RECORD2. In RECORD3 and 4, patients undergoing TKR received prophylaxis for 10-14 days. All patients were followed up for 30-35 days after the last dose of study medication. In each study, rivaroxaban regimens significantly reduced the incidence of the primary efficacy endpoint (total VTE; the composite of any deep vein thrombosis, nonfatal pulmonary embolism [PE] and death) compared with enoxaparin regimens, with similar rates of bleeding. *Aims*. Analysis of the pooled RECORD data to investigate rivaroxaban for the prevention of symptomatic VTE and death in patients undergoing THR and TKR. *Methods*. This prespecified analysis was performed on all randomized patients who received ≥1 dose of double-blind study medication to evaluate the effect of rivaroxaban on the composite of symptomatic VTE and death, and bleeding. These primary outcomes were analysed in the total treatment duration pool (planned treatment period), the total study duration pool (treatment and follow-up), both of which included the RECORD2 placebo phase, and also in the day 12±2 active treatment pool (enoxaparin-controlled period common to all studies). *Results*. Rivaroxaban significantly reduced the respective incidence of symptomatic VTE and death compared with enoxaparin regimens in the total treatment duration pool (0.57% vs 1.32%; p<0.001), the total study duration pool (0.81% vs 1.63%; p<0.001) and at day 12±2 (0.47% vs 0.97%; p=0.001). Rivaroxaban significantly reduced the composite of PE and death compared with enoxaparin in the total study duration pool (0.47% vs 0.76%, respectively; p=0.039). Respective rates for major bleeding with rivaroxaban and enoxaparin regimens were: total treatment duration pool, 0.39% vs 0.21% (p=0.076); total study duration pool, 0.44% vs 0.27% (p=0.135); day 12±2 pool, 0.34% vs 0.21% (p=0.175). Respective rates for the composite of major plus clinically relevant non-major bleeding were: total treatment duration pool, 3.19% vs 2.55% (p=0.039); total study duration pool, 3.35% vs 2.76% (p=0.064); day 12±2 pool, 2.85% vs 2.45% (p=0.186). Rivaroxaban reduced the composite outcome of death, myocardial infarction, stroke, symptomatic VTE and major bleeding in the total study duration pool compared with enoxaparin regimens. *Summary and Conclusions*. Rivaroxaban significantly reduced the incidence of symptomatic events compared with enoxaparin regimens in patients undergoing elective THR and TKR surgery. Major bleeding rates were similar for rivaroxaban and enoxaparin regimens.

0523

RESIDUAL VEIN THROMBOSIS FOR ASSESSING THE OPTIMAL DURATION OF LOW-MOLECULAR WEIGHT HEPARIN AFTER CANCER-RELATED DEEP VEIN THROMBOSIS: THE CANCER DACUS STUDY

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Background . Type and duration of anticoagulation is still matter of debate in cancer patients with acute Deep Vein Thrombosis (DVT) of the lower limbs. Residual Vein Thrombosis (RVT) has been proven to be effective for assessing the optimal duration of oral anticoagulants in non cancer patients (Siragusa S *et al.* Blood 2008:112:511-5). In the present study we evaluate the role of a RVT-based management of anticoagulation with Low-Molecular Weight Heparin in cancer patients with acute DVT. *Materials and Methods*. Cancer patients with a first episode of DVT were treated with LMWH at therapeutic dosage for 1 month followed by dose reduction of 25% in the next 5 months. At this time, they were managed according to RVT findings: those with RVT were randomized to continue anticoagulants for 6 additional months (Group A1) or to stop (Group A2), while patients without RVT stopped LMWH (Group B). Outcomes were recurrent venous thromboembolism and/or major bleeding. *Results*. Over a period of 18 months, 134 patients were evaluated across 12 centers in Italy; total duration of follow-up was 30.5 years and median duration of follow-up was 1.2 + 0.2 years. RVT was detected in 92 (68.6%) patients; recurrent events occurred in 23.4% of those who discontinued and 15.5% of those who continued LMWH (Figure 1). The adjusted Hazard Ratio (HR) for age and sex (Group A2 vs A1) was 1.58 (95% confidence interval [CI], 0.85-2.93; p=0.145). Of the 42 (31.3%) patients without RVT, one had a recurrence (2.3%) (Figure 1).

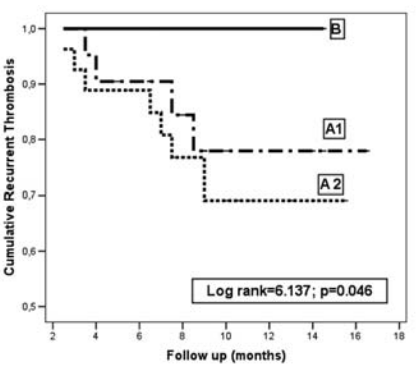

Figure 1. Kaplan-Meyer curve for recurrent events.

The adjusted HR (B vs A1) was 4.54 (CI 2.3-6.66; p=0.028). One major bleeding event occurred in each group of patients who stopped (Group A2 and B) and 2 in those who continued anticoagulation. Overall, 31 (23.1%) patients died due to cancer progression after a median follow-up of 13.2 months after randomization. *Conclusions*. The Cancer DACUS is the first study evaluating an individual marker for assessing duration of anticoagulation in active cancer population. This interim analysis shows that absence of RVT identifies a group of patients at low risk for recurrent thrombosis who can safely stop LMWH after 6 months.

0524

A PHASE III STUDY OF ENOXAPARIN VERSUS ASPIRIN VERSUS LOW-DOSE WARFARIN AS THROMBOPROPHYLAXIS FOR PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA TREATED UP-FRONT WITH THALIDOMIDE BASED-REGIMENS

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Background. The risk of venous thromboembolism (VTE) is high in newly diagnosed myeloma (MM) patients who receive thalidomide-based regimens. Anticoagulant prophylaxis is recommended. Controversies exist concerning the best thromboprophylactic regimen to be used. *Aims*. In this prospective, multicenter phase III trial we evaluated the safety and the efficacy of low-molecular weight heparin (LMWH) or low-dose aspirin (ASA) or low-fixed dose warfarin (WAR) as anticoagulant prophylaxis. *Methods*. In a GIMEMA study, newly diagnosed MM patients were randomized to VTD (Velcade 1.3 mg/m^2 d 1,4,8,11; Thalidomide 200 mg/d; Dexamethasone 320 mg/21 d) or TD (Thalidomide 200 mg/d; Dexamethasone 320 mg/21 d) or VMPT (Velcade 1.3 mg/m^2 d 1,8,15,22; Melphalan 9 mg/m^2 d 1-4; Prednisone 60 mg/m^2 d 1-4; Talidomide 50 mg/d) or VMP (Velcade 1.3 mg/m^2 d 1,8,15,22; Melphalan 9 mg/m^2 d 1-4; Prednisone 60 mg/m^2 d 1-4). In a sub-study, patients treated with VTD or TD or VMPT were randomly assigned to receive LMWH (Enoxaparin 40 mg/d) or ASA (Aspirin 100 mg/d) or WAR (Warfarin 1.25 mg/d) for the duration of the induction therapy. Patients treated with VMP did not receive any prophylaxis and were used as controls. End-points were incidence of VTE, acute cardiovascular events, sudden death, bleeding and any other serious adverse events. A total of 950 patients have been included in this study. *Results*. A total of 761 patients completed at least 3 cycles and were analyzed, 40 patients were excluded from sub-study because of indication for anticoagulant/antiplatelet therapy or high-risk of bleeding. Of the 761 evaluable patients, 193 were randomized to LMWH, 199 to ASA, 195 to WAR and 174 to the control group (VMP). Patient characteristics were similar in all groups. The incidence of VTE was 3.1% in the LMWH group, 5.5% in the ASA group and 5.6% in the WAR group (p not significant). VTEs were 2.3% in the VMP group. The rates of cardiovascular events were 0.5% in WAR, 1% in LMWH, and 1.1% in ASA group while they were 0.5% in the control group. No sudden deaths were reported. The incidence of all grades bleeding was 0.6% in the LMWH group, 0.6% in the WAR group and 1.5% in the ASA group while it was 3.7% among the controls. The distribution of the major risk factors was comparable in the 3 arms, 40% of patients had at least 2 risk factors. *Conclusions.* The overall incidence of VTE was less than 10% in all groups and was not superior to that expected during the natural course of MM. The rate of VTE was comparable among patients receiving either ASA or WAR, LMWH patients had lower risk for VTE, although no statistical difference was observed. LMWH, WAR and ASA are likely to be effective thromboprophylactic regimens. An update of these data will be presented at the meeting.

0525

SCREENING FOR OCCULT MALIGNANCY IN PATIENTS WITH IDIOPATHIC VENOUS THROMBOEMBOLISM -THE TROUSSEAU INVESTIGATORS-

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Background. Patients presenting with idiopathic venous thromboembolism (IVTE) have a ± 8% risk to develop a malignancy in the following two years. Controversy exists whether screening for occult malignancies improves prognosis. *Aims*. To investigate the influence on mortality of screening for malignancy in patients with an IVTE. *Methods*. We performed a center-controlled study in 11 hospitals in the Netherlands. In 6 centers patients underwent a standardized basic investigation only consisting of medical history, physical examination, laboratory tests and chest X-Ray (arm A). In 5 centers, an identical basic investigation was followed by thoracic and abdominal CT plus a mammography in women (armB). Outcomes were: the proportion of patients with cancer found at entry, the additional diagnostic yield of screening, the incidence of malignancies during follow up and overall mortality. *Results*.Between 2003 and 2008, 630 patients were included. The main patient characteristics were similar for both groups. In 7 of the 288 patients in arm A basic investigation resulted in the diagnosis of malignancy (2.4% 95%CI, 0.98-4.9) compared to 12 of 342 patients in arm B (3.5%, 95%CI 1.8-6.1). In arm B, 302 patients underwent CT scanning (+ mammography). In 6 patients a malignancy was found (yield 2.0%, 95%CI 0.74 to 4.3), of which 3 were stage 4. During a median follow up of 30 months, in arm A 15 new malignancies were found (5.3%, 95%CI 3.0-8.7). In arm B during a median follow up of 31 months, 12 new cancers were diagnosed (3.7%, 95%CI 1.9-6.4). In arm A 8.3% died during the study period compared to 7.6% in arm B (HR 0.87; 95% CI 0.48-1.60). *Conclusions*. The additional clinical utility of screening with spiral CT and mammography is limited after standardized basic investigation. The incidence of cancer during follow up was comparable. Extensive screening did not result in a survival benefit.

Chronic myeloid leukemia - Biology I

0526

AT THE TIME OF DIAGNOSIS, PH+ CELLS FROM BOTH CHRONIC PHASE CHRONIC MYELOID LEUKEMIA AND ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS ALREADY HARBOUR BCR-ABL KINASE DOMAIN MUTATIONS

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Background and Aims. Mutations in the Bcr-Abl kinase domain (KD) are often detected at the time of resistance to tyrosine kinase inhibitor (TKI) therapy in Philadelphia-positive (Ph+) leukemias. Mutations in rare Ph+ cells have been detected in some imatinib-naïve advanced-phase chronic myeloid leukemia (CML) patients (pts), but it is still unclear whether and with what incidence low level mutations may already be detectable at the time of diagnosis in Ph+ acute lymphoblastic leukemia (ALL) pts and in chronic-phase (CP) chronic myeloid leukemia pts. We therefore analyzed cDNA samples from 24 newly diagnosed pts with Ph+ ALL (n=13) or CP-CML (n=11) who subsequently received TKI therapy (imatinib, dasatinib or nilotinib). *Methods*. Screening for low level mutations was performed by cloning the Bcr-Abl KD (a.a. 206-524) in a bacterial vector and sequencing 200 independent clones for each pt. *Results*. All pts had evidence of aberrant KD sequences. Three to twelve different mutations were detected in each pt. Each mutation was present in two to five independent clones. A total of 115 mutations (including 41 silent, 5 nonsense, and 69 missense mutations) were observed. The vast majority (107/115, 93%) have never been reported in association with TKI resistance and are likely not to confer any advantage under TKI selective pressure. Interestingly, 103/115 (90%) mutations were transitions: G>A (n=30), A>G (n=25), C>T (n=25), T>C (n=23). Such a high prevalence of transitions (normally occurring 1.4 times more frequently than transversions) suggests that a specific mechanism generating mutations is active in Ph+ cells (activation-induced cytidine deaminase?). One of the eleven CP-CML pt received hydroxyurea for 6 months before starting imatinib therapy. In this pt, high-sensitivity mutation screening was performed again immediately before imatinib start and showed further accumulation of mutations. Eight Ph+ ALL pts and three CML pts subsequently relapsed with evidence of mutations, but only two with a mutation (T315I) that was already detectable at diagnosis. The remaining thirteen pts are in persistent remission after a follow up ranging from 12 to 52 months, although four of them were harbouring known imatinib-(H396P, D276G, E355G) or dasatinib-(F317L) resistant mutations at low levels. *Conclusions*. Our observations suggest that: a) Bcr-Abl KD mutations can probably be found at diagnosis in all CP-CML and Ph+ ALL pts; b) mutations seem to arise randomly and most of them are silent or not conferring any growth advantage under the selective pressure of TKIs; c) generation of mutations seems to be linked to Bcr-Abl-driven genetic instability; d) TKI-resistant mutations present at low levels at diagnosis do not always outgrow and lead to relapse, probably because some of them arise in cell clones with limited self-renewal capacity. This warns against high-sensitivity mutation screening of all CML and Ph+ ALL pts before the start of TKI therapy.

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0527

IDENTIFICATION OF NEW GENES SUSTAINING BCR-ABL ONCOGENIC SIGNALLING AND CML PROGRESSION THROUGH A GENETIC TOOL BASED ON HUMAN BCR-ABL TRANSGENIC DROSOPHILA MELANOGASTER (DM)

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Background. despite the role of Bcr-Abl in the pathogenesis of Chronic Myeloid Leukaemia (CML) is well established, the mechanisms responsible for CML progression are largely unknown. *Aims*. the aims of the study were to perform a genetic screening to identify new genes and pathways leading to CML progression. Methods: we developed a genetic model based on human p210 Bcr-Abl transgenic Dm. We generated a transgenic fly expressing h-p210 wt, in a tissue specific manner such as fly eyes or lymph gland, which represents the Dm haematopoietic system. A wide modifier screening was performed using 300 fly stocks carrying well characterized chromosome deletions within the whole genome. The resulting progeny was screened using eye phenotype as first read-out system. As second step, in order to clarify the role of Bcr-Abl in altering blood cells homeostasis, we drove its expression into lymph gland, in which resides the production of hemocytes, using specific drivers. Furthermore each deletion responsible for phenotype changes was analyzed either by expressing it into lymph gland as second read-out system, in order to analyzed their function into a haematopoietic background and to excluded genes involved in eye development, such as genes able to modify the eye phenotype even without being directly involved in Bcr-Abl oncogenic signalling (false positives). As final point, loss of function mutants of each gene included in the identified regions, were tested and the data obtained were validated analyzing samples from CML patients. *Results.* hBcr-Abl expression resulted into a severe eye glazed phenotype strictly dependant on p210 protein amount and induced the formation of melanotic tumors (clusters of hemocytes, clearly identifiable by their blak colors) when expressed in the lymph gland and in each hemocyte type. The analysis of eye/lymph gland-phenotypes in the progeny obtained from screening crosses, shows a first group of flies (38%) displaying a more aggressive phenotype since they lack genes encoding for Bcr-Abl negative regulators and a second group (32%) showing a mild phenotype due to the absence of genes involved in the oncogenic signalling. By now we have identified 25 new genes responsible for phenotype changes, including Fax, Disabled, Prospero, Dock and Rab5. Loss of function mutations of these genes induced a worse eye phenotype suggesting their role as enhancers of Bcr-Abl signalling. Further confirmation of their involvement in human disease comes from the analysis of their expression by Real Time PCR in 35 CML patients at diagnosis and during progression in which these genes result highly down-regulated with respect to 20 BM samples from healthy donors. Moreover, transfection of CML cell lines with gain of function constructs reduced proliferation and induced apoptosis. By contrast, loss of function of ENA, the CRKL hortologous, didn't induce phenotype changes, suggesting that ENA and presumably CRKL don't represent key regulators of Bcr-Abl signalling. Conclusions: these new genes caught with Dm system, seem to be crucial for human Bcr-Abl oncogenic signalling thus supporting the idea that Dm is a powerful genetic tool which allows the identification of new genes involved in CML development and progression.

0528

FREQUENT INACTIVATING MUTATIONS OF TET2 AND CBL ARE ASSOCIATED WITH ACQUIRED UNIPARENTAL DISOMY IN ATYPICAL CHRONIC MYELOID LEUKEMIA

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Atypical BCR-ABL negative chronic myeloid leukemia (aCML) and related myelodysplastic/myeloproliferative neoplasms (MDS/MPN) such as chronic myelomonocytic leukemia (CMML) have been associated with mutations that activate tyrosine kinase signalling; however, the molecu-

lar pathogenesis of the majority of cases remains unknown. Recent evidence has shown that acquired uniparental disomy (aUPD) is a novel mechanism by which pathogenetic mutations in cancer may be reduced to homozygosity. In this study we sought to investigate if aUPD characterizes MDS/MPN of unknown molecular etiology, and whether it could be used as a tool to help identify novel driver mutations. We performed genome wide high resolution SNP 6.0 array analysis on leukocyte DNA extracted from 121 patients with aCML (n=58) or CMML (n=63), all of whom were negative for BCR-ABL, JAK2 V617F, FIP1L1-PDGFRA and cytogenetic indicators of other tyrosine kinase fusion genes. Homozygous copy neutral SNP calls >20 Mb, considered indicative of aUPD, were seen in 32 (26%) patients. In total, 15 different chromosomes were involved with the most common recurrent abnormalities being seen at 7q (n=10), 11q (n=7), and 4q (n=6). Sequencing of candidate genes in the minimally affected regions excluded the involvement of tyrosine kinases and several other genes. Mutations in CBL, encoding a key regulator of tyrosine kinase signalling, were identified in 4 cases with 11q aUPD and analysis of 574 additional MPN and MDS/MPN revealed a total of 27 CBL variants in 26 patients with aCML (12/152; 8%), CMML (10/78; 13%), myelofibrosis (n=3/53; 6%) or hypereosinophilic syndrome (n=1/96; 1%). Most variants were missense substitutions in exons 8 or 9 (encoding the linker/RING domain) that abrogated CBL ubiquitin ligase activity and conferred a proliferative advantage to 32D cells that overexpressed FLT3. Following the identification of TET2 at 4q24 as a putative novel tumor suppressor gene of unknown function in patients with MPN and MDS (Delhommeau et al., ASH 2008), we established a sensitive high resolution melting (HRM) assay for high-throughput scanning of this large gene. Homozygous TET2 variants were found in 5/6 cases with 4q aUPD and analysis of 64 additional cases revealed variants in aCML (13/38; 34%) and CMML (13/26; 50%). In total, 38 TET2 variants were identified that were spread throughout the 6kb coding region. Of these, 21 were likely causative changes predicted to result in premature chain termination (nonsense, n=10; deletion, n=8; insertion, n=3) and 17 were missense substitutions that have not been reported as SNPs but are currently of unknown pathogenicity. We conclude that aUPD is common in atypical CML and related disorders and that inactivating mutations of TET2 and CBL are associated with aUPD at 4q and 11q, respectively.

0529

PH-NEGATIVE HEMATOPOIESIS EMERGING AFTER SUCCESSFUL TREATMENT OF CHRONIC MYELOGENOUS LEUKEMIA DYSPLAYS SEVERE AND PERSISTENT TELOMERIC LOSS AND IMPAIRED FUNCTIONAL PERFORMANCES

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Background . Most chronic myelogenous leukemia (CML) patients (pts) restore non-neoplastic hematopoiesis following treatment with tyrosine kinase (TK) inhibitors. However little is presently known on the functional and genetic integrity of Ph-negative hematopoietic cells (HC) repopulating the bone marrow after successful treatment. Indeed, the frequent detection of cytogenetic abnormalities (CA) reminiscent of those seen in myelodysplastic syndromes suggests the potential presence of functional and genetic defects. These issues have been addressed using short and long term HC cultures and telomere restriction fragment length (TRF-L) analysis, which is considered a reliable marker of proliferative and oxidative damage. *Aims and Methods*. We investigated 77 CML pts in stable complete cytogenetic remission (CR) (CR had to be documented at least one year before the analysis). 67 pts were treated with Imatinib and 10 with α-interferon associated or not to ara-C. Median age was 64 (23-88), M/F ratio was 1.5, median time from diagnosis and from complete CR were 53 (7-915), and 39 months (12-150). 33 pts had low Sokal score, 24 intermediate, and 13 high. For 7 patients it has been impossible to evaluate Sokal score. Complete and partial molecular responders were 35 and 24, respectively. 11 pts showed evidence of acquired CA in Ph-negative HC. TRF-L analysis was performed by Southern Blotting as previously described (Ladetto M et Al, Blood 2004), both on polymorphonucleates (PMN) and on monocyte-depleted PBMC (MD-PBMC) (as described by Rocci et al Exp Hematol 2007) to monitor both the myeloid and lymphoid compartment. Colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit erythroid (BFU-E) and colony forming unit-mix (CFU-Mix) along with long-term culture-initiating cells (LTC-ICs) have been so far performed on 30 patients, using bone marrow mononuclear cells as previously described (Sutherland HJ et al Blood 1994). For both TRF-L and cell culture studies a control database of 86 healthy subjects has been used for comparison. *Results*. PMN from CML patients showed a striking erosion of their telomeric DNA (Figure 1A). Also MD-PBMC showed a degree of telomere shortening although the finding was much less pronounced and not statistically significant (mean telomeric loss in PMN 1767 pb $p<0.001$; in MD-PBMC: 584 pb, $p=0.1$) We found no correlation between TRF-L and previously mentioned clinical parameters. Telomeric erosion is more severe in younger CML pts, resulting in loss of the association between TRF-L and age, typically seen in healthy subjects (Figure 1B) Telomere shortening was observed regardless of the use of TK inhibitors. When a multivariate analysis on pts and healthy controls was performed, the presence of CML resulted a stronger predictor of telomeric damage compared to age. We found no correlation between TRF-L and previously mentioned clinical and demographic parameters. Telomeric erosion show no evidence of recovery on 46 follow-up samples taken after a median time of 10 months (range 6-15). Moreover, Ph-negative HC of CML pts were functionally impaired compared to controls with reduced numbers of CFU-Mix (median 2,62 vs 4, $p=0,01$), CFU-GM (median 99,5 vs 181, $p<0,0001$) and particularly of LTC-IC (median 88 vs 198, $p<0,0001$) (Figure 1C). *Conclusions*. Ph-negative HC repopulating the bone marrow after successful CML treatment display severe telomeric DNA erosion, roughly comparable to 35 years of physiological aging. Moreover they display major defects in their functional performances. These findings underline the need of additional investigations and careful clinical monitoring of the Ph-negative haemopoietic compartment in these subjects.

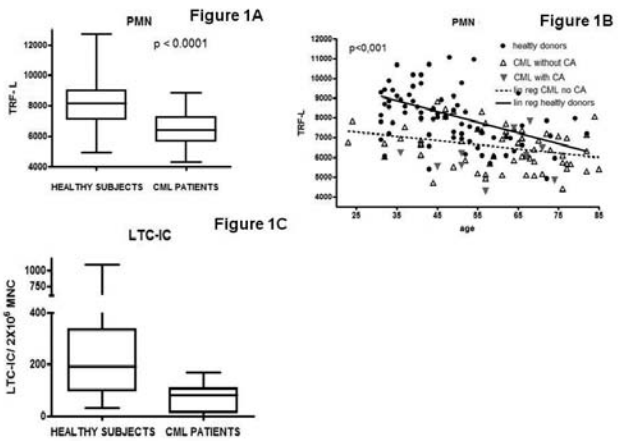

Figure 1.

0530

LONG-TERM MUTATION FOLLOW-UP OF PHILADELPHIA-CHROMOSOME POSITIVE LEUKEMIA PATIENTS TREATED WITH SECOND-GENERATION TYROSINE KINASE INHIBITORS AFTER IMATINIB FAILURE SHOWS THAT NEWLY ACQUIRED BCR-ABL KINASE DOMAIN MUTATIONS LEADING TO RELAPSE MAINLY ARISE DURING THE FIRST YEAR

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Background. Dasatinib and nilotinib are second-generation tyrosine kinase inhibitors (TKIs) active against many imatinib-resistant Bcr-Abl mutated forms. However, both dasatinib and nilotinib have been shown to retain some 'Achilles heels'. *Methods*. We have monitored Abl KD mutation status in a total of 121 pts who received dasatinib (n= 78) or nilotinib (n=43) as 2nd TKI after imatinib failure since February 2005. Results: Fifty-eight (48%) pts had chronic phase (CP) chronic myelogenous leukemia (CML), 63 pts (52%) had accelerated phase (AP) or blast crisis (BC) CML or Philadelphia-positive (Ph^+) acute lymphoblastic leukemia (ALL). Median age was 55 years (range, 18-76); median time from diagnosis was 49 months (range, 4-181); median time on imatinib was 32 months (range, 4-66). Median follow-up of all pts who received a 2nd TKI is 7 months (range, 1-38). Median follow-up of pts who are still on 2nd TKI treatment is 32 months (range, 28-38). Relapses after an initial response have so far been observed in 46/121 pts. Thirty-eight out of these 46 pts had AP/BC CML or Ph^+ ALL at the time 2nd TKI was started. Forty-one out of 121 (34%) pts have experienced relapse after an initial response during the first 12 months of 2nd TKI treatment (median time to relapse, 6,5 months; range 4-12 months), while only five of the 45 (11%) pts who were still on 2nd TKI treatment after >12 months have relapsed (at 13, 15, 18, 20, 33 months, respectively). Interestingly, none of these 5 pts had never achieved more than a minor cytogenetic response (CgR), and 4/5 pts were receiving a reduced TKI dose because of toxicity. In 36/46 (78%) cases, relapse was associated with newly acquired Abl KD mutations. In particular 26/30 (87%) pts who relapsed on dasatinib and 10/16 (63%) pts who relapsed on nilotinib had evidence of a newly acquired KD mutation presumably responsible for treatment failure. Newly acquired mutations in pts who relapsed on dasatinib as 2nd TKI were T315I (n= 12 pts) F317L (n= 8 pts) T315A (n=3 pts); V299L (n=3 pts); F317I (n=2 pts); 2 pts had multiple mutations. Newly acquired mutations in pts who relapsed on nilotinib as 2nd TKI were E255K (n=2); E255V (n=2); Y253H (n=2); T315I (n=2); F359V (n=1); F359C (n=1). *Conclusions*. a) newly acquired mutations leading to relapse in Ph+ leukemia pts receiving dasatinib or nilotinib as 2nd TKI usually arise rapidly; the likelihood of mutation selection consistently decreases over time, and seems mainly confined to advanced phase pts and to pts with no or minor CgR; b) almost all (87%) cases who developed resistance to dasatinib had newly acquired KD mutations - suggesting that the higher potency with respect to imatinib can overcome Bcr-Abl gene amplification and that Src kinase inhibition may turn off Bcr-Abl-independent resistance mechanisms; c) a lower incidence (63%) of newly acquired KD mutations was observed in pts who developed resistance to nilotinib; b) with the exception of T315I, there is little if no overlap between dasatinib and nilotinib-resistant mutants.

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Cytogenetics and molecular diagnostics

0531

IDENTIFICATION OF TET2 AS A GENE FREQUENTLY MUTATED IN MYELOID DISORDERS

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Background. Myeloid malignancies include myelodysplastic syndromes (MDS), myeloproliferative diseases (MPD) and acute myeloid leukemias (AML) that are all clonal hematopoietic stem cell disorders (HSC). The molecular mechanisms driving the MDS appearance remain poorly understood. *Aims*. We hypothesized that an early genetic event targeting the HSC could be common to all these myeloid disorders. *Methods*. TET2 coding sequence was analyzed in DNA from bone marrow samples of myeloid malignancies by PCR and sequencing. *Results*. We have previously described 3 cases of de novo (n) or secondary (s) AML and 3 MDS patients with acquired 4q24 chromosomal rearrangements. Fluorescence *in situ* hybridization (FISH) on metaphase chromosomes narrowed a common 500 kb minimal deleted region which uncovered the structure of a single gene, Ten Eleven Translocation (TET) 2. Five/6 patients harbored a deletion of a single copy of the gene while both copies were deleted in one case. We identified nucleotide changes in the sequence of the 8 coding exons and of their splice sites in 4/5 patients: 2 cases with missense mutation, one case with a C'T leading to an in frame stop codon and one with a 4 bp insertion leading to a frameshift. We confirmed that the observed changes were somatically acquired, by sequencing non-tumoral samples in 2 AML and 1 MDS. These data indicate that TET2 is a bona fide tumor suppressor gene. To establish whether mutation of TET2 could also occurs independently of a chromosomal abnormality, an additional series of 111 patients: 81 MDS, 21 sAML, 9 CMML and in 198 MPD were analyzed. Sequence abnormalities were observed in 22/111 (19.8%) MDS/sAML/CMML, and in 24/198 (12.1%), showing the high prevalence of TET2 defects in myeloid malignancies. To investigate whether the mutations could be observed in immature progenitors, sorted $CD34^+CD38^-$ and $CD34^+CD38^+$ cells from 3 MDS and 2 sAML were genotyped by PCR-restriction fragment length polymorphism (RFLP). The percentage of mutated allele in $CD34^+CD38^-$ was lower than in $CD34^+CD38^+$ cells in 3 cases, equal in one case. Sorted hematopoietic progenitors from 1 MDS were seeded at one cell per well in growth-promoting conditions. TET2 mutation was identified in 8/32 and 18/30 single cell colonies derived from $CD34^+CD38^-$ and $CD34^+CD38^+$ cells, respectively. These data demonstrate that TET2 mutations target a $CD34^+CD38^-$ cell and suggest that TET2 mutated cells have a selective advantage. *Conclusions*. We report the acquired genetic defects of TET2 as a common early event in human MDS and MDS-related AML. In MDS patients, TET2 mutations are observed in various subtypes, as previously reported for the LOH and interstitial deletions at 4q24. These events target the hematopoietic stem cell and indicate an important function for TET2 in the onset of myeloid neoplasms.

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LEUKEMIA FUSION PROTEINS ARE NOT SUFFICIENT TO INITATE THE ABERRANT METHYLATION PROFILE ASSOCIATED WITH FULL LEUKEMIC TRANSFORMATION

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Background. Genomic aberrations resulting in activation of oncogenes, inactivation of tumour suppressor genes or in the formation of novel chimeric genes are currently considered the main cause of the malignant phenotype in AML. There is now increasing evidence that in addition to genetic aberrations, therapeutically reversible epigenetic events also play a critical role in the pathogenesis of human leukemias. *Aims*. We used high-throughput methylation profiling to explore systematically the epigenomic variation underlying the biologic and clinical heterogeneity in AML. *Methods*. Using the Illumina GoldenGate Methylation Cancer Panel that spans 1,505 CpG loci, a detailed methylation profile of 116 AML patients distributed along all the cytogenetic prognostic subgroups was established. In addition, controls (BM and CB) and human progenitor cells expressing AML1/ETO, CBFB/MYH11 or MLL/AF9 fusion proteins were analysed. Unsupervised and supervised hierarchical cluster were performed, and a selection of the most significantly differentially methylated loci (delta beta (DB) of at least 0.34 and FDR <0.05) calculated as DB= (sample mean beta value) - (control mean beta value) was done. *Results*. AML samples were correctly separated from BM controls and segregated in two main categories. While one of them showed a profile (Group I) similar to the one observed in the control bone marrow samples (only 7 probes showed a mean DB>0.34), the other (Group II) presented a defined aberrant methylation signature (24 probes showed a mean DB>0.34). The distribution of the AML cases among the two methylation categories was significantly different based on their cytogenetic characterization. Eighty percent of the cases included on the adverse cytogenetic prognostic group clustered in Group I and 80% of the cases included in the good prognosis cytogenetic group clustered in Group II. In contrast, normal karyotype AML cases were evenly distributed between the two groups. No significant differences were observed for other variables such as FLT3 mutational status. Overall survival was not significantly different between AML Group I and II cases with intermediate cytogenetic prognostics, of the 60 patients with available clinical data included in these groups. Taking advantage of our model of primary human hematopoietic progenitor cells stably transduced with leukemia fusion genes[1-3] we observed that the epigenetic signature of the MLL leukemias is also observed in human progenitor cells fully transformed "*in vitro*" by the MLL-AF9 oncogene. In contrast, human cells expressing the AML1/ETO or CBF/MYH11 fusion proteins, which are immortalized upon oncogene expression but are not fully transformed by these leukemia fusion proteins, do not recapitulate the methylation signature observed in the AML primary cases. Summary and Conclusions.We conclude that a large number of epigenetically modified genes is observed in the presence of single cytogenetic abnormalities including t(8;21), t(15;17) and MLL rearrangements. However, based on our data, it appears that a full leukemic transformation is required for the acquisition of a specific aberrant methylation profile, suggesting that the presence of the recurrent fusion proteins such as AML1/ETO or CBF/MYH11 is not sufficient to initiate the aberrant methylation signature.

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REFINEMENT OF CYTOGENETIC CLASSIFICATION IN AML: DETERMINATION OF PROGNOSTIC SIGNIFICANCE OF RARE RECURRING CHROMOSOMAL ABNORMALITIES AMONGST 5635 YOUNGER ADULTS TREATED IN THE UK MRC TRIALS

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Background. Diagnostic karyotype provides a major determinant of treatment approach in younger adults with acute myeloid leukaemia (AML). There is a general consensus that presence of t(15;17)(q22;q21), t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) predicts a relatively favourable outcome and conversely that cases of AML lacking any of the aforementioned abnormalities but in which cytogenetic analysis shows inv(3)(q21q26)/t(3;3)(q21;q26), del(5q), -5, -7 or a complex karyotype generally have a very poor prognosis. However, the number of unrelated cytogenetic changes used to define a "complex karyotype" predicting a particularly adverse prognosis has been a matter of contention, differing between trial groups and ranging between 3 and 5 or more abnormalities. There has also been little consensus as to the most appropriate risk group assignment for cases with rare recurring cytogenetic abnormalities (i.e. individual incidence <2%) which together account for approximately 10% of AML and have variably been considered to predict an intermediate or adverse prognosis. In the original Medical Research Council (MRC) cytogenetic classification (Grimwade et al, Blood 1998), rarer abnormalities occurring at a frequency of ≤1% were not considered separately on statistical grounds and when combined were found to have an intermediate prognosis. *Aims and Methods*. Since it was recognized that AML cases with rare recurring cytogenetic abnormalities are likely to be heterogeneous with respect to biology and treatment response, we studied outcome of these patients, derived from 5635 cases aged 16-59 years treated in consecutive MRC AML trials (AML10, n=1238; AML12, n=2252; AML15, n=2145). *Results*. Having excluded 1462 cases with t(15;17)(q22;q21), t(8;21)(q22;q22) and inv(16)/t(16;16) and after adjusting for age, presenting WBC and type of AML (de novo/secondary), Cox regression analyses revealed no new abnormalities conferring a relatively favourable prognosis. However, the outcome of patients (n=25) with the t(3;5)(q21~25;q31~35), which is associated with the NPM1-MLF1 fusion and would have been assigned to the "adverse risk" category according to the original MRC cytogenetic classification, did not differ significantly from the normal karyotype group (5 year OS, 33% vs 43%, p=0.3). Outcome for t(11q23) patients (n=192) was significantly worse than for normal karyotype patients (p<0.00001); there was possible evidence of differences in outcomes across the t(11q23) group according to involved chromosome, with 5 year survival for the t(9;11)(p21-22;q23) comparable to that for normal karyotype, although this heterogeneity did not reach statistical significance (p=0.10). In univariate analysis, there was some evidence of poorer survival in patients (n=33) with the t(6;9)(p23;q34) as compared to those with normal karyotype (31% vs 43%, p=0.04). In multivariable analyses, various abnormalities were found to be predictive of a significantly poorer outcome (all p-values <0.0001), namely: inv(3)/t(3;3), other 3q abnormalities [excluding t(3;5)], add(5q), del(5q), -5, add(7q), -7, t(6;11), t(10;11), t(9;22), -17 and abn(17p) with other changes. Patients lacking these aberrations, but with more than 3 unrelated abnormalities also exhibited a significantly poorer prognosis (p<0.0001 adjusted for other prognostic factors). *Conclusions*. These data allow further refinement of the hierarchical MRC cytogenetic classification scheme and may facilitate development of consensus in the reporting of karyotype data allowing more reliable comparison between clinical trials involving younger adults with AML.

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CEBPA GENE MUTATIONS AFFECTING THE TAD AND BZIP REGION SHOW DIFFERERENCES IN THEIR ASSOCIATION WITH CLINICAL PARAMETERS AND PROGNOSIS: AN ANALYSIS IN 1779 ADULT PATIENTS WITH AML

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Mutations of the CEBPA gene coding for the key myeloid transcription factor CCAAT/enhancer binding protein alpha have been reported in up to 20% of patients with AML and normal karyotype. In addition CEBPA mutations appear to be associated with an improved outcome after chemotherapy. This led to the inclusion of CEBPA-mutations as provisional entity into the most recent release of the WHO classification. However, most studies published so far investigated selected patient populations or relatively small cohorts. To gain further insight and to study this abnormality in an unselected cohort, including older patients and all karyotype abnormalities, we analyzed 1779 newly diagnosed AML patients for the predominant types of CEBPA mutation, namely insertion/deletion mutations affecting the transactivation domains (TAD) or the basic-/ leucine zipper-region (bZip). Outcome was analyzed for those 1435 patients treated in the AML96 protocol of the Study Alliance Leukemia (SAL). Screening for CEBPA mutations was done on DNA isolated at the time of diagnosis using high resolution fragment analysis and direct sequencing. *Results.* Overall, 152 individual CEBPA mutations were identified in 103 of the 1779 patients (5.8%), 49 patients had combined TAD/bZIP mutations, 24 had only a TAD-mutation and 30 had only a mutation in bZip. The mutation rate was significantly higher in normal karyotype (NK) patients (84/836; 10%) compared to patients with aberrant karyotype (AK) (15/814; 1.8%; $p<0.001$). Median age of patients with CEBPA-mutations was 49 years vs. 60 years in patients with wt-CEBPA ($p<0.001$). When the different mutation types were analyzed separately, we realized that this age difference was due to the lower age of patients with bZip mutations (med. age 46 yrs.) or combined bZip/TAD (47.5 yrs.) mutations, whereas patients with only a mutation in the TAD-domain were significantly older (64 yrs.). In addition, patients with TAD-mutations differed significantly in their WBC counts, the rate of secondary AML and the frequency of additional NPM1-comutations from the other two patient groups with bZip ±TAD mutations. Overall, patients with CEBPA mutations had a significantly higher rate of complete remissions (CR CEBPA-mut: 74.7% vs. -wt: 49.4%; $p<0.001$), which translated into a significantly better overall survival (OS: CEBPA-mut: median 33.6 months vs -wt: 12.6 months; $p=0.001$). Interestingly, in patients with aberrant karyotype, CEBPA also predicted a significantly better OS (med. mutant not reached vs. 10.5 months for wt; $p=0.002$), but was not associated with improved outcome in older patients (>60 years). When analyzed separately, the OS differed significantly between patients with bZip-mutations and TAD-mutations (med.: OS: bZip: 50.2 mo vs. TAD 12.2 mo; $p=0.05$). In a multivariate analysis, CEBPA mutations represented an independent predictor of CR-rate and overall survival. *Conclusions*. Taken together, our data indicate that CEBPA mutations show a distinct association with clinical parameters and suggest a previously unrecognized association between the localization of the mutation and their effect on the leukemia, which might be due to the differential formation of the truncated p30 variant of the protein.

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A POLYMORPHIC CONSTITUTIONAL TFG-GPR128 FUSION IN HEALTHY INDIVIDUALS IDENTIFIED BY TARGETED ARRAY CGH

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Somatically acquired oncogenic fusion genes are widespread in haematological malignancies. Whilst much is known about fusions associated with cytogenetically visible abnormalities, the prevalence and nature of those arising from cryptic rearrangements remains largely unexplored. The aim of our study was to systematically search for cryptic fusion genes in patients with atypical myeloproliferative neoplasms (aMPNs), exploiting the fact that most of these abnormalities are associated with small genomic copy number changes that interrupt the target genes. To achieve this, we designed a custom comparative genomic hybridization (CGH) array using the Agilent 4x44K platform that targeted 500 candidate genes, including all tyrosine kinases, genes involved in oncogenic rearrangements and a selection of other oncogenes and haemopoietic signalling molecules. In an initial screen of 37 aMPNs, one case with hypereosinophilic syndrome (HES) showed an amplification with a breakpoint within TFG. This gene is a known target of acquired chromosomal translocations that generate fusions with ALK, NTRK1 and NOR1 in anaplastic large cell lymphoma, thyroid carcinoma and skeletal mixoid chondrosarcoma. The breakpoints were clarified by hybridization of the same sample to an Agilent 244K whole genome array, which demonstrated an amplicon of 111kb with breakpoints in intron 3 of TFG and intron 1 of the proximal gene, GPR128 (G-protein coupled receptor 128). In their normal configuration at 3q12, both genes are in identical orientations, with TFG immediately downstream of GPR128. FISH analysis showed single signals on each chromosome 3 with no evidence of episomal amplification and thus the amplification most likely results in a TFG-GPR128 fusion situated between the two normal parental genes. RT-PCR analysis confirmed the presence of an in frame chimaeric TFG-GPR128 mRNA and analysis of 575 further aMPNs revealed seven additional fusion positive cases. Unexpectedly, TFG-GPR128 mRNA remained strongly positive in a remission sample from one of these individuals. Analysis of healthy individuals revealed TFG-GPR128 at the same frequency (0.02) as that seen in MPNs, indicating that the fusion is a constitutional variant resulting from a polymorphic copy number variant (CNV) rather than an acquired somatic mutation. Analysis of POPGEN cohorts (N=1259), a German population based biobank, uncovered a further 16 positive cases with either one or two copies of TFG-GPR128, but no association with any clinical phenotype was observed. The TFG and GPR128 breakpoint regions in all cases share a region of microhomology (11/12bp identical) and flanking marker analysis indicates a single ancestral origin. Whilst it is remarkable that TFG is also the target of acquired oncogenic translocations, based on published breakpoints there is no evidence for a common mechanism of somatic and germline TFG fusion gene formation. An extensive in silico search of EST databases failed to reveal any other CNV-associated fusion transcripts, suggesting this is an uncommon event. Nevertheless, the finding of a polymorphic constitutional gene fusion adds another layer to the complexity of human genome variation.

Acute myeloid leukemia - Biology I

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RNAI-BASED IDENTIFICATION OF NOVEL SENSITIZERS TO 5-AZACYTIDINE IN MYELOID LEUKEMIAS

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Background. New therapy approaches for patients with acute myeloid leukemia (AML) are urgently needed. Aberrant methylation plays a crucial roles in leukemogenesis. Hypomethylating agents like 5-Azacytidine (5-Aza) show promise in AML. However, complimentary mechanisms, such as kinase activating events and interference with apoptosis and cell-cycle contribute to malignant transformation. Consequently, single agents activity of epigenetic therapies is limited. However, which are the targets whose inhibition augments 5-Aza's effectiveness is largely unknown. *Aims*. The aim was to identify, confirm and functionally validate novel candidate genes identified by High-Throughput RNA interference (HT-RNAi), that sensitize to epigenetic therapies in AML. Therefore, a HT-RNAi platform for transient gene silencing in acute myeloid leukemia cells was developed and a large-scale RNAi gene silencing screen (572 kinase, 500 cancer associated genes) in combination with 5-Aza was performed. *Methods*. Using lipid-based reverse transfection, cells were incubated with 2 different siRNA sequences per gene targeting 572 kinases and 500 cancer genes (i.e. anti-apoptotic, cell-cycle checkpoints etc). At 48 hrs, 5-Aza at IC15 and 30 was added and cell proliferation measured using a luminescence-based assay at 96 hrs. Data was background corrected and analyzed using the B-score method to report strength and statistical significance of growth inhibition compared to untreated controls ± siRNA. B-scores of <-2 indicate statistical significance with $p<0.05$ (>95% confidence); B-scores <-1.5 provide >87% confidence and were used as lowest cutoff given screens were focused and contain validated siRNA. 4x RNAi (4 siRNA sequences/gene) validation was performed. *Results*. Two independent RNAi screens per line in three cell lines (TF-1, ML-2, THP1) were performed with 5-Aza at the IC15 and 30 respectively. Transfection efficacy was between 65% (ML-2) to >90% (TF-1) compared to non-silencing and buffer control. Results suggest universal as well as cytogenetic and FAB subgroup specific sensitizing targets. Analysis of TF-1 and ML-2 kinome data identified 14 and 11 kinases respectively, whose silencing by 2/4 siRNA sequences at a B-score <-1.5 from both screens sensitized to 5-Aza. In ML-2 cells, 2 kinases were highly significant with a B-score for both siRNA <-2. Three kinases were common targets in both cell lines with growth inhibition for 2/4 siRNA per line of at least <-1.5, making these kinases potential universal modifiers of 5-Aza response in myeloid cells. THP-1 data will be used as independent confirmation. From the 500 cancer gene RNAi, few strong 5-Aza sensitizers emerged. Among the top hits are cyclin-dependent kinase (CDK) and anti-apoptotic BCL-2 family member proteins/genes, which have validated in secondary RNAi screens with 4x coverage. Several other high-priority candidate targets were identified and combination experiments with small molecules targeting identified genes are ongoing (presented at the meeting). *Summary and Conclusions*. Herein, we present the first large-scale RNAi screen in myeloid cells in combination with 5-Aza. Specific kinases and genes were identified that sensitize to hypomethylating agents. Functional genomics using RNAi provide a fast and attractive approach to identify molecular targets in AML and this approach holds particular promise to develop and design rational combination therapies that can be rapidly translated into the clinic.

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TWO DIFFERENT EVI1 EXPRESSING POOR-RISK AML SUBGROUPS WITH DISTINCT EPIGENETIC SIGNATURES UNCOVERED BY GENOME WIDE DNA METHYLATION PROFILING

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Background. Acute myeloid leukemia (AML) constitutes a heterogeneous group of diseases with variable clinical outcomes. The development of patient-specific therapy could potentially significantly improve therapeutic efficacy. We are interested in better understanding the biological features associated with aberrant expression of the EVI1 oncogene, which is associated with an extremely poor prognosis. EVI1 is located on chromosome 3q26. This nuclear zinc-finger transcriptional repressor interacts with HDACs, CtBPs, histone methyltransferases and MBD3. We hypothesize that abnormal EVI1 alters epigenetic programming in AML. *Aims*. We wished to elucidate whether EVI1 over-expression in human AML may act as an epigenetic regulator and is associated with specific epigenetic signatures. *Methods*. We studied genome wide DNA methylation in 295 AML patients (including 26 EVI1 AMLs) and 8 $CD34^+$ normal bone marrow controls (NBM) using the HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR), which measures with >95% accuracy the abundance of DNA methylation covering ~13,000 promoter regions. *Results*. Unsupervised analysis using hierarchical clustering segregated a cohort of 295 AMLs into 16 unique epigenetic clusters. Among these well-defined clusters we could find known subtypes of AMLs: Cluster 6 contained all inv(16) cases, cluster 1 was 100% t(15;17), cluster 4 contained only CEBPA mutant cases and cluster 8 was significantly enriched for t(8;21). The EVI1 AMLs did not cluster into any of these well-defined groups, but we rather found that 6/26 and 7/26 cases clustered in two separate clusters; the remaining cases (n=13) scattered throughout the 16 clusters. Focusing on EVI1 AMLs, hierarchical clustering (Pearson correlation distance with Ward's clustering method) readily separated the EVI1 AMLs from NBMs. Supervised analysis using a moderate t-test comparing EVI1 cases to NBM identified 303 promoter sequences differently methylated ($p<0.001$ and methylation change >1.5). Of these genes 80% were hypermethylated in EVI1 patients. Sequence analysis revealed significant over-representation of portions of the first EVI1 binding domain (TGACAAGATAAGATAA) of respectively 6 and 4 bp in the hypermethylated promoter regions. The 26 EVI1 leukemias further segregated into two distinct subgroups using hierarchical clustering: One cluster (n=14) was enriched for AML cases carrying 3q26 abnormalities (n=7). The other cluster (n=12) mainly harbored AMLs carrying 11q23 translocations (n=7) an aberration associated with EVI1 over-expression. Supervised analysis comparing these two EVI1 clusters revealed that the 3q26-enriched group featured a 122-gene signature ($p<0.001$ and methylation change >1.5) consisting entirely of hypermethylated genes. Comparing each EVI1 cluster independently to NBM controls using a moderated t-test, the 3q26-enriched group contained a hypermethylated gene signature containing 429/476 hypermethylated loci. In contrast, the MLL-enriched subgroup showed more equally distributed methylation levels, i.e. 226/384 hypermethylated genes. *Summary and Conclusions*. These data show that genome-wide methylation analysis in AML identifies novel and biologically relevant subgroups of AML. Furthermore, EVI1 over-expression is associated with specific alterations in epigenetic programming vs. normal $CD34^+$ cells. We showed that EVI1 AMLs form two epigenetically distinct AML subtypes. Specifically, the 3q26-subgroup displays a marked hypermethylation signature. This hypermethylation profile of the EVI1 3q26-subgroup AMLs suggests that these patients might benefit from treatment with DNA methyltransferase inhibitors.

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MICRORNA 29B FUNCTIONS IN ACUTE MYELOID LEUKEMIA

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Background. MicroRNAs (miRNAs) are associated with cytogenetics and molecular subtypes of acute myelogeneous leukemia (AML), but their impact on AML pathogenesis is poorly understood. *Aims*. We have previously shown that miR-29b expression is deregulated in primary AML blasts. In this work, we investigated the functional role of miR-29b in leukemogenesis. *Methods*. Using synthetic miR-29b oligonucleotides we transfected (nucleoporated) K562, Kasumi-1 cell lines (both with absent miR-29b expression) and primary AML blasts and examined cell viability, proliferation and apoptosis. A xenograft leukemia mouse model was developed to test the *in vivo* effects of miR-29b over-expression. To investigate the mechanism of miR-29b tumor suppressor function we analyzed the global transcriptome changes using Affymetrix microarrays after miR-29b over-expression in K562 cells. Microarray data was analyzed using the BRB software. Results were validated using quantitative RT-PCR, luciferase assays and Western blotting. Finally, a cohort of 45 primary AML samples was used to investigate the correlation (Spearman) between miR-29b expression and the mRNA transcriptome as detected by Affymetrix and miRNA microarrays. *Results*. Transfection of miR-29b into K562 and Kasumi-1 cells decreased cell viability, prolif-

eration and increased apoptosis by 3.4 and 2 fold respectively with respect to the controls (p=0.01 and p=0.04, t-test). Apoptosis induction by miR-29b was associated with caspase 3 and 7 activation as shown by Western Blotting. Furthermore, over-expressing miR-29b in both cell lines enhanced cytarabine induced apoptosis by 1.6 fold (p=0.007). Restoration of miR-29b in there primary AML blasts resulted in apoptosis induction and increased chemotherapy sensitivity. Next, we tested whether miR-29b could reduce tumorigenicity in a xenograft model; ~10 million viable K562 cells were inoculated subcutaneously in both flanks of immunocompromised mice. When tumors reached 50 mm3, synthetic miR-29b (left side) or scrambled oligonucleotides (right side) were injected directly into the tumors. At 10 and 14 days following the first injection, tumors injected with synthetic miR-29b (n=12) were significantly smaller than the scrambled oligonucleotide (n=12) and mock controls (n=6) (p=0.003). At day 14, the average tumor weights for control and the synthetic miR-29b inoculated mice were 0.79g and 0.089 g, respectively (p=0.001). Transcriptome analysis after ectopic transfection of synthetic miR-29b into K562 cells indicates that miR-29b target apoptosis (MCL-1), cell cycle (CDK6, CCND2) and proliferation pathways genes (IGFR, JAK2). Among the 360 genes down-regulated after miR-29b transfection, 68 genes were miR-29b predicted targets according to TargetScan. We validated the microarray results for MCL1, CDK6, CCND2 and CXXC6 using using qRT-PCR, Western blotting and luciferase assays. Last we correlated miR-29b expression with whole mRNA expression in 45 primary AML samples. A significant enrichment for apoptosis genes, including MCL-1, was found among the mRNAs inversely correlated with miR-29b expression (p=0.0007). *Conclusions*. Restoration of miR-29b in AML cell lines and primary samples induces apoptosis, increases chemotherapy sensitivity and dramatically reduces tumorigenicity in a xenograft leukemia model. Together, the data support a tumor suppressor role for miR-29b and provide a rationale for the use of synthetic miR-29b oligonucleotides as a novel strategy to improve treatment response in AML.

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ABERRANT METHYLATION OF WNT PATHWAY INHIBITORS AND ITS COOPERATION WITH OTHER GENETIC ALTERATIONS WITH DISTINCT CHARACTERISTICS IN ACUTE MYELOID LEUKEMIA

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Background and Aims. Wnt signaling pathway has been shown to play a pivotal role in stem cells self-renewal and proliferation and be crucial to the organ system development. Emerging evidences show that aberrant activation of Wnt signaling and downstream effectors is involved in several human malignancies, including acute myeloid leukemia (AML). We therefore evaluated the abnormal promoter methylation of several Wnt inhibitors in patients with AML and correlated the results with clinical and biologic features. *Methods*. We investigated the promoter hypermethylation of 8 Wnt inhibitors (Wif-1, HDPR1, sFRP1, sFRR2, sFRP4, sFRP5, DKK1, and Wnt5A) using methylation specific polymerase chain reaction (MS-PCR) in a cohort of 165 patients with newly diagnosed de novo AML at the National Taiwan University. The results were correlated with clinical features, laboratory data, immunophenotypes and other gene mutations. *Results*. Among the 165 patients, 95 were males and 70 were females. The median age was 47 years. The frequencies of hypermethylation of Wnt inhibitors at diagnosis (in descending order) were as follows: 36.9% for DKK1, 33.9% for sFRP1, 31.5% for sFRP2, 21.9% for Wif-1, 21.8% for sFRP5, 11.5% for Wnt 5A, 3.6% for sFRP4 and none for HDPR1. Taken together, 116 AML patients (70.3%) had hypermethylation of at least one (ranging from one to 7) Wnt inhibitor. Among them, 69 patients (59.5%) had 2 or more genes hypermethylation. The patients with aberrant methylation of at least one Wnt inhibitor had lower WBC count and LDH value at diagnosis (p=0.0023 and 0.0243, respectively) and tended to be older (p=0.0802) than others. The majority (75%) of patients with Wnt inhibitor hypermethylation had concurrent Class II gene mutations that affect transcription factors resulting in impaired hematopoietic differentiation. Wif-1 hypermethylation occurred more frequently in the patients with t(15;17) than in those with other chromosomal abnormalities (p=0.0459), while DKK-1 hypermethylation was preferentially demonstrated in those with t(8;21) (p=0.0087). Hypermethylation of Wif-1 and Wnt-5A is positively correlated with CEBPA mutation (p=0.0107 and 0.0239, respectively) and sFRP-2 hypermethylation was positively correlated with MLL/PTD (P=0.0334). On the other side, hypermethylation of DKK-1, sFRP-1 and sFRP-5 was negatively associated with NPM1 mutation (p=0.0015, 0.0258 and 0.0375, respectively). In term of outcome, the complete remission rate, disease-free survival (DFS) and overall survival (OS) of the patients with hypermethylation of at least one Wnt inhibitor were similar to others. However, subgroup analysis did reveal that the Wnt inhibitor hypermethylation was a poor risk factor for DFS and OS in those patients with favorable karyotype (p=0.031 and 0.05, respectively). For individual Wnt inhibitors, hypermethylation of sFRP1, sFRP4 and DKK-1 was closely associated with poor outcome in this subgroup of patients. *Conclusions*. These results provide evidence that AML patients afflicted with Wnt inhibitor hypermethylation had distinct biologic characteristics and the methylation might be associated with poor prognosis in subsets of patients. The Wnt inhibitor hypermethylation might cooperate with other genetic alterations in leukemogenesis.

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USING RNAI TO IDENTIFY NOVEL MOLECULAR VULNERABILITIES IN MYELOID LEUKEMIAS

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Background. Treatment strategies and outcomes of patients with Acute Myeloid Leukemia (AML) remain dismal. Largely, because critical targets regulating myeloid cell viability remain elusive. In order to improve treatment strategies for AML, we adapted a functional genomics approach using RNA interference (RNAi) to identify novel molecular targets that are vital to the growth of myeloid cells. Herein we report the first large-scale RNAi kinome gene-silencing screen in AML. *Aims*. A high throughput screen was developed for the efficient reverse siRNA transfection of AML cell lines in order to utilize RNAi to systematically evaluate molecular vulnerabilities in AML to advance patient care. *Methods*. Eight commercially available cationic lipid-based transfection reagents were tested for their ability to transfect myeloid cell lines with siRNA. Extensive transfection optimization experiments identified several AML lines (i.e. TF-1 and ML2) with up to 95-100 and 65-75% transfection efficiency, respectively. Three independent replicate kinome screens were performed on both cell lines using a siRNA library targeting 572 kinase genes with 2 siRNA/gene, as well as a 500 cancer siRNA gene set. At 96 hours post transfection, cell proliferation was assessed and the B-score method was used to background correct and analyze the screening data. B-scores of <-2 indicate statistical significance with p<0.05. Top hits were validated using 4x siRNA coverage (4 sequences/gene). *Results*. Several siRNA to specific kinases were identified that significantly inhibit cell proliferation of up to ~40-88%. In TF-1 the strongest hit was positive for 6/6 siRNA (both sequences) at a B-score <-2. This gene exerts essential control during the cell-cycle. Two additional genes were found to have 5/6 and 4/6 siRNA at B<-2, one of them Polo-like Kinase (PLK). In ML-2 two genes had 3/3 siRNA of a single sequence and 18 genes had 2/3 siRNA at a B-score <-2 (one of them PLK), respectively. A total of 32 hits (8%) at the least stringent criteria (2/3 same sequence siRNA, B<-2) were found in TF-1 and 18 hits (3%) in ML-2 out of 572 kinases. Two hits were overlapping between different cell lines representing potential universal myeloid growth controlling genes. Several hits were advanced into 2nd screening and the 6/6 hit validated powerfully. Confirmation of gene silencing and functional validation is currently underway for a subset of genes. Among the strongest hits are siRNA targeting PLK1, as well as siRNA targeting other kinase-genes involved in regulating cell cycle progression and checkpoints. Gene ontology (GO) analysis showed enrichment in cell cycle and cell cycle-checkpoint processes. *Summary and Conclusions*. Here, we present the successful establishment of a high-throughput reverse RNAi transfection platform in myeloid suspension cells. Excellent and specific transfection efficacy was achieved with little non-specific toxicity. Specific kinases and genes were identified, including PLK and other cell cycle genes/kinases that could be exploited therapeutically. Importantly, the RNAi approach is being used to generate a list of validated candidate targets that centrally govern myeloid cell growth and survival. These candidates represent novel molecular vulnerabilities which could serve as targets for therapeutic intervention and guide AML drug development.

Biology of B-cell disorders

0541

GENOME-WIDE ANALYSIS IDENTIFYS FREQUENT INACTIVATION OF A20 IN B-CELL TYPE MALIGNANT LYMPHOMAS

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Background. Malignant lymphomas of B-cell lineages are mature lymphoid neoplasms arising from various lymphoid tissues that consist of different pathological entities. Although the presence of balanced chromosomal translocations is among conspicuous genetic features characterizing specific histology types, unbalanced genetic changes, i.e., gains and deletions of chromosomal segments, are also common abnormalities in B-lineage lymphomas and thought to be involved in the pathogenesis of lymphoma. *Aims*. To obtain a comprehensive registry of genetic lesions in B-lineage lymphomas, and to identify the genes relevant to lymphomagenesis, we performed a SNP array analysis of 238 primary B-cell lymphoma specimens of different histologies. *Methods*. We used Affymetrix GeneChip 250K genomic microarray for 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. Three Hodgkin lymphoma (HL) derived cell line was also analyzed. Twenty four HL primary samples were also included for mutation analysis. *Results*. 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. Genetic lesions on the NF-κB pathways were common in B-cell lymphomas and found in approximately 40% of the cases, which underpinned the importance of aberrant NF-κB activation in lymphomagenesis. However, our most notable findings is that A20, a key regulator of NF-κB signaling, is a common genetic target in B-lineage lymphomas and frequently inactivated by somatic mutations and/or deletions in MALT lymphoma (21.8%) and HL of nodular sclerosis histology (33.3%), and to a lesser extent, in other B-lineage lymphomas. When re-expressed in a lymphoma-derived cell line with no functional A20 alleles, wild-type A20, but not mutant A20, resulted in suppression of cell growth and induction of apoptosis, accompanied by down-regulation of NF-κB activation. The A20-deficient cells stably generated tumors in immunodeficient mice, whereas the tumorigenicity was effectively suppressed by re-expression of A20. The cell growth and NF-κB activity that were suppressed by re-expressed A20 in the A20-deficient cells, depended at least partly on signals through cell surface receptors, including TNF receptor. *Conclusions*. We found that uncontrolled signaling of NF-κB caused by loss of A20 function is involved in the pathogenesis of subsets of B-lineage lymphomas. Considering the physiological function of A20 in the negative modulation of NF-κB activation induced upon a variety of upstream stimuli, our observations provide an intriguing insight into an association between inflammation and lymphomagenesis.

0542

MIR-17~92 UPREGULATION IN B-CELL LYMPHOMA CELLS AFTER CRYPTIC INSERTION AT BCL6

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Background. Although micro-RNA (miR) genes undergo transcriptional deregulation by genomic deletion or amplification, evidence for deregulation by chromosome translocations (which provide clues to regulatory DNA/chromatin) remains tentative. We recently described t(3;7)(q27;q21) in diffuse large B-cell lymphoma (DLBCL) whereby BCL6 is joined to a common chromosomal fragile site (FRA7H) hosting miR-29-b1 which was conspicuously downregulated (Schneider *et al.*, Leukemia 22: 1222-6, 2008) thus providing an indirect link between chromosome fragility, rearrangement and miR gene deregulation. *Aims*. We now describe a more direct link between chromosome fragility, oncogenic chromosome rearrangement and miR deregulation. *Methods*. Fluorescence *in situ* hybridization (FISH) using tyramide amplification and long distance Inverse (LDI) - PCR were used to screen and map BCL6 rearrangements in DLBCL cell lines. Reverse-transcriptase (RT) and genomic quantitative (q)-PCR, were used to quantify genomic dosage and expression. 3´-rapid amplification of c-DNA ends (RACE) was used to detect novel fusion transcripts. Stress induced duplex destabilization (SIDD) analysis which measures local GC content and DNA superhelicity was used to predict genome stability in silico. *Results*. We characterized a new BCL6 translocation t(3;12)(q27;p11) and showed it to join 5´-BCL6 to ITPR2, a gene preferentially expressed in germinal center B-cells. LDI-PCR unexpectedly revealed junction of 5´-BCL6 to chromosome 13 sequences inside the miR-17~92 host gene MIRH1 (alias C13orf25). FISH using MIRH1 clones confirmed cryptic BCL6-MIRH1 rearrangement. Despite chromosomal involvement with BCL6, 3´-MIRH1 primers RT-qPCR revealed only weak MIRH1 expression. Repeating the assay with primers covering the embedded miR-17~92 cluster region showed 5x upregulation of pri-miR and 13-18x upregulation of mature miRs. Interestingly, 3'-RACE revealed a novel MIRH1 transcript which was truncated by 3.1 kbp. Genomic qPCR and FISH both excluded miR-17~92 genomic copy number amplification. LDI-PCR showed multiple DNA cuts at 3q27, 12p11, and 13q31 - the last including a complex excision/inversion/insertion. Thus we identified a novel BCL6 fusion transcript (ITPR2-5´BCL6) from which MIRH1 sequences predicted from LDI-PCR data had been spliced out. Treatment with histone deacetylase inhibitor (trichostatin) further upregulated expression of 5´-BCL6 fusion mRNA (15x), MIRH1 (4x), and individual miR-17~92 cluster genes (2-3x), while control cell lines showed downregulation. SIDD analysis which predicts DNA fragility revealed that 6/7 breaks in the t(3;13)(q27;q31)t(13;12)(q31;p11) precisely coincide with fragility peaks. *Summary and Conclusions*. Our data document massive miR-17~92 upregulation in DLBCL cells due to chromosome rearrangement at regions of genomic fragility. Since SIDD fragility peaks host promoter regions, replication origins in yeast, and (regulatory) scaffold/matrix-attachment regions in mammalian cells, we propose that miR deregulation may involve de novo generation of regulatory DNA sequences and acetylation-responsive chromatin by chromosome rearrangement. Taken together, these data also suggest a possible role for BCL6 translocations in the deregulation of miR genes near sites of chromosome or DNA instability. Future studies will address the putative regulatory roles of superhelical DNA regions and chromatin proteins in mediating miR gene dysregulation.

0543

EBV MICRORNA EXPRESSION IN AN IN-VITRO MODEL OF B-CELL DIFFERENTIATION AND LYMPHOMAGENESIS

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Background. Lymphomagenesis is a complex process, which in part reflects the nature of the transforming event, as well as the stage of differentiation of the cell. The majority of B-cell lymphomas are derived from germinal centre (GC), or post-GC B-cells. The Epstein Barr virus (EBV) is implicated in a range of GC and post-GC derived B-cell lymphomas including Post-Transplant Lymphoproliferative Disorders and a subset of Diffuse Large B cell Lymphomas. EBV infects and activates naïve B-cells through a process paralleling the GC reaction. Interruption of this process by a transforming event may result in a clonal proliferation where differentiation of the cell is blocked at this stage. Human models of B-cell differentiation are lacking, however the use of EBV infection of isolated human naïve B-cell may provide an in-vitro model of this process. MicroRNAs (miRNAs) are small non-coding RNAs, which act as negative regulators of gene expression. miRNA expression has been demonstrated to be associated with the developmental lineage and differentiation state of several human cancers. EBV expresses at least 39 miRNAs from two clusters within the BART and BHRF1 regions of the viral genome. EBV miRNAs are differentially expressed in tumour cell lines, suggesting distinct roles during EBV-driven B-cell differentiation and lymphogenesis. We propose that EBV miRNAs may play a role in EBV-driven B-cell differentiation and lymphomagenesis. *Aims*. To determine if EBV miRNAs may be associated with the EBV-driven differentiation of naïve B-cells. *Methods*. Using high efficiency EBV infection of isolated naïve B-cells the expression levels of EBV miRNAs, along with host markers of B-cell differentiation were determined by real time RT-

PCR. The expression profile of the EBV miRNAs was then correlated with the differentiation state of the cells as determined by the expression of host marker genes. *Results*. Alterations in the expression of genes associated with the differentiation of the naïve B-cell were observed within 24 hours of infection. Levels of BCL6 were rapidly down regulated within 24 hours indicating activation of the naïve B-cell. Levels of the memory cell marker CD27 steadily increased over 24 to 96 hours, while BLIMP1 expression increased, peaking at 48 hours. Finally an increase in AID expression over 8 to 48 hours was suggestive of somatic hypermutation. These observations are indicative of B-cell differentiation taking place within four days of infection in a process exhibiting many similarities to the GC reaction. Examination of 28 EBV miRNAs revealed the majority to exhibit distinct expression profiles within 72 hours of infection. Interestingly one group (BARTs 20-3p, 4 20-5p and 1-5p) was rapidly expressed within 8 hours of infection and then down regulated after 24 to 48 hours. The only EBV miRNA not expressed during the time course examined was BART11-3p. *Conclusions*. The finding of distinct EBV miRNA expression kinetics, coincidental with several gene expression changes indicative of B-cell differentiation, suggests the possibility these regulatory molecules may be involved in this process. Current work in our lab involves the elucidation of EBV miRNA cellular targets that may play a role in EBV driven B-cell differentiation.

0544

HIGH EARLY MORTALITY AND POOR OUTCOMES FOR PATIENTS WITH AL AMYLOIDOSIS PRESENTING WITH HIGH SERUM FREE LIGHT CHAINS - A NEW RISK STRATIFICATION MODEL

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Background. The prognosis of patients with AL amyloidosis has significantly improved over the last decade with a median survival of nearly 5 years. However, a substantial proportion of patients remain at risk of early death and there is a need to identify such patients at diagnosis to allow for appropriate risk stratified treatment. Markers of organ function, ECOG performance status and cardiac biomarkers like NT-ProBNP have all been described for risk stratification. We report for significantly poorer outcomes and a very high risk of early death for patients with high presenting free light chain from a large cohort of 644 unselected of patients with newly diagnosed AL amyloidosis and should be added to the factors for AL risk stratification. *Methods*. All patients with systemic AL amyloidosis attending the UK National Amyloidosis Centre with complete data sets for FLC and conventional serum and urine monoclonal protein prior to any treatment between Jan 2001 and March 2008 were identified from the database and 644 such cases were identified. *Results*. Median age was 64 years (range 26-88), male: female ratio was 1.3:1. Median serum creatinine was 149 µmol/L (37-1079), and 24 hour proteinuria 3.9g (<0.1- 20g) with renal involvement being the commonest followed by cardiac. The FLC ratio was abnormal in 507 (79%) patients, with a lambda bias in 370 (57%) cases and a kappa bias in 137 (21%); concentration of the abnormal class of FLC exceeded 100mg/L in 308 (83%) cases for lambda and in 117 (85%) with a kappa, and median values were 256mg/L and 379mg/L respectively. In serum, a monoclonal was identified in 446 (69%) patients [median 6g/L and in 336 (52%)]. The median overall survival (OS) of the cohort was 27 months with no significant difference in the OS for patients with either kappa or lambda as the abnormal component. Patients with an abnormal FLC ratio were stratified by the absolute FLC value in three best fit cohorts according to the presenting abnormal component (aFLC). The median survival of patients with presenting aFLC <150mg/L was 63 months, with aFLC of 151-500mg/L was 22 months and those with aFLC >500mg/L was only 10 months (log rank p<0.0001). These differences in survival were especially significant for patients presenting with a creatinine clearance of >30mL/min (median survival of 14, 28 and 63 months respectively) but also for patients presenting with a creatinine clearance <30mL/min was 14, 5 and 7 months respectively. There was a very high early mortality in patients presenting with high free light chains with 30%, 55% and 70% of each group respectively dying by 2 years of evaluation. The correlation of presenting free light chains and organ involvement will be presented. *Conclusions*. The absolute presenting free light chain value is a strong prognostic factor for patients with AL amyloidosis. Patients presenting with an absolute FLC of >500mg/L have a very poor overall survival and high risk of early mortality. Free light should be used for AL risk stratification and the poor risk cohort may benefit from treatment (like bortezomib or CTD) achieving very rapid light chain responses.

0545

MULTIPARAMTER FLOW CYTOMETRY QUANTIFICATION OF BONE MARROW PLASMA CELLS AT DIAGNOSIS IS A VALID METHOD FOR PREDICTION OF MULTIPLE MYELOMA PATIENTS OUTCOME

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Background. The quantification of bone marrow (BM) plasma cells (PC) by conventional morphology (CM) in multiple myeloma (MM) patients has shown limited prognostic value, whereas the use of multiparameter flow cytometry (MFC) immunophenotyping is still considered investigational. *Aims*. To compare the BMPC quantification by CM and MFC and to assess the prognostic value of both techniques in a large series of uniformly treated MM patients.

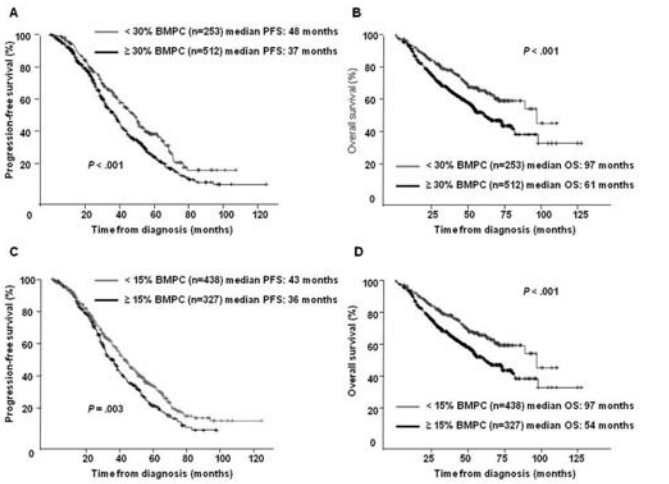

Figure 1.

Methods. A total of 765 untreated symptomatic MM patients were included in the present study, all of them uniformly treated according to the Spanish GEM2000 protocol. BM "first-pull" aspirate samples were used for morphological assessment, stained with May-Grümwald-Giemsa and using a conventional optical microscope. The PC quantification was obtained from a 200-cell differential count. For MFC analysis, BM samples were stained using a four-color direct immunofluorescence technique that allowed the identification of PC. *Results*. As expected, the median percentage of BMPC measured by CM (40%; range: 1% - 100%) was significantly higher (p<0.001) than by MFC (11%; range: 0.05% - 95%). Higher infiltration by CM was detected in the majority of patients (93%, N=709), with equal PC levels in 3% of cases (N=27), and only in 4% of cases (N=29) the percentage of PC obtained by MFC was superior to CM. Interestingly, PC enumeration showed a significant correlation between both CM and MFC techniques (R2= 0.46; p<0.001). The PC count assessment by CM as well as MFC discriminate different risk groups, with an optimal cut-off value of 30% and 15% BMPC for CM and MFC assessment, respectively. Thus, patients with <30% BMPC detected by CM at diagnosis showed significantly longer PFS (median 48 vs. 37 months; p<0.001; Figure, Panel A) and OS (median 97

vs. 61 months; $p<0.001$; Panel B) than patients with ≥30% BMPC. Considering MFC, cases with <15% BMPC had significantly longer PFS (median 43 vs. 36 months; $p=0.003$; Panel C) and OS (median 97 vs. 54 months; $p<0.001$; Panel D) than cases with ≥15% BMPC. Other baseline factors with significant impact on univariate analysis for survival were: age, anemia, high levels of calcium and serum creatinine, ISS III, percentage of PC in S-phase >1.5% and high-risk cytogenetics (t(4;14), t(14;16), or del(17p)). By multivariate analysis, only high-risk cytogenetics was selected as an independent prognostic factor for both PFS and OS (Hazard ratio, HR: 2.7; $p<0.001$ and HR: 2.6; $p<0.001$, respectively), and also age (HR: 1.6; $p=0.03$) and the percentage of PC detected by MFC (HR: 2.3; $p=0.006$) showed independent prognostic value for OS. *Conclusions.* Our results show in a very large series of patients uniformly treated that MFC immunophenotyping is a valid method for the evaluation of the PC burden in the BM of symptomatic MM patients at diagnosis, with a significant correlation with CM, and, more important, with independent prognostic value for the prediction of survival. Taken together, these results support the incorporation of MFC immunophenotyping in the diagnostic routine evaluation of all MM patients.

Allogeneic stem cell transplantation

0546

FACTORS DETERMINING SURVIVAL AFTER UNRELATED DONOR ALLOGENEIC STEM CELL TRANSPLANTATION IN PRIMARY REFRACTORY ACUTE MYELOID LEUKAEMIA

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Background. Allogeneic stem cell transplantation from an HLA identical sibling donor has the capacity to produce long term disease free survival in a significant number of patients with primary refractory acute myeloid leukaemia (AML). The increased availability of volunteer unrelated donors now permits allogeneic transplantation to be performed in patients with primary refractory AML but there has been no systematic analysis of outcome in this setting. *Aims*. We have analysed the outcome and factors determining overall survival after unrelated donor transplantation in patients with primary refractory AML. *Methods*. 186 adults with primary refractory AML who underwent an unrelated donor transplant from 1995 to 2006 in Europe were studied. All patients were refractory to induction chemotherapy having received a mean of 2.2 courses of chemotherapy (range 1 to 6). Cytogenetic status at diagnosis is available in 96 patients: 3 patients had good risk cytogenetics, 60 intermediate risk and 33 adverse risk cytogenetics by MRC criteria. The median interval from diagnosis to transplant was 4.2 months (range 2-6 months). 141 patients underwent transplantation from a 6/6 matched unrelated donor and 45 from a mismatched unrelated donor. 150 patients were transplanted using GCSF mobilised peripheral blood stem cells. 136 patients were transplanted using a myeloablative conditioning (MAC) regimen. 50 patients were transplanted using a reduced intensity conditioning (RIC) regimen of whom 30 received a low dose TBI based regimen. *Results*. The 2 year overall survival for the whole group was 31% and 43% for patients who achieved a CR post-transplant. 137 patients achieved a complete remission (CR) post-transplant. The day 100 non-relapse mortality was 16% (19% for MAC allografts v 9% for RIC allografts). In univariate analysis time to transplant (<4.2 months), presentation white blood cell count, the absence of circulating blasts at the time of transplant, and the use of a RIC regimen were associated with improved survival. Adverse risk cytogenetics at diagnosis was associated with a decreased OS. In multivariate analysis a short time from diagnosis to transplant ($p=0.05$), the absence of adverse risk cytogenetics ($p=0.02$) and the use of a RIC regimen (p=0.036) were associated with improved survival. The improved survival in patients receiving a reduced intensity transplant occurred despite their increased age at the time of transplant (54 years v 40 years). *Conclusions*. On the basis of this study we conclude that there is an important role for unrelated donor transplantation in the management of primary refractory AML-particularly if performed early after diagnosis. The encouraging results obtained using a RIC require further examination

0547

A RANDOMIZED CONTROLLED CLINICAL STUDY : COINFUSIONG OF MESENCHYMAL STEM CELLS IN HAPLOIDENTICAL HEMATOPOIETIC STEM-CELL TRANSPLANTATION CAN FACILITATE PLATELET RECOVERY BUT FAIL TO PREVENT GVHD

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Background. HSCT is an effective tool to treat hematological tumors and some non-malignant diseases. However, many of the patients can not find a HLA-matched donor. Over the past decade, it has been possible to induce the donor-recipient immunological tolerance through increasing the load of infused stem cells or by modulating cellular functions and graft composition, which greatly enhance the efficacy of haploidentical transplantation. In spite of those, the issues of graft rejection and graft-versus-host disease (GVHD) remain crucial. As confirmed by animal experiments, mesenchymal stem cells (MSC) do not only support hematopoietic functions, but also suppress the proliferation of lymphocytes in a dose-dependent fashion. Clinical studies have suggested that use of MSC may promote hematopoietic reconstitution and treat severe

acute hormone-resistant GVHD. No toxic side-effects related to MSC infusion have been reported till now. With regard to the issue of whether or not MSC could prevent the occurrence of GVHD, there were conflicting findings in animal models. *Aims*. To assess whether co-infusion with MSC in haploidentical HSCT would facilitate the hematopoietic recovery or decrease the rate of aGVHD. *Methods*. An open-label, randomized controlled phase II clinical study had been conducted. The control group received the routine institutional procedure of simple HSCT, whereas the treatment group was given a pre-infusion dose of 3-5-105 MSC cells/kg within 24 h. The study protocol was approved by the Peking University Ethics Committee. All the participating patients and donors provided written informed consent. *Results*. From June 2007 to June 2008, a total of 55 patients diagnosed with leukemia completed the entire study (27 in treatment group vs. 28 in control group) and follow-up continued until December 2008. The characteristic of patients were matched in both groups. All patients except one in the control group got myeloid engraftment. The mean time of WBC and platelet engraftment were 12 days (10-21 days) and 19.5 days (8-52 days) in treatment group, which were comparable with the 12 days (10-23 days) and 20 days (10-80 days) in control group. However, within 100 days, the time to platelets >50-109/L in the treatment group as compared with control group was markedly faster (22 days vs. 28 days; p=0.028). The accumulative occurrence rate of degree II above aGVHD were 51.8% (GVHD II : 13) and 38.9% (GVHD IV: 1; aGVHD II: 8) in treatment and control group (P=0.422). The overall accumulative occurrence rates of cGVHD were 45.4% and 61.9%, respectively (p=0.200). After a mean follow-up of 10 months (0.7-18.5 months), 1 patient in control group and 2 patients in the treatment group had the hematological relapse and 9 patients died during the study(control group:6; treatment group:3). The overall survival rate were 72.4% and 86.0% (p=0.306). Not any immediate or long-term toxic side-effects related to MSC infusion had been found. *Summary*. The use of MSC was both safe and feasible, and facilitates platelet recovery. Further studies are warranted to address the questions of whether MSC can be adopted to pretend the GVHD.

0548

A MULTICENTRIC COMPARATIVE ANALYSIS OF OUTCOMES OF HLA IDENTICAL RELATED CORD BLOOD AND BONE MARROW TRANSPLANTATION IN PATIENTS WITH BETA-THALASSEMIA OR SICKLE CELL DISEASE

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Background. Most patients with beta thalassemia major (TM) or sickle cell disease (SCD) can be cured by hematopoietic stem cell transplantation (HSCT) from either cord blood (CB) or bone marrow (BM). One advantage of CB is the absence of risk associated with donation. *Aims*. In order to compare outcomes after HSCT with CB or BM, we studied 459 patients with TM or SCD who received HLA identical sibling CB (n=70) or BM (n=389) allografts between 1994 and 2005. *Methods*. In order to avoid center and period effect, only centers that performed both types of HSCT during the same period were included. We compared the incidence of hematopoietic recovery, acute and chronic graft-versus-host disease (GvHD), survival and disease-free survival (DFS) after CB and BM transplantation. *Results*. Compared to BM, CB recipients were significantly younger (median of 6.1 y versus 8.1 y), smaller (19 kg vs 23 kg), and were transplanted more recently (in 2001 vs 1999). In the BM group, there were 130 (33%) SCD and 259 (67%) TM patients, and in the CB group, 26 (37%) SCD and 44 (63%) TM patients. The indications for transplantation in SCD were not statistically different between CB and BM groups, however, more TM patients belonging to Pesaro II-III risk classes received BM (67%) compared to CB (39%) (p=0.01). There were also differences in the conditioning regimen (more frequent use of ATG/ALG in BM group and of Fludarabine and Thiotepa in the CB group) and GVHD prophylaxis (more methotrexate-containing therapy in BM compared to CB). Moreover, the nucleated cell content was 10 times higher in BM compared to CB. The table below shows the non-adjusted univariate analysis for outcomes for all patients according to the stem cell source used. After HSCT for TM, the 5 year-DFS rates were 86% and 77% for BM and CB recipients respectively, and after HSCT for SCD, 92% and 89% respectively. In a multivariate analysis adjusted for age and type of hemoglobinopathy, DFS was not statistically different between CB and BM recipients (RR= 1,4, p=0.11). *Summary*. Patients with TM or SCD had excellent outcomes after HSCT whether they received CB or BM from an HLA identical sibling. These results strongly suggest that CB transplantation from HLA identical siblings should be pursued when possible to avoid the discomfort and risks of a bone marrow harvest.

Table 1. Non-adjusted univariate analysis of outcomes for a.

Non-adjusted univariate analysis of outcomes for all patients (TM and SCD) after HLA-identical related HSCT according to the stem cell source used

Source	Neutrophil recovery	Acute GVHD II-IV	Chronic GVHD	5y Survival	5y DFS
CB	90%	10%	5%	96%	81%
BM	93%	20%	12%	95%	88%
p value	*0.002*	*0.04*	*0.15*	*0.92*	*0.11*

0549

HIGH THROUGHPUT IDENTIFICATION OF MINOR HISTOCOMPATIBILITY ANTIGENS BY WHOLE GENOME ASSOCIATION SCANNING

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Allogeneic stem cell transplantation (allo-SCT) followed by donor lymphocyte infusion (DLI) is used as a curative treatment for patients with hematological malignancies. Donor derived T cells contribute to graft versus tumor responses by targeting minor histocompatibility antigens (mHag) that are encoded by single nucleotide polymorphisms (SNP). Applicability of immunotherapy targeting these mHag, however, is hampered due to limited numbers of known mHag. Recently, it has been demonstrated that Whole Genome Association scanning (WGAs) is a useful tool for identification of unknown mHag. From patients that displayed immune responses following allo-SCT and DLI, several mHag specific T cell clones were isolated. Panel studies revealed that the majority of these T cell clones recognized unknown mHag expressed in HLA-A*02 or HLA-B*07 with population frequencies ranging from 30% to 70%. To generate a whole genome map containing SNP genotypes, a panel of 90 HLA-A*02 and HLA-B*07 positive EBV transformed B cell lines was generated. DNA was isolated from these cell lines and 1,2 million SNP genotypes were determined using bead array techniques. This panel of SNP genotyped EBV cells was used to measure T cell recognition by 4 HLA-A*02 and 12 HLA-B*07 restricted T cell clones. The recognition pattern of each T cell clone was compared with all SNPs in the whole geno map and significance of association was calculated using Fisher's exact test. Highly significant association between recognition pattern and a defined genomic region was found in 14 out of 16 cases. In most cases, the associating region contained a single gene. Associating regions were located on chromosomes 5, 6, 7, 10, 12, 17, 19, 20 and 22. Online gene expression data were available for 12 of the identified genes. As expected, 10 genes were clearly overexpressed in EBV transformed B cells. Furthermore, overexpression restricted to hematopoietic cell types was observed in 8 genes whereas 4 genes were also overexpressed in testis derived tissues and prostate. To investigate whether the identified genes encoded the mHag, patient derived genes were specifically amplified, transfected and tested for T cell recognition. In addition, the identified genes from both patient and donor were sequenced to detect polymorphisms. T cell recognition was observed in 7 out of 11 genes tested. In all 7 cases a SNP difference between donor and patient was present. Based on HLA binding prediction algorithms, peptides spanning the SNP of interest were synthesized. Recognition of the peptides was used to confirm that these genes encoded the mHags, allowing functional studies of target tissues. In conclusion, we demonstrate that WGAs is a very efficient method for mHag discovery and therefore allows high throughput in depth analysis of clinical immune responses and selection of appropriate mHag for development of new immunotherapies.

PRESIDENTIAL SYMPOSIUM

0550

EXTRACORPOREAL PHOTOCHEMOTHERAPY FOR ACUTE AND CHRONIC GVHD, A REPORT OF 102 PATIENTS TREATED IN A SINGLE INSTITUTION

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Background. Acute GVHD and chronic GVHD remain the leading cause of morbidity and mortality of allogenic stem cells transplantation. Immunosuppressive therapy is the common practice to combat GVHD, however it heavily influences post-transplant rate of infection and is associated with secondary malignancies, affecting both morbidity and mortality. *Aims*. Exracorporal phototherapy (ECP) has been previously reported to be a useful modality treating patients with cutaneous T cells lymphoma, various autoimmune diseases and GVHD. Reported here is eight-year experience in a single institution of the use of ECP for patients with acute and chronic GVHD. *Methods*. Mononuclear cells were collected using Cobe-Spectra version 7 manual operation for adults and version 6 for pediatric patients. One and a half to 2 blood volumes were circulated through the cell separator with a product of approximately 5 x 109 mononuclear cells. When product hematocrit was less than 3%, volume was added to 300ml and when the hematocrit was more than 3%, volume was added to 500 ml. 8 Methoxy psoralen (8MOP) was added to final concentration of 0.2ug/mL. The product was transferred to irradiation bag and exposed to 2 Joule /cm^2 of UVA (365nM) (Biogenetic Vilber Lourmat). Cells were then retransfused. The treatment protocol consisted of 2 consecutive ECP performed at biweekly intervals until clinical improvement. One hundred and two patients, 66 males and 36 females, aged 1 year to 66 years (median 33 years), were treated. Patients underwent allogeneic bone marrow transplantation for the following indications: AML - 39, ALL - 28, CML - 11, CLL - 4 Thalasemia - 6, NHL - 8, MDS -5, MM -1, melanoma -1. Thirty six were treated for acute GVHD and 66 for chronic GVHD. Diffuse GVHD of skin was diagnosed in 84, liver involvement in 56, gastro-intestinal tract in 26 and joint involvement in 22 patients. Patients had from 2 to 125 ECP (median 10 for Ac-GVHD and 32 for Ch-GvHD). *Results*. 38 patients succumbed, 20/36 with Ac-GVHD and 18/66 with Ch-GVHD (p=0.005). At a median F/U of 19 months (1-108) response rate for chronic GVH was 60/66 (92%) and for acute GVH was 23/36 (64%) (p=0.001). Complete resolution of GVHD, partial response and no response were seen respectively in 33%, 60%, and 7% for patients with chronic GVHD and 0%, 56% and 44% for those with acute GVHD. Overall response rate was 46/56 (82%). Response rate for patients with GVHD grade I-III was significantly higher than for those with grade IV - 78/89 and 5/13, respectively (p=0.015). *Conclusions*. ECP is a useful modality of therapy and is most effective in patients with chronic GVHD. It is effective for both liver and skin involvement and less effective for patients with scleroderma. We suggest that this therapy should be used in early management of these patients concomitantly with immunosuppressive therapy in order to improve quality of life and reduce long-term side effects.

0551

CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II IS CAUSED BY MUTATIONS IN SEC23B GENE

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Background . CDAII, the most frequent type of congenital dyserythropoietic anemia family, is an autosomal recessive disease characterized by ineffective erythropoiesis, peripheral hemolysis, erythroblasts' morphological abnormalities and hypoglycosylation of some RBC membrane proteins. Recent studies indicated that CDAII is not a distinct glycosylation disorder but caused by a defect disturbing Golgi processing in erythroblasts. Linkage analysis located the CDAII gene in a 5 cM region on chromosome 20 but the molecular basis is still unknown. We recently investigated the cytoplasmic proteome of human red blood cells (RBCs) using a combinatorial peptide ligand library as a capturing agent to amplify the signal of low- and very-low abundance proteins: 1578 proteins, most of them unexpected, were identified allowing a deep exploration of the RBC pathways (Roux-Dalvai, 2008). *Aims*. To investigate the molecular defect of CDAII using a proteomic-genomic approach based on the recently acquired data on cytoplasmic RBCs proteins and the known chromosomic localization of CDAII locus. *Results*. The analysis of RBCs cytoplasmic proteome allowed us to identified 17 proteins codified by genes located in the chromosomic region between 20p11.23 and 20q11.23: SNX5, SEC23B, DTD1, NAT5, GINS1, BCL2-L1, MAPRE1, CHMP4B, EIF2S2, AHCY, ACSS2, GSS, EIF6, CPNE1, EPB41L1, C20orf77, TGM2. Most of them were excluded because found to be associated to other diseases, already excluded in CDAII (EPB41L1) by previous works, or because not related by function to CDAII. Among the remaining proteins we focus on SEC23B for its possible role in the endoplasmic reticulum-to Golgi trafficking and its localisation on 20p11, the region with the highest LOD-score in CDAII after the recent mapping of the markers on the current contigs (Denecke, 2009). The 20 exons and intronic flanking regions of SEC23B gene were analysed by direct sequencing in 10 CDAII patients form 8 families, finding 10 different mutations: 2 frameshift, 2 stop-codon, 1 splicing and 5 missense; all the missense mutations affected highly conserved aminoacids, and were not found in 100 normal alleles examined, ruling out the possibility of polymorphism. Patients' data are summarised on the Table 1 (* = members of the same family). *Conclusions*. We found that CDAII patients are mutated in SEC23B gene. The protein encoded by this gene has similarity to yeast Sec23p component of COPII, which is the coat protein complex responsible for vesicle budding from the ER. SEC23B is therefore implicated in vesicle trafficking and its alterations may account for the cellular phenotype observed in CDAII, with particular regard to the impaired glycosylation of erythrocyte membrane proteins. Further analysis and animal models, are necessary to unravel the role of SEC23B in the pathogenesis of CDAII.

Table 1.

Case	Sex	Origin	Hb g/dl	Retic 10^9/L	B3 deglycos	Mutation	Ex	Effects
1	F	N Italy	9.7	320	Yes	c.40 C>T c.428 A>CG	2 5	Arg14 Trp Frameshift
2	F	C Italy	11.4	3.5	Yes	c.1821delT/?	16	Frameshift/?
3	F	Bolivia	9.9	61	Yes	c.568 C>T c.1808 C>T	5 16	Arg190STOP Ser603 Leu
4	M	Albania	9.8	102	Yes	c.40 C>T c.1660 C>T	2 14	Arg14 Trp Arg554STOP
5	M	S Italy	10.4	Nd	Yes	c.325 G>A c.325 G>A	4 4	Glu109 Lys Glu109 Lys
6	F	C Italy	8.3	103	Yes	c.40 C>T Ivs6 +1g/a	2 Ivs6	Arg14 Trp Splicing
7	M	C Italy	9.7	100	Yes	c.1489C>T c. 2101 C>T	13 18	Arg497 Cys Arg701 Cys
8*	F	N Italy	9.2	121	Yes	c.325 G>A c.325 G>A	4 4	Glu109 Lys Glu109 Lys
9*	M	N Italy	11.3	115	Yes	c.325 G>A c.325 G>A	4 4	Glu109 Lys Glu109 Lys
10*	F	N Italy	11.7	63	Yes	c.325 G>A c.325 G>A	4 4	Glu109 Lys Glu109 Lys

0552

HIGH-THROUGHPUT RNA INTERFERENCE SCREENING IDENTIFIES SYNTHETIC LETHALITY BETWEEN ONCOGENIC KRAS DEPENDENCY AND SUPPRESSION OF STK33

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Background. Activating KRAS mutations are among the most common pathogenetic events in a broad spectrum of hematologic malignancies and epithelial tumors. However, oncogenic KRAS has thus far not proven to be a tractable target for therapeutic intervention. An alternative to direct targeting of known oncogenes is to perform "synthetic lethality" screens to identify genes that are selectively required in the context of specific cancer-causing mutations. Using this approach, we have discovered a synthetic lethal interaction between mutant KRAS, the most frequently mutated oncogene in human cancer, and inactivation of the gene encoding the STK33 serine/threonine protein kinase. *Aims and Methods*. To identify genes that are essential for cell viability and proliferation in the context of mutant KRAS, we performed high-throughput loss-of-function RNA interference (RNAi) screens in 8 human cancer cell lines (KRAS mutant, n=4; KRAS wildtype, n=4), representing 5 different tumor types (acute myeloid leukemia [AML], colon cancer, breast cancer, prostate cancer, glioblastoma), as well as normal human fibroblasts and mammary epithelial cells. We screened each cell line with a subset of the short hairpin RNA (shRNA) library developed by the RNAi Consortium that consists of 5024 individual shRNA constructs targeting 1011 human genes, including the majority of known and putative protein kinase genes and a selection of protein phosphatase genes and known cancer-related genes. *Results*. Suppression of STK33 preferentially inhibited the viability and proliferation of cells that were dependent on mutant KRAS, whereas STK33 was not required by KRAS-independent cells. The differential requirement for STK33 based on oncogenic KRAS dependency was confirmed in 10 additional hematopoietic (AML, n=5; T-cell acute lymphoblastic leukemia, n=3; multiple myeloma, n=2) and 7 additional epithelial cancer cell lines using *in vitro* transformation assays and human tumor xenograft models. Mechanistic studies support the hypothesis that STK33 promotes cancer cell growth and survival in a kinase activity-dependent manner by regulating the activity of S6K1 selectively in mutant KRAS-dependent cells. Molecular genetic characterization of cancer cell lines and analysis of patient-derived genomic data sets demonstrate that STK33 is not recurrently mutated or overexpressed in human tumors. 4. *Conclusions*. These observations indicate that targeting of STK33 may offer a substantive therapeutic window in a broad spectrum of cancers associated with mutant KRAS, thus providing a rationale for the development of STK33 inhibitors. Furthermore, our approach illustrates the potential of RNAi for discovering critical functional dependencies created by oncogenic mutations that cannot be identified using other genomic technologies, and represents a strategy for targeting other "undruggable" genetic alterations, such as MLL fusions in acute leukemias or mutant TP53 in lymphoproliferative and epithelial neoplasms.

0553

DOSE-INTENSIFIED COMBINED MODALITY TREATMENT WITH 2 CYCLES OF BEACOPP ESCALATED FOLLOWED BY 2 CYCLES OF ABVD AND INVOLVED FIELD RADIOTHERAPY (IF-RT) IS SUPERIOR TO 4 CYCLES OF ABVD AND IF-RT IN PATIENTS WITH EARLY UNFAVOURABLE HODGKIN LYMPHOMA (HL): AN ANALYSIS OF THE GERMAN HODGKIN STUDY GROUP (GHS

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Background and Aims. Combined modality treatment consisting of 4 cycles of chemotherapy and IF-RT is the standard treatment for early unfavourable HL. Overall survival (OS) and freedom from treatment failure (FFTF) in this group of patients was 91% and 83%, respectively, at 5 years in our prior HD8 study. Thus, the rationale for HD14 was to improve on these results by increasing dose intensity using BEACOPP escalated. *Methods*. Between January 2003 and January 2007, 1.216 patients aged 16-60 with untreated early unfavourable stage HL (CS I, IIA with one of the following risk factors: large mediastinal mass (a), extranodal disease (b), elevated ESR (c), or ≥3 nodal areas (d); IIB with risk factors c and d) were randomized to either 4 cycles of ABVD (arm A) or 2x BEACOPP escalated followed by 2x ABVD (arm B). All patients received 30Gy IF-RT after chemotherapy. Primary objective was the improvement of the FFTF. Here we present the results of the predefined 3rd interim analysis within the prespecified group sequential test design. *Results*. Of the 1.216 patients included, 1.010 were evaluable for this analysis. Patient characteristics were well balanced between both arms. At 3 years, the FFTF for arm A is 90% (95% CI: 87%-93%), and for arm B 96% (95% CI: 94%-98%). Since the observed inverse normal test statistic exceeds the critical level, the null hypothesis of equal FFTF in each arm can already be rejected. The improved FFTF is mainly due to differences in progression and early relapses (arm A 5.9% versus arm B 1.8%). Protocol adherence for chemotherapy was high and not different in both arms (arm A 98.8%, am B 97.3%). Though the chemotherapy-intensity was higher in the experimental arm, safety was comparable to the standard treatment. Secondary neoplasias occurred in 8 patients in each arm so far. *Conclusions*. Based on the significantly superior FFTF of the intensified therapy (2x BEACOPP escalated + 2x ABVD + IF-RT) compared to the prior standard (4x ABVD + IF-RT), this more aggressive treatment strategy will become the new standard for early unfavourable HL patients within the GHSG. Whether the improved FFTF translates into an improved overall survival must be awaited. Future strategies should aim at identification of those patient subgroups that profit most from this approach.

0554

DELETION OF DICER1 FROM OSTEOPROGENITOR CELLS IS SUFFICIENT TO INDUCE MYELODYSPLASIA AND HEMATOPOIETIC NEOPLASIA

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Background. Cells of mesenchymal origin are known to alter the growth of malignant and normal cells and participate in tissue development. Specific mesenchymal cell populations contribute to the specialized microenvironments or niches that regulate stem cells. In hematopoiesis, cells of the osteoblastic lineage participate in the hematopoietic stem cell niche. However, these cells have largely revealed their role as regulators of normal stem cell and hematopoietic physiology. How these cells, which have been relegated a relatively non-descript role of 'stroma,' participate in processes that result in disease is relatively understudied *Aims*. To explore the role of Dicer1, the endonuclease essential for miRNA biogenesis, in osteolineage cells in the bone marrow microenvironment and its effects on hematopoiesis. *Methods*. Dicer1 was conditionally deleted from osteoprogenitor cells by intercrossing transgenic mice expressing the GFP-Cre recombinase under the transcriptional control of the osteoblastic lineage specific osterix promoter to mice containing conditional (floxed) Dicer1 alleles. *Results*. Deletion of Dicer1 in osteoprogenitor cells induces markedly disordered hematopoiesis. Hematopoietic changes were complex, affecting multiple lineages and recapitulating key features of human myelodysplastic syndrome (MDS), including ineffective hematopoiesis with cytopenia, multilineage dysplasia, increased proliferation and intramedullary apoptosis of primitive hematopoietic cells, decreased B-cell progenitors, increased bone marrow vascularity and the propensity to develop hematopoietic neoplasms (myeloid sarcoma and acute monocytic leukemia-like disease). These changes were entirely microenvironment dependent with intact Dicer1 in hematopoietic cells. In addition, they were not observed when Dicer1 was deleted in mature osteoblasts. *Summary and Conclusions.* The data demonstrate that differentiation stage-specific perturbations in osteolineage cells can induce a complex hematological disorder and indicate the central role individual cellular elements of 'stroma' can play in tissue homeostasis. Further, they reveal that primary changes in the microenvironment can initiate secondary neoplastic disease.

0555

A COMMON CONSTITUTIONAL JAK2 HAPLOTYPE PREDISPOSES TO MYELOPROLIFERATIVE NEOPLASMS

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Epidemiological data and family studies have indicated that inherited factors may predispose to the development of myeloproliferative neoplasms (MPN). To explore the role of inherited factors in more detail, we initially analyzed JAK2-spanning SNPs in homozygous MPN cases (%V617F >50%; n=142) using pyrosequencing, which provides a quantitative readout of allele ratios. The mitotic recombination that gives rise to V617F homozygosity typically involves most of chromosome 9p and thus SNPs within this region are also reduced to homozygosity; consequently the haplotype on which V617F arose could be read directly from allele ratios that were significantly greater than the expected value of 0.5. In many cases the residual haplotype (i.e. the haplotype on which V617F had not arisen) could also be read. Strikingly, of the 142 V617F alleles, 109 (77%) had an identical haplotype (subsequently designated 46/1) within the JAK2 gene compared to only 9/74 (12%) residual wild type alleles (p= 1.4e-20, Fisher's exact test). These results indicated that homozygosity for V617F occurred preferentially when this mutation was present on the 46/1 haplotype. Analysis of data generated by the Wellcome Trust Case Control Consortium (WTCCC) showed that 46/1 is present at a frequency of 0.24 in 1500 UK healthy blood donors, and identified tagged SNPs that were then used to screen for this haplotype in further cases. V617F-associated disease was strongly associated with 46/1 in all three disease entities compared to healthy controls (PV, n=192, p=2.9e-16; ET, n=78, p=8.2e-9 and MF, n=41, p=8.0e-5) and allele-specific PCR demonstrated that V617F specifically arose on the 46/1 allele in most cases. We suggest two hypotheses to account for this observation: (i) V617F may arise randomly on all haplotypes but 46/1 is in linkage disequilibrium with an unknown constitutional functional variant that interacts with V617F in a manner that makes the development of clinically manifest disease more likely compared to V617F on a non-46/1 haplotype or (ii) there is a specific mutational mechanism by which V617F preferentially arises on a 46/1 haplotype. To investigate the possibility that JAK2 on 46/1 is functionally different from other JAK2 alleles, we tested if JAK2 haplotype influences myeloid colony formation in hematologically normal individuals (n=56). Healthy individuals that carried at least one 46/1 allele (n=26) grew significantly fewer peripheral blood CFU-GM than individuals without 46/1 (n=30), consistent with the hypothesis that JAK2 on 46/1 is functionally different from other JAK2 alleles. Furthermore, compared to controls 46/1 was more frequent in MPNs with JAK2 exon 12 mutations (N=50; P=0.003), MPL 515 mutations (N=157; p<0.001) as well as those that tested negative for all mutations (n=318; p=0.003). In summary, our data indicate that 46/1 is a strong predisposition factor for development of V617F associated diseases (OR=3.7; 95% CI 3.1-4.3; RR=2.6; 95% CI 2.3-2.9) as well as being relatively weakly but significantly associated with other MPNs. We estimate that this haplotype accounts for approximately 50% of the population risk of developing an MPN described in epidemiological studies.

POSTER SESSION II

Growth factors, receptors and signaling

0556

THE SOMATOSTATIN RECEPTOR 2 INDUCES A REVERSIBLE NEUTROPHILIC DIFFERENTIATION BLOCK

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Background. Previous studies have shown that the cannabinoid receptor 2 (cnr2), a GPCR abnormally expressed in 47% of the AML samples studied, could induce migration and a complete and reversible block in neutrophilic differentiation *in vitro*. Both effects were mediated by Gai and MEK/ERK. To assess whether other GPCRs would induce the same effects, we studied multiple receptors, including sstr2. The somatostatin receptor 2 is a seven transmembrane receptor normally expressed in immature CD34$^+$ human hematopoietic cells. It has previously been shown that, similarly to cnr2, stimulation of sstr2 with octreotide results in migration of hematopoietic cells. However, it remains unknown whether activation of the sstr2 might also interfere with the normal neutrophilic differentiation. *Aims*. To investigate whether stimulation of the somatostatin receptor 2 by octreotide may alter normal hematopoiesis *in vitro*. *Methods*. Sstr2-expressing cells. The human sstr2 gene was cloned into pLNCX. Site-directed mutagenesis was used to mutate the DRY motif into the DRA and DAY motifs. 32D/G-CSF-R cells were infected with virus containing these constructs, and single clones were obtained. Migration. Migration assays were performed using trans-wells with or without 1 nM octreotide placed in the lower chamber. The migrated cells were counted after 4 hours of incubation. Neutrophilic differentiation. The 32D/G-CSF receptor cell line was cultured in RPMI 1640 medium supplemented with 10% Fetal Calf Serum and murine IL-3 (10 ng/mL) or human G-CSF (10 ng/mL). Ligands were added to the cultures to a final concentration of 1 nM octreotide, 100 ng/mL pertussis toxin, and 25 mM PD98059. The cell density was daily readjusted to 2x10^5 cells/mL. Cell morphology was determined by microscopy on May-Grünwald-Giemsa stained cytospins at day 3, 5 and 8. *Results.* The expression of the sstr2 was functionally determined by migration assays. When 32D/G-CSF-R/sstr2 cells were cultured in the presence of G-CSF and octreotide, differentiation was fully blocked, whereas in the absence of octreotide or the receptor, cells differentiated into mature neutrophils at day 5. Neutrophilic differentiation was reestablished by PD98059, a MEK1 inhibitor, and by pertussis toxin. These ligands showed no effect on control cells. Finally, only the sstr2 DAY-mutants were not capable to migrate upon octreotide stimulation nor could they interfere with G-CSF induced differentiation following exposure to octreotide, indicating G-protein dependency of the two effects of sstr2 in myeloid cells. *Summary and Conclusions.* In this study we show that neutrophilic differentiation could be blocked upon stimulation of the sstr2. Similarly to cnr2, sstr2-induced effects were mediated by Gai and MEK/ERK. However, it remains unclear whether activation of MEK/ERK involves Gbg or occurs in a G-protein-independent fashion. In addition, our results suggest that the arginine aminoacid present in the DRY motif is crucial for sstr2 functions. Possibly, the non-blocking GPCRs would block differentiation upon stimulation with the correct agonist. Given the similar effects induced by cnr2 and sstr2, it is tempting to look at the underlying mechanism of GPCR pathways involved in leukemic transformation.

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WITHDRAWN

0558

SUSTAINED INCREASE OF CD69 ON ACTIVATED LYMPHOCYTES MEDIATED BY MESENCHYMAL STROMAL CELLS AND ITS POTENTIAL IMMUNOMODULATORY ROLE

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Background. Mesenchymal stromal cells (MSCs) exert an immune regulatory function and suppress T-cell proliferation. One of the ways by which MSCs may modulate immune responses is by the induction of regulatory T cells (Treg). TGF-B is known to induce the expression of FOXP3 and to drive the generation of Tregs. Despite the importance of secreted factors, cell to cell contacts promote increased lymphocyte immune modulation. Interestingly, TGF-B is known to induce the expression of TGFBI, a secreted extracellular matrix adaptor protein, whose expression is higher on hematopoietic stem cells adherent to MSC. *Aims.* To uncover new potential immunomodulatory mechanisms on lymphocytes, mediated by MSC. *Methods*. Peripheral blood CD3$^+$ T cell 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), were profiled by whole genome microarrays. Additionally, peripheral blood mononuclear cells (PBMC) from 6 individuals where similarly activated and cultured, and collected 1, 3 and 5 days after activation. Percentage of CD69$^+$ cells and proliferation of activated lymphocytes were evaluated by flow cytometry. Transcripts levels of TGF-B, TGFBI, FOXP3 and IL10 were determined by RT-PCR. *Results*. As expected, proliferation of lymphocytes co-cultured with MSC was significantly inhibited. Microarray analysis revealed many differentially expressed genes involved in immune response. Interestingly, among these, CD69 and components of the NFkB pathway were at higher levels on co-cultured lymphocytes. CD69 is the earliest activation marker of T cells and is only transiently expressed. As expected, activation of lymphocytes from PBMC cultured alone was evidenced by the expression of CD69 in 5% of cells in the first day (mean percentage), followed by a decrease in subsequent days (4% and 3%, respectively). In line with microarray results, lymphocytes co-cultured with MSC displayed a completely different pattern, with a similar initial activation (7%) followed by a significant increase in the 3rd and 5th days (16% and 14%, respectively). Interestingly, the expression of CD69 is controlled by NFkB and recent literature indicates that this receptor may modulate the inflammatory response, by inducing TGF-B production. Accordingly, TGF-B and FOXP3 levels in the 5th day were significantly higher on PBMC co-cultured with MSC, compared to PBMC cultured alone. Furthermore, TGFBI and IL10 were both expressed at significantly higher levels on PBMC co-cultured with MSC, in all days evaluated, compared to PBMC alone. In addition, their transcript levels decreased faster on PBMC cultured alone. In the other hand, *Conclusions*. We demonstrate for the first time that MSC promotes a sustained increase of the CD69 marker on CD3+ lymphocytes, which is accompanied by increased levels of TGF-B, TGFBI, FOXP3 and IL10 on total PBMC. Our results are in line with the proposed immunoregulatory role of CD69. In addition, higher TGFBI levels on PBMC may increase lymphocyte adherence to MSC, thus favoring immune modulation. Supported by FINEP,CNPq and FAPESP.

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MACROPHAGES AS NOVEL TARGETS FOR ERYTHROPOIETIN

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Background. The immunomodulatory effects of erythropoietin (EPO) on the cellular and humoral compartments of the immune system were originally described by our group in multiple myeloma patients and have been further elucidated in murine experimental models (Mittelman, 2001; Prutchi-Sagiv, 2006; Katz, 2005; 2007). However, the mechanism of action by which EPO affects lymphocyte number and function are still unkown, particularly since lymphocytes do not seem to carry EPO receptors (EPO-R). Our search for possible mechanisms led us to the novel discovery that dendritic cells (DCs) express the EPO-R, and that stimulation with EPO enhances their survival and function (Prutchi-Sagiv, 2008; Lifshitz, 2009). Here we focus on macrophages as an additional EPO target, since in analogy to DCs, macrophages are also antigen presenting cells, and serve as key effectors of the innate immune response. *Aims*. This study was designed to explore the possibility that macrophages are either direct or indirect targets of EPO. *Methods*. The in-vitro effects of EPO were investigated in a model of murine bone marrow derived macrophages (BMDMs) treated with recombinant human EPO (rHuEPO). These cells were subjected to biochemical analysis, phenotyping, and analysis of phagocytic activity and cytokine secretion. The *in vivo* effects of EPO on macrophages were assessed by analysis of macrophage phenotype molecules in rHuEPO-treated mice and in human EPO-overexpressing transgenic tg6 mice, in comparison to their control counterparts (diluent-injected and wt littermates, respectively). *Results*. Relying on murine models, we found that BMDMs express EPO-R mRNA, as detected by RT-PCR. *In vitro* stimulation of the BMDMs with rHuEPO activated multiple signaling pathways. EPO treatment of the BMDMs up-regulated their surface expression of CD11b, F4/80 and CD80, as well as enhanced their phagocytic activity. EPO treatment of LPS- stimulated BMDMs decreased IL-10 secretion. These results are supported by in-vivo experiments in rHuEPO-injected mice, as well as in tg6 mice. EPO treatment was associated with an increase in the size of the splenic macrophage population, as detected by F4/80 expression, and an increase in the number of macrophages expressing CD11b, CD80 and MHC class II. *Summary*. Our results show that macrophages are direct targets of EPO and that EPO treatment enhances their maturation and function. These findings point to the multifunctional role of EPO and may advance its clinical applications as an immunomodulator.

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MEMBRANE AND SOLUBLE CD40 LIGAND HAVE DIFFERENT EFFECTS ON BCL-6 EXPRESSION AND PRODUCE DIFFERENT PROTEIN/DNA COMPLEXES AT A SITE IN EXON 1 OF THE BCL-6 GENE

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Bcl6 is a zinc finger transcription factor that is highly expressed in germinal centre B-cells and is a potent transcriptional repressor. It is essential for germinal centre formation and T-dependent antibody responses. Deregulated expression of this gene has been associated with the development of non-Hodgkin's lymphomas including lymphocyte predominant Hodgkin's lymphoma and other tumours such as breast cancer. Tight regulation of Bcl6 is, therefore, essential but there are large gaps in our understanding of the signals involved. The inhibition of Bcr-Abl by the tyrosine kinase inhibitor, Imatinib, is known to up-regulate Bcl6 in Ph^+ lymphoid cell lines. We demonstrated that this effect is due to p38 MAPK. We also demonstrated that p38 is, in part, responsible for Bcl6 expression under basal conditions in the Burkitt lymphoma cell line, Ramos. Bcl6 was further up-regulated in Ramos cells by soluble CD40 ligand, but membrane bound CD40 ligand reduced Bcl-6 expression by a mechanism involving NF-kB mediated IRF4 up-regulation. Soluble CD40 ligand did not activate NF-kB to the same extent as membrane CD40 ligand although both activated p38, thus supporting a role for p38 in driving Bcl6 transcription. Next we sought to find out the sequences responsible for mediating the effects of p38. Utilizing Anisomycin we showed that a 300bp sequence around exon 1 was responsive to p38. Gel shift assays demonstrated that membrane bound CD40 ligand induced a different protein/DNA complex at a site within this 300bp region, corresponding to the terminal portion of exon 1, from soluble CD40 ligand. We conclude that p38, activated by CD40 ligand, up-regulates Bcl6. However, the effects of CD40 ligand depend on whether the soluble or membrane bound form of the molecule is used. It is possible that a repressive complex is formed at a site in exon 1 in response to membrane CD40 ligand stimulation.

0561

ALTERNATIVE USE OF EXON 1 AND DIFFERENTIAL REGULATION OF EXPRESSION OF DOCK10 ISOFORMS BY INTERLEUKIN-4

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Background. The Dock or CZH proteins are a family of non classical guanine exchange factors (GEF) for Rho GTPase proteins. We identified Dock10, one of the eleven members of the CZH family, as an Interleukin 4 (IL4)-inducible gene in B lymphocytes and chronic lymphocytic leukemia (CLL) cells. In addition, we obtained the 5'-end cDNA sequence from a Dock10 transcript by RACE-PCR, and cloned a full length cDNA by RT-PCR (accesion no. EU236710, encoding for 2180 amino acids; Yelo *et al.*, Mol. Immunol. 2008; 45: 3411-3418). This cDNA differs from the validated Dock10 transcript (accesion no. NM_014689.2, encoding for 2186 amino acids) in the sequences corresponding to exon 1, suggesting the existence of two isoforms of the human Dock10 gene. *Aims* To confirm the existence of two transcripts of the human Dock10 gene and to describe the effect of IL4 stimulation on their expression. To check whether equivalent transcripts of the ortolog murine Dock10 gene are expressed. *Methods*. B lymphocytes were isolated from the peripheral blood (PB) of CLL patients by using a RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies) followed by ficoll centrifugation. Total RNA was obtained from a sample before culture and after 24 hours of culture in the presence of 10 ng/mL of IL4 (BD Pharmingen). Expression of the Dock10 transcripts was studied by RT-PCR. To this purpose, forward oligonucleotide primers were designed from the respective exons 1, designated hDOCK10.45 (for our novel isoform) and hDOCK10.104 (for the validated isoform), and a reverse oligonucleotide primer from exon 5, designated hDOCK10.50, common for both isoforms. The amplicons were cloned by TA cloning and sequenced. Their levels of expression were compared by quantitative RT-PCR. Homology searches between human and mouse Dock10 sequences were conducted using the LFASTAn-LALIGNn program. RNA obtained from the mouse hematopoietic pluripotent cell line FDCP1 was used to check by RT-PCR whether a mouse Dock10 transcript equivalent to the novel human Dock10 isoform exists. Forward and reverse oligonucleotide primers, designated mDOCK10.4 (exon 1) and mDOCK10.8 (exon 6), respectively, were designed for this PCR. *Results*. Two isoforms of the human Dock10 gene were expressed in CLL cells, which differ in their exon 1. Within the Dock10 gene, exon 1 of the validated isoform is 95 kb upstream of exon 1 of the novel isoform, which in turn is 15 kb upstream of the common exon 2. Culture with IL4 strongly induced the levels of the novel isoform, but did not affect the levels of the validated one. These data suggests that expression of the Dock10 gene is controlled by two different promoters which drive expression of two isoforms, each one with a different exon 1, therefore encoding different amino terminal sequences. The structure of the mouse Dock10 gene ressembles that of the human Dock10 gene. A homologous sequence to exon 1 of the novel human Dock10 isoform was found 93 kb downstream of exon 1 and 16 kb upstream of exon 2 of the mouse validated Dock10 isoform. We confirmed by RT-PCR the existence of a novel isoform of mouse Dock10 in FDCP1 cells. *Summary and Conclusions*. Human and mouse Dock10 ortolog genes share a similar structure and produce two isoforms which arise from the alternative use of a different exon 1 for each isoform. IL4 induced expression of only one of the isoforms, suggesting that expression of Dock10 isoforms is driven by two promoters: an upstream promoter unresponsive to IL4, and a downstream promoter inducible by IL4.

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BIOEQUIVALENCE OF A NEW BIOSIMILAR FILGRASTIM AND NEUPOGEN: PHARMACOKINETIC, PHARMACODYNAMIC AND SAFETY DATA FROM TWO RANDOMISED, PHASE I, HEALTHY-VOLUNTEER STUDIES

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Background. Since its launch in 1991, recombinant human granulocyte colony-stimulating factor (G-CSF; filgrastim) has been established as an integral part of supportive therapy across multiple clinical indications. Consequent to patent expiry 3 years ago, Hospira has developed a biosimilar form of filgrastim, which could provide a bioequivalent alternative to Amgen's Neupogen. *Aims*. To compare the pharmacokinetic, pharmacodynamic and safety profile of Hospira filgrastim with that of Neupogen in healthy volunteers. *Methods*. Two phase I, single-centre, randomised, crossover trials were undertaken. In the first study (GCF061), which had a primary objective to demonstrate equivalence in pharmacokinetic characteristics, 48 healthy volunteers were randomised to intravenous (i.v.) or subcutaneous (s.c.) dosing and then further randomised to order of treatment. Subjects in each of the two dosing groups received a single 10 µg/kg dose of Hospira filgrastim followed by 10 µg/kg Neupogen or vice versa. In the second study (GCF062), which had a primary objective to demonstrate equivalence in pharmacodynamic characteristics, 50 healthy volunteers were randomised to one of two dose levels (5 or 10 µg/kg) and further randomised to order of treatment. All subjects received five daily s.c. doses of Hospira filgrastim or Neupogen (at 5 or 10 µg/kg), with subsequent crossover to the alternative treatment (at a matching dose level). Bioequivalence was evaluated by analysis of variance. If the estimated 90% confidence intervals (CIs) for the ratio of *test* to reference treatment means were within the conventional equivalence limits of 0.80-1.25, then bioequivalence was concluded. In both studies, subjects provided written informed consent before undertaking any study-specific procedures. *Results*. In GCF061, 46 subjects completed the study. The geometric mean AUC0-tlast for plasma G-CSF (primary endpoint) was similar in subjects treated with Hospira filgrastim and Neupogen following either i.v. (ratio of means: 0.96; 90% CI: 0.90-1.02) or s.c. (ratio: 1.02; 90% CI: 0.95-1.09) dosing. In GCF062, 48 subjects completed the study. The geometric mean absolute neutrophil count (ANC) AUC0-tlast at day 5 (primary endpoint) was comparable in subjects who received Hospira filgrastim or Neupogen in both the 5µg/kg (ratio: 0.98; 90% CI: 0.92-1.05) and 10 µg/kg (ratio: 0.97; 90% CI: 0.93-1.01) dose groups. In both studies, 90% CIs for the ratio of *test* to *reference* treatment means associated with the primary endpoints were within the predefined range necessary to demonstrate bioequivalence. With regard to secondary endpoints, bioequivalence of the two treatments was demonstrated for AUC0-infinity, Cmax, Tmax and T1/2 in the single-dose study following both i.v. and s.c. dosing, and for ANCmax, ANCmin and $CD34^+$ cell counts at day 5 in the multiple-dose study at both dose levels; thus, confirming the biological similarity of the two drugs. Hospira filgrastim was well tolerated and there were no additional or unexpected safety concerns associated with its use. *Conclusions*. These two studies support the bioequivalence of Hospira filgrastim and Neupogen, and provide a rationale for further clinical evaluation of the biosimilar filgrastim.

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AKT AND PKC AS REGULATORS OF DIFFERENTIATION AND TELOMERASE ACTIVITY IN LEUKEMIC CELLS

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Background. Several mechanisms for the regulation of telomerase have been reported, including transcriptional, translational, and post-translational mechanisms, suggesting that telomerase activity is controlled in a complex manner. *Aims*. To determine the mechanisms that modulate telomerase activity during granulocytic and monocytic differentiation of hematopoietic cells. *Methods*. A human acute myeloblastic leukemia cell line (HL60) was induced to undergo monocytic differentiation by exposure to VD3, while granulocytic differentiation was induced by exposure to ATRA. Changes of several signaling proteins during differentiation were examined and the *in vitro* kinase assay was performed. The effect of RNA interference with some signaling proteins was also assessed. Furthermore, the level of telomerase activity and the expression of human telomerase reverse transcriptase (hTERT) protein and mRNA were examined. *Results*. Rapid down-regulation of telomerase transcription occurred during early differentiation of HL60 cells into both lineages prior to G1 arrest. Each differentiation agent caused a significant increase of several signaling proteins (including Akt, mTOR, p70S6K, p21, PKC, Raptor, and Rictor) and a decrease of 4EBP1 at 3 days after the induction of differentiation. In addition, recombinant AKT or PKC-α caused dose-dependent activation of telomerase derived from serum-starved HL60 cells. Telomerase protein disappeared before activation of AKT or PKC-α during the late stage of differentiation, which might explain the repression of telomerase activity in differentiated cells. To further examine the role of the active form of AKT or PKC-α in differentiated cells, rictor was knocked down. The results suggested that mTOR/rictor kinase activity was essential for phosphorylation of both AKT and PKC in differentiated cells. Preincubation with a PI3K inhibitor (LY294002) or a PKC inhibitor (bisindolylmaleimide) did not alter the expression of surface markers, but led to a decrease of NBT reduction and esterase activity. *Conclusions*. There was a decrease of telomerase activity and hTERT (protein and mRNA) expression during granulocytic or monocytic differentiation stimulated by ATRA or VD3, respectively. The active forms of Akt, PKC-α, p70S6K, mTOR, and mTOR-associated rictor protein showed an increase on day 3 after the induction of differentiation. It has been reported that Akt promotes the transcription of hTERT and post-translational activation of telomerase. PKC was also reported to activate telomerase via a post-translational mechanism, and we found that recombinant PKC-α and AKT caused a dose-dependent increase of telomerase activity in HL60 cells. In differentiated cells, telomerase protein disappeared before the activation of AKT and PKC-α occurred, suggesting that these proteins contribute to cell function after differentiation. The present findings indicate that telomerase activity is regulated by at least two mechanisms during granulocytic and monocytic differentiation, with one being transcriptional and the other being post-translational and involving both Akt and PKC.

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A PHASE II RANDOMIZED STUDY COMPARING PEGFILGRASTIM VS FILGRASTIM AFTER HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS PERIPHERAL BLOOD STEM CELL SUPPORT

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Background. HDC with peripheral autologous stem cell support is widely accepted as standard care in many hematological malignancies and some solid tumors. The ASCO guideline recommends the use of growth factor after PBSC infusion. The use of Pegfilgrastim (PEG) after high dose chemotherapy (HDC) and peripheral blood stem cell (PBSC) PBSC has been evaluated in retrospective, single-arm phase II study or case-control study, all suggesting that PEG worked as well as filgrastim (FIL) to accelerate the hematological reconstitution rate. *Aims*. The aim of this study was to demonstrate the non-inferiority of one fixed dose of PEG compared to daily FIL, in patients (pts) receiving HDC and PBSC. *Methods*. Criteria for elegibility were: at least 3×10^6/kg $CD34^+$ reinfused; age >18 years; normal cardiac, renal, liver and pulmonary function; signed written informed consent. Pts were randomized to receive on

day+1 subcutaneously (sc) fixed-dose PEG (6 mg) or sc FIL 5 mcg/kg/d until an absolute neutrophil count (ANC >1.0×10⁹/L) was reached. Primary end points were number of days with an ANC <0.5×10⁹/L and the number of days to achieve an ANC > 0.5×10⁹/L. Secondary end points were: number of days to achieve an ANC >1.0×10⁹/L, number of days with fever >38°C, duration of antibiotic therapy, and number of documented infections. *Results*. The analysis was performed on 76 patients (36 pts in each arm). The mean number of CD34⁺ reinfused was similar. Considering the primary endpoint it can be claim that PEG is non-inferior to FIL because the upper two-side 95% CI for the difference in the duration of ANC <0.5×10⁹/L was less than the non-inferiority margin of delta=1.67, presenting a mean value of 0.18 with a CI 95% of -0.83; +1.19. The same results were found considering time to reach an ANC more than 0.5×10⁹/L, time to ANC >1.0×10⁹/L, days with fever, and days to discharge. Regarding platelet recovery and extra-hematological toxicities no statistical differences were observed between the two groups. *Conclusions*. This phase II randomized study shows that PEG was not inferior to FIL in terms of hematological reconstitution (primary end point). PEG could be safely used after PBSC infusion.

Table 1. Patient characteristic.

	FIL** N (%)	PEG** N (%)
	40 (100)	40 (100)
Median age (range)	48 years (21-72)	45 years (20-72)
Sex (M/F)	22/18	23/17
Disease		
Multiple Myeloma	6 (15)	6 (15)
Non-Hodgkin lymphoma	19 (48)	16 (40)
Hodgkin lymphoma	11 (27)	14 (35)
Acute leukemia	2 (5)	1 (2)
Solid tumors	2 (5)	3 (8)
Disease status		
Complete remission	30 (75)	29 (73)
Partial remission	8 (20)	9 (22)
Progressive disease	2 (5)	2 (5)
Conditioning regimens*		
BEAM	17 (43)	22 (55)
PAM200	16 (41)	12 (30)
Busulphan + Cyclophosphamide	3 (8)	6 (15)
Others	3 (8)	0 (0)
Mean CD34 reinfused (10^6/kg) (IC 95%)	4.6 (4.2-5.1)	5.7 (4.6-6.8)

* 1 patient, randomized in FIL group, did not receive HDC for progression
** No significant correlations were found for each variable

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COMPARATIVE EFFECT OF FILGRASTIM VS. PEGFILGRASTIM AFTER CHEMOTHERAPY ON HIGH GRADE NON HODGKIN LYMPHOMA

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Background. The use of growth factors may reduce the duration of neutropenia induced by chemotherapy. New pegylated form may be of further benefit for movilization of haemopoietic precursors and is under evaluation associated to chemotherapy. *Aims*. We performed a comparative study to evaluated the capacity to prevent fever associated to neutropenia and the recover of highly myelosuppressor post-chemotherapy neutropenia with haematopoietic growing factor (Filgrastim: 300µg/dayx5days) and the pegylated form (Pegfilgrastim 6mg:). Haematopoietic Progenitors Cells (HPC) were determined by two different methods: quantification of CD34⁺ cells by flow cytometry (FCM) and in the Sysmex XE-2100 auto-analyzer. *Material and Methods*. 56 cycles administered in patients with high grade NHL were analyzed. Median age was 63 years (47-76). Male/female proportion was 62,5% and 37,5% respectively. Treatment regimens were CHOP-R14 and EDOCH-R14 (VP-16 at 50mg/ m² /d x 4d, Adriamicine 10mg/ m²/d x 4 d, Vincristine 0.4 mg/m²/d x 4 d, Cyclophosphamide 750 mg/ m²/d x 1 d, Dexamethasone 40 mg/m²/d x 5d y Rituximab 375 mg/m²/d). Selection was based on age and IPI. Patients were randomized to receive Filgrastim 5 µg/Kg/day sc during 5 days vs. Pegfilgrastim 6 mg SC, in unique dose in the day +4. Feverish neutropenia and side effects were examined in both branches. Periodic determinations of HP were performed on days 6-7, 8-9, 11-12 post-QT, with the intention of analyzing neutropenia nadir and the exit of it in the cycles. The number of CD34⁺ cells circulating in peripheral blood was analyzed by FCM with FASCALIBUR, and counting HPC in the IMI channel with the Sysmex XE-2100 analyzer. *Results*. Neutropenia episodes: 16/56 (28%), feverish neutropenia admittances: 5/56 (9%), (3 received Peg-filgrastim (1CHOP-R and EDOCH-R) and 2 Filgrastim (1 CHOP-R). Neutropenia median (<500) on the day +11 for EDOCH-R and +10 in CHOP-R. Most of neutropenia episodes (12/16, 75%) were after EDOCH-R. Feverish neutropenia episodes with no admittance were only 2/56 (3,5%). Number of delays in the neutropenia cycle administration were 2/56 (3,5%). Only one case of severe bone pain was reported after administration of Pegfilgrastim. HPC were not detected with any of the two methods before day +11. HPC were detected by both methods. The coefficient of correlation between HPC counts by Sysmex XE-2100 and CD34⁺ cells by FCM was r=0.68. *Conclusions*. 1. No significant differences to prevent febrile neutropenia were observed between the two haemopoietic growing factors in this study. 2.No difference in the neutropenia nadir was observed. 3.The measure of the HPC demonstrates its presence in the cycle nadir (day +10 and +11), by both methods.

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AN ERYTHROPOIETIN AND CYCLOPHOSPHAMIDE COMBINATION ADDITIVELY ENHANCES IMMUNOGLOBULIN PRODUCTION IN MICE

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Background. Erythropoietin (EPO) is an important component in the treatment of cancer related anemia, and it is usually combined with chemotherapy. Cyclophosphamide (CP) is a known cytotoxic alkylating as well as immune suppressing agent, widely used in cancer chemotherapy. The anti-neoplastic activity of CP at low doses is attributed to enhancement of cellular and humoral immunity. We have previously shown that EPO displays anti-neoplastic activity (Mittelman, 2001; 2004) and that EPO treatment is associated with enhancement of both the humoral and cellular immune responses (Prutchi-Sagiv, 2006; Katz, 2007). We have recently examined a murine model of anti DNP-KLH immunization, in order to assess the effect of EPO on immunoglobulin (Ig) production. We have shown that administration of high doses of recombinant human EPO (rHuEPO) (180u x 3/wk) to DNP-KLH-injected BALB/c mice resulted in an increase in anti-DNP IgG1 production (Katz, 2007). *Aims*. Here we focused on the humoral immunomodulatory effects of combined CP+EPO treatment both administered at low doses, thus simulating clinical conditions. *Methods*. Low dose CP (12.5mg/kg) was administered for 2 days before antigen (DNP-KLH) administration, followed by rHuEPO injection (90u x 3/wk). We compared anti-DNP Ig serum levels in DNP-KLH-injected C57BL mice that were treated with either EPO or CP alone, or with CP+ EPO combination. *Results*. CP treatment alone resulted in increased anti-DNP IgG1 serum levels (CP-O.D.450 nm = 0.38±0.06 vs. non-treated-O.D.450nm = 0.18±0.06, $p<0.05$). In contrast, EPO treatment alone enhanced IgG2a levels (EPO-O.D.450 nm = 0.47±0.09 vs. non-treated-O.D.450nm = 0.18±0.07, $p<0.05$). Moreover, the combined CP+EPO treatment maintained the increase in both IgG1 and IgG2a subtype levels (CP+EPO-O.D.450nm = 0.38±0.06, CP+EPO-O.D.450nm = 0.49±0.1, respectively). With respect to anti-DNP total Ig, while neither CP nor EPO alone conferred any significant effect, the combined CP+EPO treatment resulted in markedly increased total Ig serum levels (CP+EPO-O.D.450nm = 0.48±0.05 vs non-treated-O.D.450 nm = 0.28±0.07, $p<0.05$), most likely reflecting the higher levels of IgG1 and IgG2a. Hence, the combined CP+EPO treatment additively improves Ig production, compared to treatment with either CP or EPO alone. *Summary*. We thus demonstrate that in the context of chemotherapy treatment, EPO can enhance the humoral immunity in addition to its erythropoietic activities. Our findings emphasize the potential role of EPO as an immunomodulator, particularly when given as a part of a combined treatment.

0567

ATYPICAL WHIM SYNDROME ASSOCIATED WITH GRANULOMATOSIS: REPORT OF TWO CASES

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We report on two cases of young females presenting with extended granulomatosis, warts, recurrent infections, severe B-cell lymphopenia, and neutropenia sensitive to granulocyte-colony stimulating factor. One patient granulomatosis was successfully treated with upfront interferon α2-a. Three years later, the patient developed severe post-cytomegalovirus hemophagocytic syndrome. The other patient died of mycobacterium avium septicemia, two years after the initial presentation. These two patients were suspected of WHIM syndrome, a rare immunodeficiency disorder associated to dysfunctions of the CXC chemokine receptor 4, which combines Warts, Hypogammaglobulinemia, Infections and Myelokathexis. Genetic analysis of CXCR4 concluded with a wild type genotype whereas functional analyses of CXCR4 revealed a defective internalization upon CXCL12 stimulation. However, WHIM diagnosis could not strictly be asserted due to the absence of myelokathexis on bone marrow histology. These two clinical observations of granulomatosis with immuno-hematological disorder related to CXCR4 dysfunctions could be assimilated to an atypical WHIM syndrome.

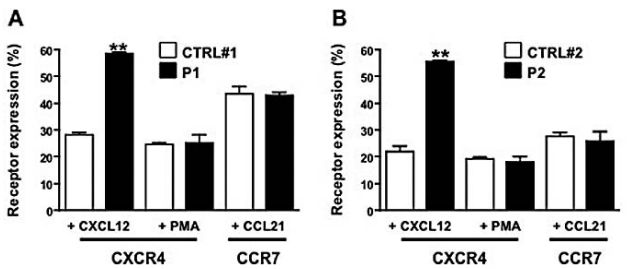

Figure 1. Impaired CXCL12-induced internalization of CXCR4.

0568

THE PHYSICOCHEMICAL PROPERTIES OF A BIOSIMILAR FILGRASTIM ARE COMPARABLE TO THOSE OF NEUPOGEN

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Background. Recombinant human granulocyte-colony stimulating factor (rhG-CSF; filgrastim) is a therapeutic protein used primarily to reduce the incidence and duration of severe neutropenia and associated complications. Following patent expiry, Hospira and PLIVA have worked together to co-develop a biosimilar filgrastim (Hospira filgrastim) that is highly comparable to Neupogen®, the filgrastim originally produced by Amgen. The complexity of manufacturing a biopharmaceutical such as filgrastim is much greater than that of conventional, low-molecular weight generic medicines. Thorough comparability analyses are required to demonstrate similarity to the reference medicinal product before regulatory approval is granted. *Aims*. To assess the physiochemical similarity of Hospira filgrastim to Neupogen®. *Methods*. An extensive characterisation study was performed to compare Hospira filgrastim to Neupogen®. Both drugs were supplied in 1mL glass, single-use, prefilled syringes (five batches of each product at 480 μg/0.5mL and one batch of each product at 300 μg/0.5mL). State-of-the-art analytical methods were used to evaluate physicochemical properties and molecular characteristics (appearance, colour, clarity, pH, intact molecule mass, protein concentration, isoelectric point, secondary protein structure, amino acid sequence and disulphide bridges position), assay, purity and biological activity. A side-by-side comparability exercise was performed on samples stored in long-term storage conditions (2-8°C), as well as on stressed samples (40°C), in order to compare degradation impurity profiles between Hospira filgrastim and Neupogen®. All methods were validated in accordance with ICH or Ph. Eur. guidelines. *Results*. All tested batches of Hospira filgrastim and Neupogen® stored at long-term storage conditions were within the acceptance criteria for all parameters tested. All batches of the two drugs complied with the circular dichroic and fluorescence spectra for the secondary and tertiary structure of filgrastim. The amino acid sequence and disulphide bridges position in all tested batches were consistent with those previously reported. SDS-PAGE and isoelectric focusing showed that Hospira filgrastim and Neupogen® have comparable molecular masses and isoelectric points. All detected impurities in both products were at similar levels. The biological activity of Hospira filgrastim and Neupogen® were similar and within the accepted range. There were no significant differences in profile and quantities of the product-related impurities between Hospira filgrastim and Neupogen® following storage for 12 weeks under stress conditions. *Conclusions*. These data, acquired using rigorous analytical methodology, show that the physicochemical profile of Hospira filgrastim is highly comparable to that of Neupogen®. If these similarities are borne out in clinical studies, Hospira filgrastim may prove to be a valuable and cost-effective alternative to Neupogen®.

Table 1.

Assessment	Hospira filgrastim	Neupogen
Identification by SDS-PAGE and isoelectric focusing	Principal band in similar position to reference solution	Principal band in similar position to reference solution
Impurities by SDS-PAGE and isoelectric focusing	No band is more intense than reference solution	No band is more intense than reference solution
Impurities with molecular mass higher than that of filgrastim by size exclusion chromatography	<0.20%	<0.20%
Filgrastim assay by RP HPLC†	0.89–0.94mg/mL	0.84–0.85mg/mL
Impurities by RP HPLC#	1.82–2.20%	1.51–2.37%
Impurities by ion chromatog.#	<0.20%	0.30–0.46%
pH‡	4.0–4.1	3.9–4.0
Protein (UV/Vis)‡	0.92–0.94mg/mL	0.88–0.91mg/mL
Intact molecule determination‡	18,800Da	18,800Da
Biological activity assay‡	95.8–98.2x10^6 IU/mL	78.9–93.5x10^6 IU/mL

†For 480μg/0.5mL presentation, excluding expired batches
#Results for both presentations, excluding expired batches
‡Data from independent data set of three batches each of Hospira filgrastim and Neupogen

Drug resistance and pharmacology

0569

THE PROTEASOME INHIBITOR BORTEZOMIB MODULATES THE EFFECT OF GLUCOCORTICOID TREATMENT IN PREDNISONE-RESISTANT CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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Background. The response to initial glucocorticoid (gc) therapy in childhood acute lymphoblastic leukemia (ALL) reliably predicts the response to multi-agent chemotherapy. In a recent study, we identified the valosin-containing protein (VCP), part of the ubiquitin proteasome degradation pathway (UPDP), as a differentially expressed protein in prednisone good (PGR) and poor responder (PPR) patients. *Aims*. The aim of the study was to investigate the association of differential VCP expression and glucocorticoid response in childhood ALL in order to find ways to overcome prednisone resistance. *Methods*. To investigate whether treatment of ALL cells with the proteasome inhibitor bortezomib acts synergistically with glucocorticoid treatment, human B-cell precursor leukemic cell lines MHH cALL 2 (PPR) and MHH cALL 3 (PGR) were treated with prednisone (6.2 µM) and various concentrations of bortezomib (1.5 nM-12 nM) up to 96 hours. Cells were sampled every 24 h for subsequent analyses. To quantify the amount of VCP expression, real-time PCR and Western blot analysis using the mouse monoclonal anti-VCP antibody (MA3-070, Dianova) were performed. Viability was determined by WST-1 analyses and trypan blue stainings. *Results*. Both cell lines showed a similar response to single bortezomib treatment after 96 h, but compared to single prednisone treatment the decrease in viability of the PPR cells was earlier and stronger, e.g. with 7 nM bortezomib as a single treatment, PPR cells showed a 13-fold higher decrease in viability compared to prednisone single treatment. In combined bortezomib and prednisone treatment, the loss of viability was even 21-fold higher than with prednisone alone. The effect of prednisone was potentiated dose-dependently by bortezomib treatment, which caused a significantly higher decrease of viability in the PPR cells within 48 h compared to PGR cells. Untreated PPR cells showed a 1.5-fold higher VCP RNA expression than PGR cells. Within 72 h after prednisone treatment, VCP RNA expression increased to 1.5-fold compared to the initial level in the PPR cell line, whereas, in PGR cells, the VCP amount remained unchanged. However single prednisone treatment decreased viability in PPR cells to 70% only after 72h, but just 10% viable cells were detectable after combined prednisone and 7nM bortezomib treatment. *Conclusions*. The results of this study suggest that VCP, an important member of ubiquitin proteasome degradation pathway, modulates prednisone response in childhood ALL. In addition, proteasome inhibitors like bortezomib seem to be able to sensitize glucocorticoid-resistant childhood ALL cells for prednisone treatment. Therefore, drug targeting the proteasome may be a novel therapeutic option in resistant ALL.

0570

IMATINIB INDUCES AUTOPHAGY THROUGH INCREASING THE EXPRESSION LEVELS OF BECLIN-1 AND ATG-5 GENES IN BOTH PARENTAL SENSITIVE AND IMATINIB-RESISTANT K562 CELLS

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Background. Chronic myeloid leukemia (CML) is a hematological malignancy resulting from a translocation between the chromosomes 9 and 22 which generates a strong oncogenic BCR/ABL protein. BCR/ABL regulates cell growth and proliferation, migration, adhesion and differentiation. Imatinib is a chemotherapeutic agent that specifically binds to the ATP-binding pocket of the BCR/ABL protein and inhibits phosphorylation of downstream targets. Autophagy is the biological event in which the components of the cell are degraded through activation of the lysosomal machinery. Autophagy plays important roles in programmed cell-death, maintenance of the homeostasis, resistance to chemical stress and nutrient deprivation. Formation of autophagosomes involves several genes belonging to ATG family. The most known members of this family are ATG6 (Beclin-1) and ATG5 (autophagy related 5 homolog). *Aims*. In this study, we aimed to examine the autophagic effects of imatinib on parental sensitive and 3 µM imatinib-resistant K562 cells and the possible roles of autophagy in imatinib-induced cell death and/or drug resistance. *Methods*. Philadelphia chromosome positive (Ph(+)) K562 cells were cultured in the increasing concentrations of imatinib step by step starting from the initial concentration of 50 nM. The cells that were able to grow in the presence of the drug were selected gradually in the culturing and the final cells which are resistant to 3 µM imatinib (K562-IMA3) were maintained. IC10 values for both sensitive and resistant cells were determined by XTT cell proliferation assay. Drug concentrations were picked according to IC10 concentrations. Total RNA was isolated at the end of 72 hours by using Nucleospin RNA Isolation Kit and reverse-transcriptase PCR was conducted by using beclin-1 and atg5 primers with the internal control of β-actin. Products were then visualized by the agarose gel electrophoresis. *Results*. XTT data was plot in the inhibitory concentration (IC) graph. IC50 values of K562 and K562/IMA-3 0.24 µM and 14,6 µM, respectively. There were 60 times increase in resistance to imatinib in K562/IMA-3 cells comparing to parental cells. According to the XTT data IC10 and smaller concentrations were picked as follows: 0.1- 1- and 10 nM imatinib for parental sensitive cells; and 10- 100- 500 nM imatinib for the resistant cells. mRNA analyses of beclin1 and atg5 genes by reverse transcriptase PCR techniques have shown that there were significant dose-dependent increase in the expression levels of these genes in both sensitive and the resistant cells. *Summary and Conclusions.* In this study, we have shown for the first time that imatinib induces autophagy in CML cells. These data provides an insight into the relationship between autophagy and Ph^+ CML. Dose-dependent increase in expression levels of autophagy related genes is encouraging for our further plans of experiments with more advanced techniques such as real-time PCR, western blotting and GFP-tagging. In this context, the mechanistic role of autophagy will have been elucidated in Ph^+ CML in response to imatinib. It might play a role in either imatinib-induced cell death or the drug resistance. Gaining more information about autophagy might provide novel targets for an effective CML therapy by promoting the autophagy.

0571

DEVELOPMENT OF A PERSONALIZED MEDICINE TEST FOR HAEMATOLOGICAL MALIGNANCIES

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Background. We have developed a cell-based screening platform, called ExviTech, which incorporates both automated sample preparation and automated evaluation by flow cytometry, in conjunction with proprietary analytical software and a database structure geared for rapid data acquisition, analysis and reporting of results. The ExviTech platform has been used to test samples, either peripheral blood or bone marrow, extracted from patients diagnosed with B-cell chronic lymphocytic leukaemia (B-CLL). The patient's biological sample is tested against all drugs approved for the treatment of B-CLL, and results are obtained by assessing the ability of each drug to induce apoptosis in the leukemic cell population. The complete patient sample is diluted and plated, retaining the erythrocyte population and serum proteins. B-CLL is a clinically heterogeneous disease in which the individual prognosis of patients is extremely variable. We propose that screening such targets in their biological context will add valuable data to aid in the prognosis and treatment of this disease. *Methods*. Working in collaboration with four hospitals in Spain, samples are extracted from patients diagnosed with B-CLL, having first obtained informed consent. The experimental assay is setup within 2-6 hours of obtaining the sample. The sample is diluted to achieve a leukemic cell concentration of approximately 4,000 cells/µm, then 45 µL of the suspension are added to each well of 96-well plates that contain the pharmacological agents. Sample extraction from the patients and the experimental setup are all done under sterile conditions. The compound plates are then incubated for 24, 48 or 72 hours at 37oC with 5% CO_2. After incubation, the erythrocytes are lysed and Annexin V-FITC, monoclonal antibodies anti-CD45-APC and anti-CD19-PE, are added to each well. The plates are then transferred to an automated flow cytometry system where the contents of each well is aspirated and analyzed by a CyAn flow cytometer. *Results*. The ability of individual cytotoxic drugs to induce apoptosis in the leukemic cell population varies greatly from patient to patient, even among patients with similar prog-

noses. The maximum level of apoptosis for a compound can range between 20% and almost 100% among the patients. Additionally, the kinetics of the induction of apoptosis can differ between patients for a given cytotoxic drug. *Conclusions*. By testing patient samples directly we are looking at the effectiveness of a given drug *in vitro* to do exactly what it is expected to do *in vivo*. The large level of variability between patients illustrates what is already known, that CLL patients do not always respond well to treatments given according to standard protocols. We are expanding the range of drugs we are testing to include the polypharmacy that is inherent in the treatment of haematological malignancies. Using our automated ExviTech screening platform we are able to screen each patient sample against all drug combinations in the accepted protocols, at concentrations similar to the reported plasma concentrations. Personalized medicine offers the capacity to respond to this disease on a case by case basis, identifying which treatment will be the most effective for each individual.

0572

EFFECTS OF THALIDOMIDE, ROFECOXIB AND CAPTOPRIL ON VASCULAR ENDOTHELIAL GROWTH FACTOR IN EHRLICH ASCITES CARCINOMA

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Background. The growth and metastasis of tumors are dependent on their development of a vascular supply. An array of factors that regulate angiogenesis has been isolated, with the most pivotal positive regulator being vascular endothelial growth factor (VEGF). *Aims*. The study was conducted to evaluate the effects of thalidomide, rofecoxib and captopril on the angiogenic marker, VEGF, when used alone or in combination with cisplatin in Ehrlich ascites carcinoma (EAC)-bearing Swiss albino mice. *Methods*. The EAC cells were implanted subcutaneously at 2 sites bilaterally to produce solid tumors on the lower ventral side of Swiss mice. These tumors were used to evaluate the effects of thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) as individual treatments or in combination with cisplatin (2 mg/kg, i.p.) on VEGF. Inhibition of VEGF is proposed as a possible mechanism through which these drugs exert their anti-tumor and anti-angiogenic effects. All treatments were started 24 hours after tumor cells inoculation. VEGF was measured as a time course on days 7, 14 and 21 both in plasma (using ELISA) and tumor tissue (using immunohistochemistry). The tumor weight was evaluated as a criterion for the anti-tumor activity. *Results*. Individual treatments with thalidomide or rofecoxib (not captopril), alone or combined with cisplatin, reduced plasma levels of VEGF on day 7. Comparing the plasma levels of VEGF on days 14 and 21 post inoculation, one-way ANOVA showed a non-significant effect among all groups. EACs in the control group showed a strong expression of VEGF (VEGF-rich tumors). Individual treatments with thalidomide, rofecoxib or captopril could reduce the percentage of VEGF-rich tumors to reach 42.9%, 57.1% and 66.6% of the control, respectively. The combination of cisplatin with thalidomide, rofecoxib or captopril has produced a further reduction in the percentage of VEGF-rich tumors to reach 28.6%, 42.9% and 57.1%, respectively. A significant reduction in tumor weight was observed after individual treatments.

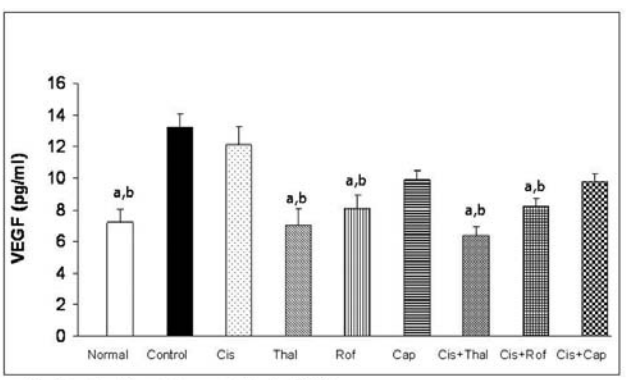

Figure 1. Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) on plasma levels of VEGF on day 7 in EAC-bearing female Swiss albino mice.

Conclusions. The data indicate that EAC is a VEGF-producing tumor and VEGF is essential for initial but not continued *in vivo* growth of EAC cells. Thalidomide and rofecoxib proved to exert their anti-tumor and anti-angiogenic effects through the inhibition of VEGF, while the anti-tumor effect of captopril might be mediated through a different mechanism. The results suggest a beneficial role of anti-angiogenic agents as an adjuvant treatment to chemotherapy.

0573

A VERY SENSITIVE AND RAPID METHOD FOR BCR-ABL T315I MUTATION DETECTION BY PEPTIDE NUCLEIC ACID DIRECTED PCR CLAMPING

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Background. BCR-ABL kinase domain (KD) mutations are the major cause of resistance to tyrosine kinase inhibitors (TKIs). Among different mutations identified the frequently observed T315I is of particular concern being not effectively targeted by any TKIs so far available. Currently, the recommended method for BCR-ABL mutation detection is the sequencing the KD. This is time consuming, available in few centres and allows to reach a maximum sensitivity of 15-20%. The latter point represents a limit as frequently mutated clones may be present at a lower percentage. The availability of a simple, sensitive and quick assay, allowing a rapid detection for the T315I mutation is therefore crucial, as the detection of this mutation represents an important element in clinical decision making for CML patients. We report a novel method allowing easy, rapid and sensitive detection of the mutation affecting the codon 315 (T315I). *Rationale and Methods.* The innovative strategy we propose is based on a variant of a single tube PNA-PCR clamping. Peptide Nucleic Acid (PNA) is a potent DNA mimic in terms of sequence specific hybridization. PNA/DNA is thermally more stable than DNA/DNA or DNA/RNA duplexes, but PNA sequences cannot be extended by DNA polymerase (5). As consequence, PNA/DNA duplex suppresses DNA amplification. Furthermore, a single base pair mismatch discrimination is greater for PNA/DNA than for the corresponding DNA/DNA duplex. Based on this premise we attempted to develop a novel and sensitive detection assay aimed to quickly and easily identify T315I mutation in CML patients making use of primer exclusion PNA directed PCR clamping. The experimental design forecasts that both PNA and PCR primer target sites overlap, thus leading to a direct competition towards complementary template DNA. When perfect matching occurs PNA-template hybridization is favoured, in comparison to template-primer duplex, and DNA amplification is suppressed. Conversely, a single mismatch destabilizes the PNA-template duplex, favouring the hybridization between template and primer thus allowing template amplification. Competitor PNA sequence was designed to perfectly match wild-type (WT) template sequence. Therefore, when a single base pair mismatch occurs (like in the case of T315I) PNA-template stability is strongly impaired and DNA amplification favoured. *Results and Conclusions.* cDNA synthesized from total RNA isolated from patients harboring either wt or mutated Bcr-Abl alleles have been amplified by nested PCR. During the first step a specific pair of primers designed to discriminate between the kinase domain of c-Abl and Bcr-Abl have been utilized. The second step involves the use of sequence specific 19-mer PNA, matching within the region around the codon 315 (T315I), in a *PNA primer exclusion PCR clamping* reaction. Initially, we aimed to study patients previously analyzed by sequencing in which either the wt or the mutated allele were to be predominant. Encouraging results show amplification in patients harboring the point mutation at position 315 which determine the substitution of a threonine for isoleucine (T315I) indicanting the bona-fide of this novel method. These results were further corroborated by carrying-out a *blind analysis*, in which samples were subsequently sequenced. Afterwards, we tested the sensitivity of the method. Serial dilutions with WT and mutated T315I templates were performed keeping constant the total template amount. Surprisingly, the method displayed a quite high sensitivity, allowing to detect amount of mutated template as low as 0.5%, which are not identified by classical sequencing allowing the identification of T315I mutation even when present at low amount. We suggest that this approach could be extended to few others relevant and frequent BCR-ABL mutations (i.e. P-loop mutations) thus allowing to drive the clinical decisions after imatinib failure on the use of the most appropriate second generation TKIs or directly to switch to stem cell transplantation.

0574

DEVELOPMENT OF A NOVEL METHOD TO EVALUATE THE EFFICACY OF MOLECULAR TARGETED THERAPY IN CHRONIC MYELOGENOUS LEUKEMIA BY BIO-IMAGING SYSTEM

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Background. Imatinib, a potent inhibitor of the oncogenic tyrosine kinase BCR-ABL, has shown significant clinical achievement in treatment of chronic myelogenous leukemia (CML). However, some patients have shown imatinib resistance and several second-generation ABL kinase inhibitors have also been developed to overcome this problem. Thus, an assay system to examine the drug efficacy for each patient is strongly desired. *Aims*. Our purpose is to develop a bio-imaging system to evaluate BCR-ABL kinase activity in living cells. Here we utilized fluorescence resonance energy transfer (FRET)-based biosensing. *Methods and Results.* CrkL is a main substrate of BCR-ABL in CML cells. We first constructed the probe molecule, which consisted of YFP (yellow fluorescent protein), a part of CrkL and CFP (cyan fluorescent protein), named Pickles. When CrkL is phosphorylated at Tyr207, an intramolecular binding of Src-homology 2 (SH2) domain to phosphorylated Tyr207 induced conformational change of CrkL, brought YFP closed to CFP, and yielded a FRET between YFP and CFP. The FRET efficiency within the cells was evaluated by fluorescence microscopy, which enables the evaluation of the cells one by one. When Pickles is co-expressed with BCR-ABL in 293F cells. FRET efficiency was increased by expression of BCR-ABL in dose-dependent manner. This FRET efficiency was decreased in response to imatinib. When imatinib-resistant type of BCR-ABL mutant was expressed, the FRET efficiency was retained. We also expressed Pickles in K562 cells, a CML-derived cell line. FRET efficiency was increased, and this efficiency was inhibited by imatinib. Finally we expressed Pickles in primary mononuclear cells from *de novo* CML patients. Similar to the results of cell lines, we clearly detected increased FRET efficiency in samples from CML patients. This efficiency was not detected in mononuclear cells from healthy volunteers. *Conclusions*. 1) Pickles, a FRET-based biosensor, enables the evaluation of BCR-ABL kinase activity in living cells. 2) The drug efficacy was assessed by the decrease in FRET efficiency compared to that of control cells. 3) This assay would be applied not only for screening to assess the sensitivity of molecular targeted drugs against CML cells but also for detecting drug-resistant cells.

0575

MULTIDRUG RESISTANCE TESTING WITH ABC TRANSPORTER ASSAY

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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 hematological malignancies. The studies focused on rapidly progressing acute leukaemia's, with the emphasis being on acute myeloid leukaemia (AML). There are numerous technical approaches to study multidrug resistance, which came into the centre of interest; however correlating results derived from different methods raised serious difficulties. We have developed two major forms of ABC transporter assays to investigate the MDR phenomenon: dye efflux assays using whole cells expressing the transporter of interest and ATPase and vesicular transport (VT) assays using purified membrane vesicles. Both methods could be used to determine whether a compound acts as a transporter substrate (EC50) and/or inhibitor (IC50). *Aims*. Aims of this study were to test the interactions several drugs and ABC efflux transporters and to measure function of these transporters on the cell lines. We have intended to develop a new fluorescent assay, in order to extend it for the measurement of BCRP/ MXR activity as well. *Materials and 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 three clinically highly relevant transporters (MDR1, MRP1, BCRP) and different chemotherapeutics (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. Dye efflux assays are based on determining fluorescence intensity differences in a flow cytometer after a short *in vitro* incubation of the cell suspension with a fluorescent dye such as the calcein-acetoxymethyl ester (calcein AM) for MDR1 and MRP1 with or without the addition of selective inhibitors of MDR1 and MRP1 and Hoechst 33342. The BCRP arm of the MDQ kit utilizes mitoxanthrone as dye and Ko134 as BCRP-specific inhibitor. *Results*. We have characterised 35 drugs for their interaction with the MDR1, MRP1 and BCRP transporters. In general, good correlations have been found between the methods employed. Some novel, highly transporter specific interactions have been found (e.g. leflunomide and it's active metabolite teriflunomide specifically interacts with BCRP. Data on the new, improved functional assay measuring the multidrug resistance activity of the three, clinically most relevant efflux transporters, such as MDR1, MRP1 and BCRP in clinical specimens will also be presented. *Conclusions*. It's known, that several review focused drug-transporter interactions but this study demonstrates full particulars regarding transporter profile of selected chemotherapeutic drug. These transporter assays have more capabilities and they are useful for the testing of different parameters: (1) new drug-candidates in drug resistant cell lines and prepared membranes (2) multidrug resistance of the cell lines. These assays and results might have potential role in theranostic application.

0576

ABCB1 SINGLE NUCLEOTIDE POLYMORPHISMS IN *DE NOVO* AML WITH NORMAL KARYOTYPE - IMPLICATIONS ON DRUG RESISTANCE AND SURVIVAL

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Background. Multidrug resistance and expression of the ATP-dependent drug transporting protein ABCB1 is a clinically relevant problem in the treatment of acute myeloid leukaemia. Single nucleotide polymorphisms (SNPs) altering the function and activity of ABCB1 might influence the clinical outcome in AML and predict individual differences in response to therapy with ABCB1 substrates. *Aims*. To investigate the impact of ABCB1 SNPs in exon 11, 12, 21 and 26 on treatment response and survival in AML patients. *Methods*. PCR and Pyrosequencing were used to determine the genotype of the SNPs G1199T/A, C1236T, A1308G, G2677T/A and C3435T in 97 *de novo* acute myeloid leukaemia, patients with normal karyotype treated at Linköping University hospital or Huddinge University Hospital. Almost all patients were treated with one anthracycline and Ara-C during the induction regime. The genetic variants in ABCB1 affect on survival were analysed using Kaplan-Meier Log-rank tests. Patients receiving transplantation were censored at that point in the analysis. A Nordic reference material of 130 healthy volunteers of equal age and sex distribution was also included. *Results*. The survival of the AML patients were significantly correlated to the ABCB1 genotypes G1199T/A (p=0.002), C1236T (p=0.02) and G2677T (p=0.02, A-allele excluded from analysis due to low frequency). Only the wild type of A1308T was found in the material and C3435T did not correlate to survival. Comparison of allele frequencies between AML patients and healthy volunteers showed no significant difference. *Conclusions*. Our findings suggest that ABCB1 SNPs do not affect the development of the disease but the survival after chemotherapy. The correlation between ABCB1 genotype and the overall survival of AML patients might provide useful information for treatment strategies and individualized chemotherapy.

0577

ANALYSIS, PHARMACOKINETICS AND BIODISTRIBUTION OF THE NEW CDK INHIBITOR-CR8

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Background. Roscovitine is cyclin-dependent kinase (Cdk) inhibitor that has been shown to be effective against several tumors, and is passing Phase II clinical trials in cancer patients. The drug has limited side effects and toxicity compared to conventional chemotherapy. However, roscovitine was found to have short half life and rapid metabolism, leading to suboptimal exposure in the clinical trials. Recently, CR8 was introduced as the second generation of roscovitine. CR8 was shown to be about 50-fold more potent compared to roscovitine in inducing apoptosis in different tumor cell lines including ALL and CLL.[1] Despite these promising results still the pharmacokinetic profile of CR8 is not investigated prior to clinical investigation. In the present paper we have estab-

lished an analytical method and evaluated the pharmacokinetics of CR8 in mice. *Aims*. 1- To develop and validate analytical method for CR8 in the biological matrix. 2- To study the pharmacokinetics, oral bioavailability and distribution of CR8 to the bone marrow (BM) in female bulb/C mice. *Methods*. We have developed a simple and rapid liquid chromatographic method using UV-detection for the determination of CR8 in plasma. CR8 was administered both orally (75 mg/kg) and I.V. (50mg/kg), animals were killed at 5, 10, 20, 30 min, 1, 2, 3, 4, 6, 8, 12 and 24 hours. Blood was collected and plasma separated, both femurs were flushed using 0.5 mL PBS. The pharmacokinetic parameters e.g. area under the concentration-time curve (AUC), distribution volume, half life, the maximum concentration, clearance and oral bioavailability were calculated using WinNonlin, Version 5.2. *Results*. The retention time of CR8 was 5 minutes. The lower limit of quantification was 0.10 μg/mL and the linearity was within the range 0.10 - 10 μg/mL (r2 > 0.998). The accuracy and precision were more than 86%. The recovery from plasma was more than 90%. CR8 was stable at RT, +4°C and -20°C for 2 months. CR8 pharmacokinetics after IV and oral administration were found to fit a two-compartment open model with biphasic elimination. CR8 was rapidly absorbed. The exposure to CR8 expressed as AUC was about 85-90 μg/mL.hr and the elimination half life was about 2.5hr which is longer than 2 fold compared to roscovitine. CR8 concentrations in the bone marrow were less than 1% of that observed in plasma. The oral bioavailability of CR8 was found to be 100%. *Conclusions*. CR8 has 100% bioavailability and longer half life compared to roscovitine that allow systemic exposure higher than the IC50 for duration of about 10 hours. The low distribution to BM may indicate lower myelosuppressive effect which in turn can benefit patient treatment. CR8 has pharmacokinetics pattern that may allow further *in vivo* studies.

Reference

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0578

EXPRESSION OF PHOSPHORYLATED HISTONE H2AX AND ITS POTENTIAL TO MODULATE ADRIAMYCIN RESISTANCE IN K562/A02 CELL LINE

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Background. Histone H2AX phosphorylation (ser139) (γH2AX) plays an important role in DNA damage repair, however, whether it is involved in drug resistance remained unknown. *Aims*. To investigate the reversal effect of γH2AX on adriamycin resistance of K562/A02 cells and related mechanisms. *Methods*. We cultured K562/A02 cells with adriamycin and (or) LY294002 which could reduce γH2AX and then analyzed the proportion of cells expressing γH2AX by flow cytometry; the sensitization to adriamycin by MTT assay; the level of DNA double strands breaks (DSBs) by Single-cell gel electrophoresis assay and the expression of P-glycoprotein, γH2AX and BRCA1 proteins by Western blot. *Results*. γH2AX expression increased by adriamycin in a concentration dependent manner (p<0.01); and treated with the same concentration, γH2AX expression of K562/A02 was lower than that of K562 cells (p<0.01). After K562/A02 cells treated with LY249002, the γH2AX expression diminished (p<0.01); the IC50 decreased significantly with a high reverse time of 5.98; the level of DSBs which was judged by tail moment and tail DNA% in K562/A02 enhanced significantly (p<0.01) and the expression of P-gp and BRCA1 suppressed. *Conclusions*. It is feasible to use γH2AX to predict the sensitivity of tumor cells to chemotherapy agents. Depressed H2AX phosphorylation by LY294002 has a reversal effect on the adriamycin resistance of K562/A02 cells through reducing repair of DSBs, and down-regulating BRCA1 and P-gp expression.

Anemia, aplastic anemia - PNH

0579

GENETIC ANALYSIS OF THE SHELTERIN COMPLEX COMPONENTS (POT1, RAP1, TPP1, TRF1 AND TRF2) IN DYSKERATOSIS CONGENITA

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Background. Dyskeratosis congenita (DC) is a multi-system syndrome which displays marked clinical heterogeneity. Patients present with a wide range of haematological and non-haematological features including nail dystrophy, abnormal skin pigmentation, leucoplakia and aplastic anaemia (AA). Six genes have been implicated in DC, all involved in telomere maintenance either by telomere elongation (telomerase) or telomere protection (shelterin). Mutations within these genes account for approximately 50% of cases of DC, with the remainder being genetically uncharacterised. *Aims*. The shelterin complex is comprised of six proteins: telomeric-repeat binding protein 1 (TERF1, TRF1), telomeric-repeat binding protein 2 (TERF2, TRF2), TRF1-interacting nuclear factor 2 (TINF2, TIN2), TERF2-interacting protein (TERF2IP, RAP1), TIN2-interacting protein 1 (ACD, TPP1) and protection of telomeres (POT1, POT1) (gene and protein name abbreviation, respectively). Only TIN2 has been previously screened in DC patients. The aim of this study was to screen the other members of the shelterin complex for any potential mutations that could be associated with DC in a subset of the remaining uncharacterised patients. *Methods*. 47 patients with genetically uncharacterised DC were screened for possible mutations in all the remaining shelterin complex genes. Each gene was analysed by heteroduplex analysis using denaturing high performance liquid chromatography following PCR amplification. Any abnormal elution patterns were sequenced to determine the nucleotide change involved. All coding changes observed were compared to known SNPs on the SNP database at www.ncbi.nlm.nih.gov. *Results*. From all the analyses performed 6 changes were identified that alter the coding sequences of the genes investigated (Table 1). Two of these were novel: POT1c.820G>A p.Gly274Arg and TPP1 c.1383T>G p.Phe461Leu. The POT1 mutation did not segregate with disease and the affected individuals in this family also had mutations in TIN2 (Arg282Cys) There were no additional family members available to perform segregation analysis of the TPP1 mutation. *Conclusions*. The data obtained from this large genetic screen of the remaining proteins of the shelterin complex indicates that there is little involvement of any of these proteins in the pathophysiology of dyskeratosis congenita. This result is surprising as mutations in TIN2 account for approximately 10% of DC. The unclassified variant seen in POT1 is unlikely to be pathogenic due to a lack of segregation within the family whereas it was not possible to draw a conclusion regarding the TPP1 mutation. In conclusion, although TIN2 mutations have been found to play a significant role in the pathophysiology of DC, this does not appear to be case for the other shelterin proteins.

Table 1. Coding changes identified in shelterin complex.

Gene	No. exons	Coding SNPs	Novel coding changes
POT1	15	Gly 404 Val	Gly 274 Arg
RAP1	3	Lys 324 Glu	0
TPP1	12	Val 518 Ala	Phe 461 Leu
TRF1	10	Glu 55 (8-10)	0
TRF2	10	0	0

0580

IMMUNOSUPPRESSIVE THERAPY WITH RABBIT ANTITHYMOCYTE GLOBULIN MIGHT BE COMPARABLE TO HORSE ANTILYMPHOCYTE GLOBULIN IN CHILDHOOD SEVERE APLASTIC ANEMIA WITHOUT HLA-IDENTICAL SIBLING DONOR

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Background. Antilymphocyte globulin or antithymocyte globulin has a major role in immunosuppressive therapy (IST) for children with severe aplastic anemia (SAA), who are not suitable donor for hematopoietic stem cell transplantation. *Aims*. We investigated long-term clinical outcome in in children with SAA, who received IST with horse ALG (H-ALG) or rabbit ATG (R-ATG). *Method*. We retrospectively analyzed 112 children with SAA who were treated with either H-ALG 1mL/kg/day (n=46) or R-ATG 2.5mg/kg/day (n=66), and cyclosporin A. There was no difference in demographic characteristics of both groups, except for the administration of oxymetholone (82.6% in H-ALG vs. 13.6% in R-ATG). *Results*. The overall response rate was higher in H-ALG than R-ATG at post-IST 6 month (p=0.02), and complete response higher in H-ALG than R-ATG at post-IST 6 month (p<0.01). The adverse effects during IST showed similar incidence in both groups. However, relapse rate was higher in H-ALG than in R-ATG (p<0.01), with similar time from response to relapse in both groups. The higher response rate in H-ALG might be contributed by administration of oxymetholone because relapse in H-ALG was developed after stop of androgen. The overall survival (OS) was 69.7%(median: 6 years), and associated with severity at diagnosis, and response on post-IST 6 month (p<0.01). The OS was not different in both groups. The causes of death were mainly infection (66.7%), bleeding (25.9%) and cardiomyopathy due to iron overload (7.4%), and not different between both group. The failure free survival (FFS) was related to response on post-IST 6 month (p<0.01), and similar between H-ALG and R-ATG groups. *Conclusions*. Our results suggest that the front line IST with R-ATG is comparable to H-ALG in childhood SAA. We propose that treatment with R-ATG is accepted as a front line IST for childhood SAA. We need clinical trial for dose modification of R-ATG.

0581

EFFICACY OF THE COMPLEMENT INHIBITOR ECULIZUMAB IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS NEVER TRANSFUSED

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Background. Hemolysis of paroxysmal nocturnal hemoglobinuria (PNH) is due to the lack of the complement inhibitors CD59 and CD55 from the cell surface, leading to complement-mediated intravascular hemolysis and subsequent anemia. The release of free hemoglobin during hemolysis causes a number of debilitating symptoms, which include hemoglobinuria, sever anemia requiring transfusions, disabling fatigue, abdominal pain crises and dysphagia, as well as life-threatening morbidities, such as thromboembolisms, kidney dysfunction and pulmonary hypertension. The terminal complement inhibitor eculizumab (Ecu) has proven effective for the treatment of hemolysis in PNH patients requiring minimal transfusions. However, many patients with PNH do not require transfusions but continue to demonstrate marked hemolysis and its downstream clinical consequences. *Aims*. We assessed efficacy and safety of eculizumab in a cohort of 9 PNH patients who had received no transfusions before starting the anti-complement therapy and in a subset of patients previously enrolled in clinical trials who had been only minimally transfused. *Methods*. The Italian Early Access Program included untransfused PNH patients with at least one of the following conditions: i. severe anemia due to intravascular hemolysis: ii. frequent paroxysmal crises; iii. severe symptoms due to intravascular hemolysis; iv. life-threatening thromboses. Eculizumab was given at the standard dose: 600 mg weekly for 4 weeks; then 900 mg every two weeks, preceded by anti-meningococcal vaccination. We compared our results to a subset of 21 patients included in three previous eculizumab trials, who had received zero or one transfusions during the year prior to the treatment. *Results.* Six of the 9 untransfused patients had a long disease history, while 3 had been recently diagnosed. Even in the absence of transfusion needs, patients had recurrent hemolytic crises, often associated with severe symptoms of PNH (haemoglobinuria, abdominal pain, dysphagia). All patients had moderate anemia, with the exception of two with Hb ≥10 g/dL who were eligible to receive eculizumab because they had experienced brain and/or abdominal thromboemboli. Following eculizumab treatment (median duration 16 months), all patients showed dramatic reductions of hemolysis measured as LDH level, from a median of 1,500 U/L to 356 U/L (upper normal limit 230 U/L; p=0.008). Overall, there was a significant increase in median Hb level (from 9.0 to 10.7, before and after treatment, respectively, p=0.0003), with a median increase of 2.0 g/dL. Eculizumab treatment prevented any further hemolytic crisis and associated serious morbidities in these patients, resulting in improvement of quality of life. With a cumulative exposure of 127 months, no severe adverse event or thromboembolic event has been observed. These data are in agreement with those relating to the 21 patients minimally transfused and treated by eculizumab in the setting of the registration trials (Table). *Conclusions*. This report indicates that eculizumab provides substantial clinical benefit even to hemolytic PNH patients who are not transfusion dependent because of compensated anemia. Besides the immediate effect of eculizumab treatment in increasing Hb level and abrogating all signs and symptoms of intravascular hemolysis, such a treatment promises to result in reduction of long-term morbidity and mortality.

Pre-Ecu Transfusions	N	Treatment Duration (Months)	LDH, (U/L) Median		Hb (g/dL) Median			FACIT-Fatigue score Median	
			Pre-Ecu	During Ecu	Pre-Ecu	During Ecu	Change	Pre-Ecu	Change During Ecu
0*	9	16	1,500	356	9.0	10.7	+2.0	--	--
0-1§	21	6	2,030	336	9.0	10.7	+1.7	29.0	+8.9

Ecu-eculizumab; LDH-lactate dehydrogenase; Hb-haemoglobin; FACIT - Functional Assessment of Chronic Illness Therapy-Fatigue Instrument
* No transfusions ≥ 2 years prior to ecu treatment
§ 0 or 1 transfusions 1 year prior to ecu treatment (from previous eculizumab trials; Blood (ASH Annual Meeting Abstracts) 2007 110: Abstract 840).

Figure 1.

0582

HOME INFUSION OF ECULIZUMAB: A UNIQUE AND INNOVATIVE MODEL OF DRUG DELIVERY TO REDUCE TREATMENT-ASSOCIATED BURDEN AND ENHANCE QUALITY OF LIFE FOR PATIENTS WITH PNH

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Background. Paroxysmal Nocturnal Haemoglobinuria (PNH) is an acquired clonal stem cell disease, characterised by intravascular haemolysis, bone marrow failure and thrombosis. Symptoms of PNH include haemoglobinuria, fatigue, anaemia, thromboses, recurrent pain, renal impairment, erectile dysfunction and pulmonary hypertension. Patient care is complex and challenging, as many patients experience chronic symptoms with periods of acute exacerbations. Historically the management of PNH included bone marrow transplant, blood transfusion and supportive care, all necessitating frequent hospital visits. Eculizumab is a monoclonal antibody that binds to the C5 complement component inhibiting terminal complement formation and preventing haemolysis. Clinical trials of eculizumab demonstrated the resolution of the majority of symptoms and complications of PNH and resulted in its approval in the UK in June 2007. Eculizumab is administered as a 30 minute intravenous infusion every 14 days, and under the terms of its current EU licence, must be administered by a healthcare professional. Due to the rarity of PNH there are relatively few specialist centres resulting in patients travelling long distances for review and treatment. *Aims*. To develop a unique safe method of drug delivery for patients at home to reduce their frequency of hospital visits whilst enhancing patient qual-

ity of life. *Method*. In the UK, Leeds Teaching Hospitals with Healthcare at Home have developed a home infusion programme that ensures safe administration of eculizumab in the patient's home at a time convenient to them, leading to enhanced treatment-associated convenience for patients and their families. Patients then only attend the PNH centre every 3 months to ensure appropriate monitoring and patient education. *Results*. A recent survey of patients reports a reduction in treatment-associated burden for PNH patients and their families when receiving infusions at home. 46 patients responded to the survey with just over half receiving eculizumab. Of the 21 patients at the time receiving home infusions 19 found this more convenient than the hospital. Home treatment allows flexibility and for some, the return to full-time employment, with the associated financial benefits and improvement in psychological well-being. Of the 21 patients on home care 7 stated there ability to work was transformed with a further 10 having great improvement. Whilst the purpose of the survey was not to address financial burden, the home infusion programme has reduced the financial burden on the patient and their family by eliminating the need for time off work, allowing return to full-time employment, and removing the cost of frequent travel to and from the hospital. No patients reported negative impact, including effect on social life and family relationships, whilst 15 experienced improvement or complete transformation in both areas. The patients reported confidence in the homecare programme, knowing that a very close working relationship existed between the expert hospital and homecare teams. *Summary*. This innovative programme of medication delivery by a dedicated home nursing team allows patients who have previously struggled to cope with their illness to lead a near normal life with an associated enhancement in quality of life. Patients are able to carry on with activities of daily life, including work, recreational activities and holidays, whilst at the same time ensuring compliance with treatment and therefore allowing maximum therapeutic benefit.

0583

USE OF FLAER-BASED ASSAY OF WHITE BLOOD CELLS (MONOCYTES AND GRANULOCYTES) IN THE PRIMARY SCREENING OF PNH CLONES

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Background. Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired stem cell disorder caused by a somatic mutation in the X-linked PIG-A gene. This leads to a partial/complete loss of all GPI-linked proteins and clinical features of chronic intravascular hemolysis, thrombosis and marrow failure. Diagnosis and follow-up of PNH improved with flow cytometry-based assays that involved the analysis of CD55 and CD59, typically on red cells and neutrophils. However, the ability to accurately detect PNH RBCs is compromised by prior hemolysis and/or the presence of transfused RBCs. Aplastic Anemia (AA) and Myelodysplastic Syndrome (MDS) patients may also show the presence of PNH clones that are not readily detectable by RBC-based flow assays. We recently described a sensitive multi-parameter fluorescent Aerolysin (FLAER)-based flow assay utilizing CD45, CD33 and CD14 that accurately identified PNH monocyte and neutrophil clones in PNH, AA and MDS patients. The assay could detect small PNH clones (0.5-1%) in samples up to 48 hours post-draw and required only 40 minutes from sample receipt to result availability. Analysis could be performed on a wide range of 4-colour instruments and specialised software was not required. The assay also has utility in detecting other (non-PNH) hematologic abnormalities in samples submitted for PNH screening. *Aims*. In this study, we compared the efficiency of detection of PNH monocyte and neutrophil clones using the FLAER assay with a CD59-based assay on RBCs upon samples submitted for PNH screening at our Institution from October 2005 to December 2008. *Results*. Of 536 evaluable samples, PNH clones were detected in 63 (11.75%) with the FLAER assay, while PNH RBCs were detected in only 33 (52.4%) of these PNH clone-containing samples. Of the samples containing PNH clones by both assays, clone size was always larger with the FLAER assay. When PNH clones were detected in samples with the CD59 test only, repeat analysis of both assays demonstrated the RBC-derived data to be in error. The explanation for this discrepancy in bona fide PNH cases is that the FLAER assay is unaffected by hemolysis and/or prior transfusion. In cases of aplastic anemia and myelodyplasia, almost all patients had prior RBC transfusions, further reducing the utility of the RBC assay. We also stabilized a fresh PNH blood sample and showed that the qualitative and quantitative aspects of the FLAER assay were unaffected by the stabilization process and significant differences were not detected up to 12 weeks post stabilization. These data indicate that it will be possible to generate material suitable for Quality Controlling and Proficiency Testing this assay, an important aspect of clinical assay design. *Summary and Conclusions.* Our results suggest that the FLAER assay on WBC's is a much more sensitive and robust primary screening assay for the detection of PNH clones in the clinical flow laboratory than are RBC-based assays. Since the probability of a thrombo-embolic event is directly related to the PNH WBC clone size, this new assay should improve the identification of patients at risk for this life-threatening complication.

0584

EFFECT OF ECULIZIMAB THERAPY ON THROMBOCYTOPENIA IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Background. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder resulting from a mutation in the PIG A gene of the hematopoietic stem cell. This defect results in the loss of the GPI membrane anchored proteins such as CD 59, a membrane inhibitor of reactive lysis. CD59 deficient cells are more susceptible to complement lysis. All hematopoietic bone marrow derived cells, including the megakaryocyte and platelets, are affected. The disorder is characterized by chronic hemolysis, leucopenia, thrombocytopenia, and high incidence of thrombosis. The association with bone marrow failure syndromes such as Aplastic Anemia, is well recognized. Eculizumab, an inhibitor of C5 activation, reduces red cell hemolysis and the risk of thrombosis in patients with PNH. However, no effect on thrombocytopenia or leucopenia has been reported. *Methods*. We recently reported the preliminary results of an ongoing study (Weitz *et al.* Blood 2008; 112: Abst 407) on the effect of Eculizumab therapy on markers of inflammation and thrombin generation in patients with PNH. Patients were treated by the FDA-approved protocol with blood samples obtained prior to treatment on day 1 and prior to each dose on days 8, 15, 22, 29, 43, and 90. In addition to plasma samples for studies of markers of inflammation and thrombin generation, complete blood counts were also obtained. Treatment resulted in sustained and statistically significant reductions in D-Dimer, thrombin-antithrombin complex (TAT) and IL-6 levels were seen and did not correlate with hemolysis as assessed by measurement of LDH. Since thrombin in a potent platelet agonist, we hypothesized that some patients would demonstrate significant increases in platelet count with Eculizumab treatment. *Results*. At the present time 11 patients have been enrolled in this study with treatment follow-up ranging from 2 to 20 months. 7/11 (64%) patients had thrombocytopenia with platelet counts below 100×10^9/L (range: 26 to 88×10^9/L) prior to treatment. Of the 7 patients, 2 patients had aplastic/hypoplastic bone marrows, but large WBC PNH clones; 2 patients had classical paroxysmal PNH and 2 patients had splenomegaly due to a pre-existing Budd Chiari syndrome. A sustained platelet recovery above 100k occurred in 4/7 (57%) patients with Eculizumab therapy. D-Dimer and TAT were elevated in all of the patients with thrombocytopenia prior to treatment, but decreased with Eculizumab therapy. *Summary*. The results of this ongoing prospective study suggest that the improvement in platelet counts may be occur is some patients with PNH due to a reduction in complement-mediated thrombin generation and thrombin-induced platelet activation and aggregation.

0585

EFFICACY OF THE TERMINAL COMPLEMENT INHIBITOR ECULIZUMAB USED CHRONICALLY IN A PATIENT WITH COLD AGGLUTININ DISEASE

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Background. Previously, we reported initial efficacy data of the terminal complement inhibitor eculizumab in cold agglutinin disease (CAD). Here, we report the chronic use of eculizumab in a patient with CAD refractory to previous treatments. In CAD, IgM autoantibodies can activate the complement system in colder areas of the body (e.g. extremities), resulting in red blood cell hemolysis. Prednisone is typically ineffective and only on rare occasions can chemotherapy suppress autoantibody production. Some cases are mild and self-limited, but others require hospitalization and can be life-threatening. Supportive transfusions of red blood cells may be necessary. Eculizumab, a monoclonal antibody targeting complement factor C5, has shown efficacy in patients

with paroxysmal nocturnal hemoglobinuria (PNH) through inhibition of terminal complement activation. Based on the pathogenesis of CAD, blockade of terminal complement by eculizumab could be a new possible therapeutic approach. *Aims and Methods.* To test for continued efficacy of eculizumab in a transfusion-dependent patient with chronic CAD refractory to extensive previous treatments (including several cycles of rituximab) and experiencing elevated hemolysis for at least 1 year prior to treatment. Eculizumab was dosed as follows: 600 mg IV every 7 days x 4; 900 mg 7 days later; and then 900 mg every 14±2 days. Prior to treatment, the patient was vaccinated against Neisseria meningitidis as eculizumab treated patients are at an increased risk for meningococcal infection. Transfusion requirements as well as clinical and biochemical indicators of hemolysis were monitored. *Results*. Hemolysis, as measured by lactate dehydrogenase (LDH) against time, was significantly reduced 47% from 848±41.2 U/L (mean±SE) to 446 ± 8.3 U/L (12 months pre-treatment vs. 20 months post-treatment). The chronic control of hemolysis resulted in an improvement in anemia (median of 9.9 g/dL 12-months pre-treatment vs. 11.6 g/dL 20-months post-treatment) and reduced transfusion requirements from 18 PRBC units during a 12 month pre-treatment period to 0 during 20 months of treatment. Furthermore, the patient continues to report a sustained improvement in fatigue and quality of life and no longer suffered from angina pectoris at 20 months of treatment. There were no drug-related adverse events and ongoing therapy with eculizumab continues to be safe and well tolerated in this patient. *Conclusions*. Here we present a long-term follow-up of our initial report of eculizumab efficacy in a CAD patient. Importantly, since the initiation of eculizumab treatment, hemolysis has been controlled with no exacerbations of the disease. Chronic treatment with eculizumab remains safe and highly effective for over 20 months. With limited available effective treatment options, this promising result with a well-tolerated antibody provides a strong rationale for a planned clinical trial for the use of eculizumab in patients with CAD.

0586

ANEMIA DURING PEGYLATED INTERFERON-RIBAVIRIN THERAPY RESULTS FROM BOTH INCREASE SUPPRESSION OF ERYTHROID DIFFERENTIATION AND HEMOLYSIS

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Background. Anemia is a common side effect of Pegylated Interferon (PegIFN) and Ribavirin (RBV) combined therapy in patients with chronic hepatitis C (HCV). Hemolysis as consequence of RBV concentration in erythrocytes is the most accepted underlying mechanism; however, since the degree of treatment related anemia is highly variable, other pathogenic mechanisms could be hypothesized. *Aims*. To evaluate the prevalence of hemolysis in patients treated with the combination of PegIFN and RBV compared to patients on monotherapy with PegIFN and to assess other possible mechanisms underlying anemia. *Methods*. We studied 18 patients with chronic hepatitis C HCV-2 treated with PegIFN-α2a (180 mcg/week) plus RBV (800 mg/day) for 24 weeks and 10 patients with chronic hepatitis B treated with PegIFN-α2a 180 mcg/week monotherapy for 48 weeks. Routine hematologic parameter, reticolocyte count and serum LDH and haptoglobin levels were monitored. The therapy effect on erythropoiesis was evaluated *ex vivo* through peripheral erythroid progenitors cell cultures and gene expression analysis during treatment. Peripheral blood mononuclear cells were plated in methylcellulose-supporting media at a concentration of 2×10^5 cells/mL and colony formation (BFUe and CFU-GEMM) was analyzed after 14 day of culture. Expression of gamma-globin and GATA2 gene was evaluated with quantitative real-time PCR. *Results*. All the patients developed anemia after 24 weeks of treatment, although the mean hemoglobin decrease was higher in HCV patients (2.77 vs 1.82 g/dL at week 24, p=0.03), particularly at week 4 (1.86 vs 0.51 g/dL, p=0.007). Only 3 out of 18 HCV patients (16%) developed hemolytic anemia, documented by a marked increase in reticulocytes (3,4%) and in LDH levels (502 U/L) and by a significant decrease in haptoglobin levels (40,3 mg/dL). These patients showed a sharper and faster decrease of Hb compared to the remaining 15 HCV patients (week 4: 3.40 vs 1.55 g/dL, p=0.01). None of the HBV patients on treatment developed hemolytic anemia. HCV patients with hemolysis had an increase in BFUe number at week 4, whereas patients without signs of hemolysis showed a decrease in colonies formation at week 4 (baseline: 15.5 to 7.5 colony/105 cells). A decrease in BFUe number was also observed in HBV patients along PegIFN treatment period. In all the HCV and HBV patients the reduction in BFUe number was associated with an increase in undifferentiated CFU-GEMM colonies (BFUe: 87 to 76% in HCV, 91 to 64% in HBV; CFU-GEMM: 13 to 24% in HCV, 9 to 36% in HBV) and in primitive erythropoiesis specific genes (gamma-globin and GATA2 genes) expression. *Conclusions*. These observations suggested that the inhibitory effect of PegIFN on erythroid differentiation plays a major role in causing anemia even in combined treatment with RBV. The hemolysis as cause of anemia so far attributed to RBV is apparently restricted to few cases and deserves further investigation.

0587

MODIFICATION OF THE STANDARD ECULIZUMAB DOSE TO SUCCESSFULLY MANAGE INTRAVASCULAR HAEMOLYSIS BREAKTHROUGH IN PATIENTS WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

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Background. Paroxysmal nocturnal haemoglobinuria (PNH) is characterised by a lack of CD59 on erythrocytes rendering cells susceptible to intravascular haemolysis. Eculizumab blocks terminal complement resulting in reductions in haemolysis, thrombotic events, renal impairment and transfusion requirement and improvement in quality of life. The standard dosing regimen for eculizumab is 600 milligrams per week for 4 weeks; 900 milligrams one week later; and then 900 milligrams every 14±2 days. This regimen maintains eculizumab levels >35 micrograms per millilitre which consistently blocks complement-mediated haemolysis. 4/195 (2%) patients in PNH clinical trials were not consistently blocked with this regimen and developed breakthrough intravascular haemolysis, with haemoglobinuria, abdominal discomfort and dysphagia, 12-13 days after an eculizumab infusion. *Aims*. An increased dose of 1200 milligrams of eculizumab every 2 weeks was evaluated for effectiveness and safety. *Method*. LDH, pharmacokinetics (PK), and clinical signs of complement breakthrough were monitored.

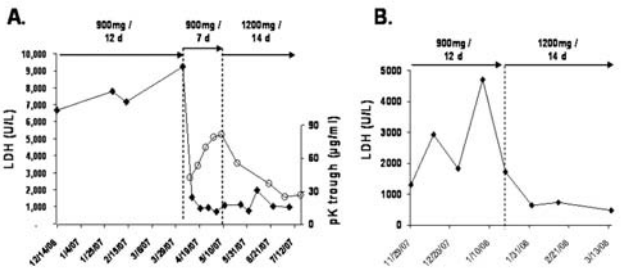

Figure 1. Patient LDH levels in response to 1200 mg dosing.

Results. Patient 1 was managed for 6 months with 900 milligrams every 12 days before experiencing complement breakthrough (Figure, panel A). LDH levels (closed diamonds) reached 9234 U/L (ULN, 430-450 U/L) and breakthrough symptoms occurred 2 days prior to the next dose. The patient was re-induced with 900 mg eculizumab every 7 days for 5 weeks followed by 1200 milligrams every 14 days. Trough levels of eculizumab increased (open circles) each week during the induction phase (42.7 - 81.8 µg/mL) resulting in an immediate reduction in LDH to near normal levels. A maintenance dose of 1200 mg every 14 days resulted in sustained complement blockade with normal LDH levels for 1 year before further breakthroughs occurred. A further 1200 milligrams dose in between the 14 day dosing interval every 4-5 doses provides complement blockade. Patient 2 experienced breakthrough haemolysis after 19 months of standard dosing. Complement breakthrough occurred during a post-cholecystectomy infective endocarditis. An adjustment to 900 mg every 12 days did not control complement breakthrough (Figure 1, panel B) and the dose was changed to 1200 mg every 14 days. Further episodes of haemoglobinuria and other symptoms of haemolysis were not observed. Patients 2 and 3 experienced breakthrough haemolysis after 4 and 19 months of standard dosing respectively and were changed to 1200 milligrams every 14 days without re-induction. No further episodes of breakthrough haemolysis have occured. Patient 4 received

900 milligrams every 12 days for 58 months after breakthrough before changing to 1200 milligrams every 2 weeks for convenience. *Summary*. It is important to recognise the phenomenon of breakthrough from complement blockade as these patients can be effectively managed with a higher dose of eculizumab. We demonstrate good correlation between eculizumab and LDH levels, suggesting that complement breakthrough can be monitored by LDH levels near the end of the dosing interval. It appears that using the standard dosing regime that approximately 2% of patients will break-through and this appears to occur within the first 12 months of eculizumab therapy.

0588

DIAGNOSING IRON DEFICIENCY ANEMIA IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Background. Iron deficiency anemia (IDA) in patients with rheumatoid arthritis is difficult to diagnose with certainty. *Aims*. The aim of our study was to explore the value of hepcidin, reticulocyte hemoglobin equivalent (Ret-He), erythrocyte hemoglobin equivalent (RBC-He) and delta hemoglobin equivalent (Delta-He) in diagnosing iron deficiency anemia (IDA) among patients with rheumatoid arthritis in comparison to the conventional diagnostic parameters mean cell volume, ferritin and transferrin saturation. *Methods*. One-hundred-and-six patients with rheumatoid arthritis participated in this explorative cross-sectional study. Fresh EDTA-anticoagulated blood samples of all patients were analyzed for Ret-He, RBC-He and Delta-He on a Sysmex XE-5000 automated hematology analyzer. Serum hepcidin levels were measured by weak cation exchange chromatography followed by mass spectrometry. The presence and type of anemia (IDA, anemia of chronic disease or both) was determined by conventional laboratory tests. *Results*. Receiver operator characteristic analysis showed that hepcidin had the highest discriminating power in the diagnosis of IDA (either with or without anemia of chronic disease), with an area under the curve (AUC) of 0.85 (95% confidence interval = 0.71-1.00). RBC-He had only a slightly lower performance, with an AUC of 0.83 (0.67-0.95). Also Ret-He turned out to be useful for this diagnostic purpose (AUC = 0.77; 0.59-0.99), but Delta-He did not lead to a better identification of IDA as coincidence would do. The percentage total agreement between the conventional classification and the classifications based on hepcidin or RBC-He measurement alone was 75% and 72%, respectively. *Summary and Conclusions*. Our results show that both hepcidin and RBC-He are useful for the differentiation between the different types of anemia in patients with rheumatoid arthritis.

0589

IN VITRO EFFECT OF RIBAVIRIN AND PEGYLATED INTERFERON ON ERYTHROPOIESIS

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Background. The antiviral drug ribavirin (RBV) is widely used in combination with pegylated interferon (PegIFN) for the treatment of hepatitis C virus (HCV) infection. A major side effect of RBV is a reversible anemia. The mechanism underlying anemia in RBV treated patients remains speculative although hemolysis was suggested. *Aims*. To obtain more insights on this issue, we evaluated *in vitro* the effect of RBV and PegIFN on pheripheral CD34 proliferation and erythroid differentiation. *Methods*. After informed consent, the mononuclear cells derived from pheripheral blood of healthy volunteers were enriched for $CD34^+$ cells by positive selection using anti-CD34-tagged magnetic beads. $CD34^+$ cells were cultured for 14 days with a specific medium containing erythropoietin to induce erythroid differentiation. RBV (0.5 mM and 0.1 mM) and PegIFN (100U and 1000U) were added alone or in combination at different days of culture: precisely at day 0 corresponding to CD34 stage and at day 7 corresponding to proerythroblast stage. Cells growth and viability were evaluated by tripan blue exclusion; erythroid differentiation was investigated by cytofluorimetric analysis of Glicophorin A (GPA) expression, by morphological analysis on benzidine-May-Grunwald-Giemsa stained smears and by erythroid specific (gamma-globin and GATA2) gene expression analysis with real-time PCR. *Results*. RBV inhibited cell growth and differentiation with reduction of GPA^+ cells in a time and dose dependent manner; drug added at day 0 of culture had a stronger inhibitory effect than addition at day 7 as well as RBV 0.5mM versus RBV 0.1 mM (Addition at day 0: RBV 0.1 mM: 8.5 and 1.6-fold reduction in cells growth and GPA+ cells respectively vs untreated cells; RBV 0.5 mM: 12 and 30-fold reduction in cells growth and GPA^+ cells vs untreated cells. Addition at day 7: RBV 0.1 mM: 2.4 and 0.7-fold reduction in cells growth and GPA+ cells respectively vs untreated cells; RBV 0.5 mM: 3.6 and 2.3-fold reduction in cells growth and GPA^+ cells vs untreated cells). Addition of PegIFN both at day 0 and day 7 of culture delayed erythroid differentiation with reduction of GPA^+ cells and orthocromatic erythroblasts and increase of more undifferentiated polychromatophilic erythroblasts and primitive erythropoietic genes expression (GPA^+ cells: 61% untreated cells, 50% PegIFN100U and 47% PegIFN1000U; orthocromatic e.: 55% untreated cells, 56% PegIFN100U and 23% PegIFN1000U; polychromatophilic e.: 17% untreated cells, 37% PegIFN100U and 57% PegIFN1000U; gamma gene expression: 1 untreated cells, 1.75 PegIFN100U and 2.39 PegIFN1000U). The combination of PegIFN and RBV both at day 0 and day 7 of culture affected cell growth and erythroid differentiation less than RBV alone; however cells treated with the combination of PegIFN and RBV were more undifferentiated than cells treated with PegIFN alone. *Conclusions*. All these data confirmed the inhibitory effect of PegIFN on erythroid differentiation and strongly suggested an inhibitory action of RBV on cell growth, particularly on undifferentiated stem cells. These observations provide possible explanation for the pathophysiology of anemia observed with ribavirin treatment.

0590

ANTI CD 20 (RITUXIMAB) IN PEDIATRIC AUTOIMMUNE HAEMOLYTIC ANAEMIAS. LONG FOLLOW UP IN A SINGLE CENTRE

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Introduction. Pediatric autoimmune haemolytic anaemia (AIHA) usually responds to steroids therapy. However, in some cases, especially in infants and adolescents, it can be resistant. In these patients failure of second and third line treatments is frequent. In the past years the chimeric monoclonal antibody that targets CD 20, expressed in B lymphocytes surface, has been used in the management of autoimmune diseases. Recently it has been implemented in pediatric autoimmune anaemia. *Aims*. To communicate a single centre experience with rituximab as a salvage treatment of pediatric autoimmune haemolytic anaemia. *Methods*. Children and infants with refractory or steroid-dependent AIHA were included in this series. Rituximab was administered as a third-line treatment (375 mg/m^2/week, 4 doses). Prophylactic iv immunoglobulins and cotrimoxazole were administered to all patients. *Results*. Table 1.

Table 1.

n	age	DAT	Δ FLT-RTX	days to normal Hb	relapse (n)	2 nd RTX (t)	BL recovery	follow up
1	11mo	IgG warm	3.5mo	26	no	no	3mo	52mo
2	3mo	IgG warm	1.5mo	39	no	no	5mo	46mo
3	13yrs	IgG warm	2.5mo	26	yes	yes (8mo)	no	36mo
4	18mo	IgG/C3 warm	11mo	45	no	no	3mo	18mo
5	4yrs	IgG warm	4.5mo	60	yes	yes (8mo)	4mo	16mo
6	5yrs	IgG warm	8mo	pr	no	no	no	4mo

FLT: first line treatment; RTX: rituximab; BL: B-lymphocytes; pr: partial response; FLT-RTX: interval between FLT and RTX; (t) time from first dose of previous course.

Conclusions. In this series, children and infants with AIHA achieving successful results with rituximab are described. All of them were treated with steroids and immunoglobulins as a first line therapy. No serious side effects or opportunistic infections were observed in a long follow up

period with a median of 27 month (range 4-52 mo). Rituximab was effective in controlling refractory disease in all patients but two, who achieved partial response. Responders took 4-8 weeks to attain normal haemoglobin values. Disease may recur but it can be responsive to a second course of rituximab. The immunosuppression and potential life-threatening opportunistic infections reported in other series with rituximab have to be balanced against the harmful complications of prolonged corticosteroid use and other immunosuppressant agents in this young population.

0591

LONG-TERM OUTCOME OF ACQUIRED APLASTIC ANEMIA: COMPARATION BETWEEN IMMUNOSUPPRESSIVE THERAPY AND BONE MARROW TRANSPLANTATION

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Background. Both immunosuppressive therapy (IST) and bone marrow transplantation (BMT) are accepted treatments for patients with aplastic anemia (AA). Choosing one of these therapies depends not only on donor availability but also on such factors as patient age. The aim of this study was to compare survival rates and long-term complications after IST or BMT in patients with AA and to identify prognostic factors associated with improved survival. *Patients and Methods*. between 2/1986 and 2/2009 48 patients with newly diagnosed AA were treated either with allogeneic BMT (18 patients) or with IST (32 patients). The median time from diagnosis to therapy was 2 months (range 1-9) in IST and 3 months (range 1-16) in BMT group. There was no statistical difference between 2 treatment groups in sex, severity of AA, disease duration, previous transfusion support, except age (patients in BMT group were younger). Eighteen allogeneic BMT were performed in 16 patients. All donors were HLA-identical sibling (1 donor was identical twin). Source of stem cells was bone marrow in 15 (2 with second transplants) and peripheral stem cell in 3 BMTs. Conditioning regimens were based on cyclophosphamide (CY) with antithymocyte (ATG) in 15 and Flud with CY and ATG in 3 BMTs. Nineteen patients received combined IST with ATG or ALG (antilymphocyte globuline), cyclosporine A and steroids and 13 patients ATG with steroids (from which 5 were splenectomized). *Results*. engraftment was documented (median time 16 days) in 15 transplanted patients (93.8%). In the first 100 days died 3 (18.8%) transplanted patients: one without engraftment (6.3%) on +23 days with gram-negative sepsis, the other developed steroid-refractory aGvHD grade 3-4 with invasive fungal infection and died on 78 days and the third died on 60 days with pneumonitis interstitialis (CMV+). Two patients (12.5%) who rejected allograft after 6.5 months, were retransplanted: 1 successfully, the other died 8 days following the second BMT. One female patient (8.3%) developed cGvHD. In IST group responded 25 patients (78.1%) and 6 of them (24%) had two cycles IST. Five patients (15.6%) from IST group died, major causes of death were infection and hemorrhage. Aplasia recurred in 6 patients (24%) with partial response following IST in 24 months. Long-term complications of IST were: an evolution in MDS/AML after 76 months at 2 patients (8%), avascular necrosis of hip at 5 patients (20%) and Ca prostate gland (after 150 months) at 1 patient (4%). Between groups did not difference in probability 12-years overall survival: 65% in BMT and 71% in IST group. Difference in survival was found in patients with/without very severe neutropenia (p<0.05) and with/without response/engraftment (p<0.001). An improvement of peripheral blood counts was faster following BMT (median 16 days) related to IST (median 4 months) (p<0.001). *Conclusions*. This study indicates that over 65% of patients with AA can be successfully treated with either BMT or IST. Long-term overall survival does not differ in the two treatment groups.

0592

THALASSEMIA INTERMEDIA: AN EGYPTIAN EXPERIENCE

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Background. Thalassemia Intermedia (TI) includes a wide clinical spectrum of varying severity.A number of clinical complications commonly associated with thalassemia major (TM) are rarely seen in (TI). There are no clear guidelines for initiating and maintaining transfusions in TI for prevention of complications. *Aims.* To assess the phenotypic variability of Egyptian children, adolescents and young adults with TI, their clinical characteristics as well as frequency of complications in comparison to age and sex matched β TM patients. The clinical and haematological response to various therapeutic modalities in TI patients were assessed. *Study Design*. Sixty TI patients (aged 4-25 years, mean 12.48± 5.43 years) were compared to forty β-TM patients (aged 4-25 years, mean 13.22±5.97 years). All patients were subjected to full history taking and clinical examination stressing on transfusion protocol, chelation therapy and presence of complications. Investigations included complete blood count, haemoglobin electrophoresis,serum ferritin, as well as abdominal ultrasound, two dimensional M- mode echocardiography, with Echo-Doppler, and Dual Energy X-ray Absorptiometry for measurement of bone mineral density(BMD).TI patients were subdivided into 3 groups according to therapy: group A(n=20) received hydroxyurea (mean dose of 20.7±3.58 mg/kg/day),group B(n=20) received hydroxyurea (mean dose of 18±5.2 mg/kg/day)with L-carnitine (mean dose of 45±10.2 mg/kg/day),and group C (n=20) received L-carnitine (mean dose of 50±4 mg/kg/day). All patients were prospectively followed up for 12 months for clinical and haematological response. *Results*. Compared to TM patients, TI patients had higher incidence of left atrial dilatation (p=0.008),right ventricular dilatation (p<0.001)and pulmonary hypertension (p=0.001). However, TM patients had higher incidence of left ventricular dilatation (p=0.001), restrictive left ventricular filling pattern (p=0.03), and impaired left ventricular contractility (p=0.01), with an overall higher incidence of heart disease compared to TI patients (45% versus 17.5%, p=0.001). Higher incidence of osteoporosis was present in TM compared to TI patients (40% versus 15%, p=0.006).TI patients had higher incidence of fractures compared to TM patients (5% versus 2.5%, p=0.03).Compared to TM, TI patients had lower prevalence of hepatitis B viral infection (p=0.04),diabetes mellitus (p=0.03),delayed puberty (p=0.01) and short stature(p=0.005). However TI patients had higher incidence of cholelithiasis and thrombocytosis compared to TM patients although did not reach statistical significance. Therapy with hydroxyurea alone in TI was associated with significant decrease in transfusion index, increase in mean haemoglobin and mean haemoglobin F values (p<0.001),with improvement in quality of life. L-carnitine therapy used alone or when added to patients already on hydroxyurea treatment did not have significant effect on transfusion frequency or haemoglobin F (p>0.05). *Conclusions.*TI patients differ from TM in the frequency and distribution of complications.The use of hydroxyurea in paediatric TI patients to minimize or even eliminate the need for blood transfusion is recommended. L-carnitine therapy should be individualized according to the patient response.

0593

SUCCESSFUL TREATMENT OF COMPLEMENT MEDIATED REFRACTORY HAEMOLYSIS ASSOCIATED WITH COLD AND WARM AUTOANTIBODIES USING ECULIZUMAB

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Background. In about 10% of patients, chronic lymphocytic leukemia (B-CLL) is complicated by autoimmune haemolysis. Approximately 80% of cases are caused by autoantibodies of the IgG type (warm autoantibodies) while about 20% of cases are mediated by autoantibodies of the IgM type (cold autoantibodies). Destruction of RBCs by cold autoantibodies is mediated by complement activation and subsequent formation of the membrane attack complex on the cell surface. Eculizumab blocks the cleavage of complement protein C5 and is a potent inhibitor of terminal complement activation. Eculizumab is approved for the treatment of paroxysmal nocturnal haemoglobinuria (PNH). *Aims*. We describe a case of autoimmune haemolytic anemia due to both cold and warm autoantibodies that was successfully treated with eculizumab. *Case report.* A 50 year old male with chronic lymphocytic leukemia (B-CLL) and chronic hepatitis B presented with severe autoimmune mediated haemolytic anemia. Laboratory tests were as follows: haemoglobin 7.2 g/dL, reticulocytes 342×10^9/L, LDH 372U/L, haptoglobin undetectable, and total bilirubin 101 µmol/L. Serologic testing revealed cold and warm autoantibodies as well as erythocyte allo-antibodies type anti-Cw and anti-Lua. The patient was also direct antiglobulin test (DAT) positive with polyspecific antiglobulin serum, monospecific anti-IgG, anti-IgA, anti-C3c and anti-C3d antibodies. Serum levels for complement factors were low: C3c 0.51g/L (normal range 0.90-1.80) and C4 <0.01g/L (0.10-0.40). He required up to 8 PRBC transfusions/week. Various therapeutic approaches failed: 1) prednisolone (beginning with 2 mg/kg/day over a period of 4 months), 2) polyvalent human immunoglobulins (2 g/kg), 3) rituximab (4 cycles of 375 mg/m^2) and cyclophosphamide (500

mg), and 4) alemtuzumab (90 mg/week s.c.) and rituximab (375 mg/m^2/week i.v.) for 8 weeks. Thus we decided to use the complement inhibitor eculizumab off label. The patient gave informed written consent. One month after ending the alemtuzumab/rituximab-combination therapy, eculizumab was given 4 infusions of 600 mg/week followed by 900 mg every 2 weeks. Meningococcal vaccination was given as per label. DAT became negative for anti-IgA and anti-C3c during treatment. Indirect antiglobulin test showed a decrease of free autoantibody in the serum from a titer of 1: 32 to 1:4 during therapy. No change in the titers of erythocyte allo-antibodies type anti-Cw and anti-Lua was observed. The number of PRBC units transfused has dropped from 131 during the 7 months before eculizumab therapy to 12 during 7 months of eculizumab treatment and haemoglobin levels have remained stable. The patient has been transfusion independent for the last 4 months and continues on eculizumab. *Conclusions*. We report a case of severe refractory autoimmune haemolytic anemia due to both cold and warm autoantibodies that was successfully treated by the complement inhibitor eculizumab. We conclude that eculizumab could be an effective treatment for complement mediated autoimmune haemolysis. Clinical trials confirming our results are warranted.

0594

EFFECT OF DIHYDROARTEMISININ ON HUMAN ERYTHROID CELL DIFFERENTIATION: IMPLICATIONS FOR MALARIA TREATMENT IN PREGNANCY

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Background. Severe malaria in pregnancy causes anemia, low birth weight and increased mortality of both mother and infants. WHO recommends few antimalarials due to safety problem; artemisinin combination therapy is the first line treatment; however artemisinin derivatives showed animal embryotoxicity with a reduction of embryonic erythrocytes when treatment is performed on certain days of gestation. *Aims*. To investigate the effect of dihydroartemisinin (DHA), the metabolite of artemisinin, on an *in vitro* model reproducing human erythropoiesis and to characterize the target erythroid stage, in order to predict the window of susceptibility to DHA in human pregnancy. *Methods*. The mononuclear cells derived from pheripheral blood of healthy volunteers were enriched for CD34$^+$ cells by positive selection using anti-CD34-tagged magnetic beads. CD34$^+$ cells were cultured for 14 days with a specific medium containing erythropoietin to induce erythroid differentiation. DHA at 0,5 or 2 µM, according to the dosages of previous animal experiments, was added for the first time at day 0 (on isolated stem cell), at day 2 (on early erythroid progenitors), at day 4 (in presence of both early progenitors and pro-erythroblasts), at day 7 (on basophilic erythroblasts) or at day 11 (polychromatic erythroblasts) and then continuously every 3 days up to 14 days, because of its short half life. Cells growth and viability were evaluated by tripan blue exclusion; erythroid differentiation was investigated by cytofluorimetric analysis of Glicophorin A (GPA) expression, by morphological analysis on benzidine-May-Grunwald-Giemsa stained smears and by erythroid specific globin gene expression analysis with real-time PCR. *Results*. DHA added on stem cells or early erythroid progenitors (day 0 and 2 of culture) caused a transient inhibition of both cell growth and erythroid differentiation (p<0.05) up to day 7, but then the treated cells started growing and completed their erythroid differentiation at day 14 of culture. When DHA was added on more differentiated basophilic erythroblasts (day 4 and 7 of culture) a significant and long lasting decrease in proliferation as well as a delay in erythroid differentiation was observed up to day 14. With DHA added on mature stages (polychromatic erythroblasts, day 11 of culture), only a small reduction of cell growth has been observed without any consequence for erythroid cell differentiation. *Conclusions*. These data suggest that DHA's specific target is the basophilic erythroblast since DHA added at this stage causes a significant inhibition of erythroid differentiation. Based on these *in vitro* results, we hypothesize that DHA could affect human primitive erythropoiesis, which occurs during the late phase of human secondary yolk sac erythropoiesis (weeks 4-8 of gestation), when foetal blood is formed of only primitive erythroblasts. This means that if the treatment with DHA or artemisinin derivatives is performed during the first trimester of human pregnancy, toxic effects on embryo could be expected.

0595

OSTEOPROTEGERIN AND RECEPTOR ACTIVATOR OF THE NF-KB LIGAND (RANKL) IN EGYPTIAN PATIENTS WITH THALASSEMIA-INDUCED OSTEOPOROSIS

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Background and Objectives. Osteoporosis represents an important cause of morbidity in thalassemic patients, many etiologic factors were accused. The aim of the study was: to characterize the possible role of the osteoprotegerin (OPG) and receptor activator of the NF-κB ligand (RANKL) system & Viral hepatitis-C infection (HCV) in Egyptian thalassemic patients with evident bone loss. *Individuals and Methods*. The study group: the study included 35 thalassemic patients (19 males and 16 females), mean age 17.3 years ± 5.57; range(9-25) year old; The control group: included 35 healthy age and sex matched individuals. *Methods*. medical history, clinical examination, abdominal ultrsonography were carried out to all individual included in the study. Complete blood count, serum ferretin, calcium, phospshorus, alkaline phosphatase measurements; HCV antibodies by Elisa and RT-HCV PCR followed by serum concentrations of OPG and RANKL, were carried out. In addition to lumbar spine bone mineral density (BMD) by DEXA. *Results*. Thalassemic patients had significantly lower levels of OPG compared with healthy controls (1.41±0.74 vs. 3.31±1.95, respectively; p<0.05) and higher not statistically significantly, serum levels of s-RANKL (2.11±0.75 vs. 1.96±0.97, respectively; p<0.05) with a consequent significantly lower OPG/RANKL ratio (0.75±0.23 vs. 2.37±1.93, respectively; p<0.05). Also, thalassemic patients displayed a lower bone mineral density values (detected by DEXA) than the healthy controls group. HCV infection was detected in62.1% of the thalassemic patients; the incidence of osteoporosis was numerically increased in thalassemic patients infected with HCV (27.8% vs. 9.1% with HCV negative patients; p value > 0.05). Differences between HCV infected thalassemic patients and HCV non infected thalassemic patients regarding OPG, RANKL and OPG/RANKL ratio were statistically significant (p>0.05). ROC curve analysis showed that an OPG value ≤1.5 can be used to detect osteopenia/osteoporosis among thalassemic patients with 67% sensitivity (95% CI=44.7-84.3) and 76% specificity (95% CI=58.8-89.2) and an OPG/RANKL ratio ≤0.95 can be used to detect the presence of osteopenia/osteoporosis with 80% sensitivity (95% CI=57.8-92.8) and 65% specificity (95% CI=46.5-80.2). *Conclusions*. Our data suggest that, in thalassemic patients, an altered modulation of the OPG/RANKL system resulting in the decrease of OPG could contribute to the enhanced osteoclastic bone resorption and bone loss characteristic of thalassemic patients. HCV infection could be considered as a potential osteoporotic aggravating factor in these group of patients.

0596

A CHILD WITH DYSKERATOSIS CONGENITA AND RECENTLY DESCRIBED TINF2 MUTATION

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Introduction. Dyskeratosis congenita (DC) is a multisystem bone marrow failure syndrome characterized by a triad of mucocutaneous abnormalities (skin pigmentation, nail dystrophy, leucoplakia) and a predisposition to cancer. The genetic basis of DC remains unknown in more than 60% of patients. Mutations have been identified in components of the telomerase complex (dyskerin, TERC, TERT, NOP10, and NHP2), and recently in one component of the shelterin complex TIN2 (gene TINF2). *Case report*. A 26 month-old boy had gum bleed during an upper respiratory infection and his complete blood count showed anemia and thrombocytopenia. He was the second child of the family. There was no consanguineous marriage and parents and brother were healthy. Patient born at term and had good features except dystrophic nails. There was no abnormal skin pigmentation. Complete blood count showed a Hb 9.4 g/dL, MCV 95.5 fl, WBC 8050/mm^3, ANC 1000/mm^3, PLT 50700/mm^3 and reticulocyte count 29000/mm^3. Bone marrow aspiration and biopsy were hypocelular. Hb electrophoresis showed increased HbF (17%). There was no increase in chromosomal breakage with DEB and nitrogen mustard. Cytogenetic study showed a normal karyotype (46 XY). Viral serologic study of hepatitis A, hepatitis B, hepatitis C, EBV, CMV and parvovirus showed no acute viral infection. Informed consent of the parents was obtained for the genetic study of DC. A mutation analysis showed TINF2 mutation c.845 G>A amino

acid substitution:p.Arg 282His. This gene encoding a component of the sheltrin complex has been screened by a combination of denaturing HPLC and direct sequence analysis. The patient was heterozygous for the mutation. Mother did not carry the mutation. The patient could not undergo a hemotopoietic stem cell transplantation due to unavailability of a donor. He is in the 40th month of the follow-up and on androgen (1-2 mg/kg/day) and metil prednisolon (0.25 mg/kg/day) therapy. He is followed as outpatient without any severe infection so far but packed red cells are infrequently transfused. He also had epiphora in the last months due to stenosis of lacrimal canal which is an associated finding in DC. *Discussion*. TINF2 mutations account for approximately 11% of all DC cases. Mutations affect amino acid 282, changing arginine to histidine or cysteine. Telomere lengths in patients with TINF2 mutations were the shortest compared with other DC subtypes, but TERC levels were normal. Patients with TINF2 mutations have severe disease, with most developing aplastic anemia by the age of 10 years. TINF2 is generally a *de novo* mutation. Physical anomalies of the patients with aplastic anemia must be carefully evaluated. Dystrophic nails must not be overlooked. Patients with DC may not have typical skin findings. Useless treatments as immunotherapy must not be tried in patients with congenital bone marrow failure syndromes. *Acknowledgement*. Thanks to Tom Vulliamy, Inderjeet Dokal and coworkers from Center for Paediatrics, Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry for performing the mutation analysis of the patient.

0597

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN ADOLESCENTS AND ADULTS. UNDERESTIMATED PROBLEM?

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Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening syndrome very rarely described in adults and usually not even mentioned in manuals of internal medicine. Its initial presentation may resemble sepsis and patients are initially admitted to infectious diseases departments and then unsuccessfully treated for this condition. After the first such patient was referred to our Department because of pancytopenia, within 4 years we identified additional seven who have met the diagnostic criteria for HLH. Description of their clinical characteristics, treatment and outcome constitutes this report. *Materials and methods*. Eight patients, age between 15 and 41 years, 5 of them male and 3 female are included. They have been evaluated for HLH symptoms using the Histiocyte Society Criteria excluding sCD25 and cytotoxicity assays for NK cells that were not available. All of them meet at least 5 out of 6 remaining criteria. *Results*. All patients presented with fever, splenomegaly, hyperferritinemia (8514-76315 ng/mL), hypertriglyceridemia, all except one had also hypofibrinogenemia. All had cytopenias of normal cells, including 3 who had very severe pancytopenia. In all hemophagocytosis could be demonstrated, 3 in the bone marrow aspirate, 3 in trephine biopsy, and in one only in a lymph node biopsy. Other abnormalities included hepatomegaly in all patients, skin changes (3 patients panniculitis, 4 patients maculopapular rash, 1 patient without skin involvement), in 7 patients fluid in either peritoneum, pleura or pericardium, coagulopathy with very high D-dimer level (16600-58700 ng/mL), and 3 patients with both a very prolonged prothrombin and APTT time, highly elevated CRP, LDH, β2-microglobulin (serum), moderately elevated transaminases, and decreased albumin level. One had concomitant blastic phase of chronic myelocytic leukemia as initial presentation associated with pruritus. One patient had a history of moderate lupus erythematodes, and one had clearly EBV-associated HLH (2910 EBV DNA copies in the peripheral blood). In the remaining patients there was no obvious trigger for the syndrome, but all had elevated levels of some anti-EBV antibodies (VCA, EA, EBNA) without clear and uniform pattern. EBV PCR was positive only in 1 of remaining 4 tested patients. In the patient with lupus erythematodes, EBV was detected in the excised lymph node by EBV-LPM staining. Three patients, transferred to our hospital in grave condition, died without specific treatment within few days of admission and were diagnosed postmortem. Five patients started treatment according to the HLH-2004 protocol (etoposide, cyclosporine A, corticosteroids) and one died day 18 of Enterococcus sepsis with ferritin level already decreased 10-fold. Of the remaining four, three achieved complete remission that has been maintained between 10 and 18 months, and one is in partial remission maintained on cyclosporine A. *Conclusions*. Treatment with etoposide, cyclosporine A and corticosteroid s has allowed for control of the syndrome for all four patients who survived long enough to achieve remission. Identification of eight cases in relatively short time in a single department may suggest that this syndrome may occur more frequently in adults than usually anticipated.

0598

TAFI (THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR) AS A MARKER OF HEMOSTATIC ALTERATION IN PATIENTS WITH β-THALASSEMIA

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Background. Profound hemostatic changes have been observed among thalassemic patients. Thrombin activatable fibrinolysis inhibitor (TAFI) is newly discovered protein which potentially attenuate fibrinolysis. *Aims*. We aimed to investigate plasma level of TAFI in splenectomized and non-splenectomized β-thalassemia patients and to correlate its level with clinical severity and hemostatic alteration. *Methods*. 51 patients (mean age 10.79±5.59 years)(21 splenectomized thalasemia major patients, 18 non splenctomized thalassemia major patients, 12 non-splenectomized thalassemia intermedia) were recruited from Pediatric Hematology Clinic, Ain Shams University, Cairo, in addition to 32 healthy age and sex matched controls(mean age 10.31±5.58 years)were included. In addition to clinical assessment, laboratory investigations included complete blood count, prothrombin time, activated partial thromboplastin time, liver function tests, viral hepatitis markers, serum ferritin and plasma TAFI levels. *Results*. Our study shows significant increase in prothrombin time and activated partial thromboplastin time in thalassemic patients compared to controls. Significant reduction in TAFI levels was shown in thalassemic patients compared to controls ($p<0.0001$), in splenectomized compared to nonsplenectomized thalassemia group ($p<0.0001$) and in thalassemia major compared to intermedia group ($p<0.0001$). Negative correlation was present between TAFI levels and liver enzymes and serum ferritin levels ($p<0.05$).Thalassemic patients suffering from bleeding showed lowest mean TAFI levels. *Conclusions*. Marked alteration in TAFI levels was observed in thalassemic patients with splenectomy, altered liver functions and poor chelation who therefore might be at a higher risk for altered hemostasis.

Granulocytes

0599

NAMPT IS ESSENTIAL FOR THE G-CSF-INDUCED MYELOID DIFFERENTIATION

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Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting protein in NAD+ biosynthesis by converting nicotinamide to nicotinamide mononucleotide, which is further converted into nicotinamide adenine dinucleotide (NAD+). NAMPT also known as pre-B cell colony enhancing factor (PBEF), was originally identified as a gene product expressed in activated peripheral human lymphocytes and was found to be implicated in the maturation of B cell precursors. We identified that NAMPT is an essential enzyme, which mediate granulocyte colony-stimulating factor (G-CSF)-triggered granulopoiesis in healthy individuals and in individuals with severe congenital neutropenia (CN) (Skokowa *et al.*, Nature Medicine, 2009). CN is a hematopoietic disorder characterized by a *maturation arrest* of granulopoiesis in bone marrow at the promyelocyte stage, by absence of neutrophilic granulocytes in peripheral blood and by severe bacterial infections. G-CSF treatment increases blood neutrophil numbers in more than 90% of individuals with CN. A definitive mechanism for this G-CSF-induced differentiation remains unknown. We found that intracellular NAMPT and NAD+ amounts in myeloid cells, as well as plasma NAMPT and NAD+ levels, were increased by G-CSF treatment of both healthy volunteers and CN patients. Additionally, exogenously added NAMPT or lentivirally transduced NAMPT complementary DNA induced myeloid differentiation in progenitor cells with concomitant increased expression of NAD+. The activity of NAMPT was dependent on signaling through G-CSF receptor (G-CSFR), and inhibition of NAMPT with the specific inhibitor FK866 substantially abrogated G-CSF-induced myeloid differentiation. We postulate that NAMPT induces a G-CSF autoregulatory loop via the following sequential mechanism. First, G-CSF promotes increased extra- and intracellular NAMPT expression. Second, high levels of NAMPT lead to increased NAD+ production and activation of the granulocyte specific transcription factors C/EBPα and C/EBPβ. Last, these transcription factors bind the promoters of the genes encoding G-CSF and G-CSFR and activate their expression, leading to a positive feedback regulation of G-CSF as well as NAMPT synthesis and activity. Further we administered oral vitamin B3 (nicotinamide), a substrate of NAMPT, to six healthy individuals (10-20 mg per kg per day) for 1 week. In all subjects who received vitamin B3, we found a significant increase in neutrophil count over a seven-day period, with a decline to physiologic levels after vitamin B3 discontinuation. Vitamin B3 treatment was associated with increased amounts of NAD+ and elevated C/EBPα and C/EBPβ RNA levels in bone marrow progenitors. Based on our data, we conclude that NAMPT and its product NAD+ are essential factors in neutrophil granulopoiesis. Moreover, our findings present new perspectives in clinical and experimental medicine, supporting the therapeutic application of vitamin B3 for the treatment of clinical syndromes associated with chronic or acute neutropenia.

0600

REGULATION OF GRANULOPOIESIS BY NAMPT/NAD+/SIRT1 DEPENDENT ACTIVATION OF C/EBP, IN PATIENTS SUFFERING FROM SEVERE CONGENITAL NEUTROPENIA

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Granulopoiesis is a tightly regulated process and C/EBP transcription factors plays important role in the regulation of granulopoiesis *in vitro* and *in vivo*. C/EBPα is considered to be the master regulator of *steady state* granulopoiesis via upregulation of several myeloid genes (e.g. ELA2, CSFR3, etc.). Recently, C/EBPβ has been shown to be essential for cytokine induced emergency granulopoiesis *in vivo* in absence of C/EBPα (Hirai H *et al.*, Nature Immunology, 2006). We were able to show that in patients with congenital neutropenia (CN) harbouring HAX-1 or ELA2 mutations C/EBPα is severely abrogated secondary to defective expression of LEF-1 transcription factor (Skokowa J *et al.*, Nature Medicine, 2006). Therefore, we were interested, whether other transcription factors are capable of substituting C/EBPα, since these patients respond to G-CSF with slight increase in neutrophils from less than 200/uL to above 1500/uL depending on the dose of G-CSF. Indeed we found that C/EBPβ mRNA was upregulated 2.8-fold in $CD33^+$ myeloid cells from CN patients by G-CSF treatment, as compared to healthy individuals. It was associated with upregulation of G-CSFR mRNA and protein expression as well as ligand binding to G-CSFR in myeloid cells, and elevated levels of biologically active G-CSF in serum from CN patients. Recently we found that levels of NAMPT, a protein involved in biosynthesis of NAD+, were significantly higher in myeloid cells and in plasma of CN patients treated with G-CSF, as compared to healthy individuals (Skokowa *et al.*, Nature Medicine, 2009). We also measured elevated levels of NAD+ in myeloid cells and plasma of CN patients, that correlated with induction of SIRT1. SIRT1 is an enzyme involved in deacetylation of several histone and non- histone proteins by utilising NAD+. We asked if induced SIRT1 can positively regulate C/EBPβ to induce granulopoesis in CN patients having abrogated levels of C/EBPα. Indeed, we observed that ectopically expressed SIRT1 protein interacts with both C/EBPα and C/EBPβ proteins. Moreover, endogenous SIRT1 interacts with endogenous C/EBPβ in HL-60 cells. We clearly demonstrated the activation of the promoters of the genes encoding G-CSF and G-CSFR containing C/EBP binding sites in presence of NAD+ or after transfection with SIRT1. In presence of specific siRNA against SIRT1, neither NAMPT nor NAD+ significantly activated these gene promoters. Further we demonstrated that the promoters of the genes encoding G-CSF and G-CSFR harbouring mutations in C/EBP binding sites also do not get significantly activated in presence of NAMPT or NAD+. These results suggest that G-CSF induced NAMPT regulates G-CSF and G-CSFR expression through SIRT1- dependent activation of C/EBPα and C/EBPβ in a NAD+ dependent manner. Based on this we conclude that since in CN patients C/EBPα is not available for binding to SIRT1 and the steady-state granulopoiesis is abrogated, SIRT1 dependent activation of C/EBPβ mediates emergency granulopoiesis leading to sufficient numbers of neutrophils in these patients.

0601

IS THE RISK OF DEVELOPING AML OR MDS INCREASED IN X-LINKED NEUTROPENIA

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Background. In 2001, we described X-linked neutropenia (XLN) as a novel subtype of severe chronic neutropenia (SCN) with an L270P gain-of-function mutation in the Wiskott-Aldrich Syndrome protein (WASP). Since then, two other cases have been described. *Aims*. to discover new XLN cases and to investigate the association of XLN with myeloid leukemia. *Methods*. PCR and dHPLC were used for WAS mutation screening. Exon 17 of CSF3R was sequenced to detect truncating CSF3R mutations in leukaemic samples of XLN patients. *Results*. We discovered 3 more XLN families. The first family is a large kindred with an activating I294T WASP mutation, in which we described additional features of the phenotype: a variable degree of neutropenia, not correlating with the severity of infections, low-normal IgA levels and low NK cells. The second new XLN family had an L270P mutation, and is probably related to our originally described XLN family. The third case was discovered with an L270P mutation when screening 16 Irish ELA2 negative XLN patients. The risk of transformation to MDS/AML in XLN remains unclear. One of two cases reported by Ancliff *et al*. reportedly presented as MDS and two out of five L270P male cases of the original family developed MDS/AML, with (-7), after a prolonged disease course and under G-CSF. Acquired truncating CSF3R mutations were found in leukaemic samples. We screened exons 7-10, encoding the GTP-ase binding domain (GBD) of WASP in 253 patients with MDS (23%) or AML (77%), including 25% with (-7). No relevant mutations were observed in the GBD of WAS in 253 paediatric and adult MDS/AML cases. *Conclusions*. two cases of MDS/AML have been observed in the L270P XLN family, but no malignancies were documented in a large kindred with I294T XLN. The cases in the L270P XLN might be related to more liberal use of G-CSF. This is further supported by the presence of CSF3R mutations in the leukaemic phase. Our findings indicate that the risk of

progression to leukaemia might be increased in XLN, like in other SCN syndromes, most likely via a final common pathway involving CSF3R and (-7). In contrast, we found no cases initially presenting as MDS or AML.

0602

STUDY OF THE QUANTITATIVE AND FUNCTIONAL CHARACTERISTICS OF T REGULATORY CELLS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Background. Functional and/or quantitative changes of T-regulatory (T-reg) cells have been implicated in the pathophysiology of autoimmune diseases. CIN is a disorder of granulopoiesis characterized by increased apoptosis of bone marrow (BM) granulocytic progenitor cells due to the presence of activated T-lymphocytes. The role of T-regs in CIN has not been studied. *Aims*. To investigate the frequency and function of T-regs in CIN patients and probe the underlying mechanisms implicated in their abnormalities, if any. *Methods*. We studied 50 patients fulfilling the previously defined diagnostic criteria for CIN and 20 age- and sex-matched healthy controls after informed consent. We evaluated the proportion of FOXP3$^+$ cells within the CD4$^+$/CD25^{high} (T-reg) cell fraction in the peripheral blood (PB) and BM using flow-cytometry and we also assessed the FOXP3 mRNA levels of immunomagnetically sorted patient and normal CD4$^+$/CD25$^+$ T-cells by means of real-time PCR. For the evaluation of T-reg cell function, we performed suppression assays using anti-CD3 activated normal CD4$^+$/CD25$^-$ effector cells labeled with the fluorescence dye CFSE, in the presence of patient or normal CD4$^+$/CD25$^+$ T-cells. Fluorescence intensity was measured by flow-cytometry. Because the pro-inflammatory Th17 cells display opposite functions with T-regs and interleukin(IL)-17 levels reflect Th17 numbers, we evaluated cytokine levels in serum and long-term BM culture (LTBMC) supernates of patients and controls using an enzyme-linked immunosorbent assay (ELISA). *Results*. In keeping with our previously reported data, CIN patients displayed statistically significant lower number of total lymphocytes (1632±493) and CD4$^+$ T-cells (765±281) compared to healthy controls (2575±559 and 1096±308 respectively) (p<0.001 and p<0.001, respectively). The percentage of FOXP3$^+$ cells within the CD4+/CD25high T-cell fraction was significantly lower in CIN patients (57.77±15.77%) compared to controls (72.95%±12.23%, p<0.01). In accordance with flow-cytometry data were the real-time PCR results; the FOXP3 mRNA levels of patient CD4$^+$/CD25$^+$ cells were significantly lower compared to healthy subjects (p=0.0293). The parallel measurement of T-reg proportion in PB and BM of CIN patients showed a statistically significant increased percentage of FOX3$^+$ cells within the CD4$^+$/CD25high T-cells of BM (68.51±11.88%) compared to PB (52.20±12.77%, p=0.0005) suggesting a possible accumulation of T-regs in the BM. Patient CD4/CD25$^+$ T-regs sufficiently suppressed CFSE-labeled CD4$^+$/CD25$^-$ effector T-cells as was demonstrated by the CFSE fluorescence in day-3 and day-5 of co-culture. Serum IL-17 levels did not differ significantly between CIN patients (5.41±7.98 ng/mL) and healthy controls (5.99±9.40 ng/mL, p=0.8788). IL-17 levels, however, in LTBMC supernatants were significantly increased in patients (4.09±6.20 ng/mL) compared to controls (0.69±1.82 ng/mL, p=0.0268). *Summary and Conclusions*. CIN patients display decreased number of T-regs in the PB as was demonstrated by the low expression of FOXP3 within the CD4$^+$/CD25$^+$ cell fraction. Compared to healthy subjects, CIN patients display increased levels of IL-17 in the BM indicating increased number of Th17 cells. The increased number of T-regs in patients' BM compared to PB indicates probably an accumulation of these cells in the BM in an attempt to suppress the local immune reactions mediated by activated T- cells and pro-inflammatory Th17 cells.

0603

ABNORMALITIES OF SERUM IMMUNOGLOBULIN LEVELS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA: RESULTS FROM A COHORT OF 295 PATIENTS

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Background. Activated oligoclonal T-lymphocytes with immunosuppressive properties have been implicated in the pathophysiology of chronic idiopathic neutropenia (CIN) through overproduction of pro-inflammatory cytokines and pro-apoptotic mediators that induce the apoptotic death of the granulocytic progenitor cells. *Aims*. Because immunoglubulin production by B-cells is under the surveillance of the T-cell system either through interactions of CD40/CD40-Ligand (CD40L) or through the production of a variety of cytokines that may affect the immunoglobulin class/subclass switching, we sought to evaluate the immunoglobulin levels in CIN patients and probe the mechanisms associated with the underlying abnormalities, if any. *Methods*. We studied 295 patients fulfilling the previously defined diagnostic criteria for CIN and 82 healthy subjects after informed consent. Patients neutophil counts were lower than 1800/μL (mean 1484±314, range 288-1799). We evaluated (a) the levels of serum IgM, IgA, IgG1, IgG2, IgG3, IgG4 by means of nephelometry, (b) the expression of CD40 on CD19$^+$ cells by quantifying both the proportion of CD19$^+$/CD40$^+$ cells and the mean fluorescence intensity by flow-cytometry, (c) the induction of surface CD40L on T-cells by flow-cytometry following incubation of cells with PMA (10ng/mL) and Ionomycin (500ng/mL), (d) serum levels of transforming growth factor (TGF)-β1 by means of an enzyme-linked immunosorbent assay (ELISA). *Results*. Patients with CIN displayed statistically significant increased serum levels of IgM (160.4±90.7 mg/dL) compared to healthy subjects (103.4±30.9 mg/dL; p<0.001). In contrast, serum levels of IgG3 and IgG4 were significantly decreased in the patients (40±19 mg/dL and 46.4±22.8 mg/dL, respectively) compared to controls (55±21 mg/dL and 75.1±31.2 mg/dL, respectively; p<0.001 and p<0.001 respectively). Serum IgG1, IgG2, and IgA levels did not differ significantly between CIN patients and controls. Interestingly, an inverse correlation was observed between the levels of IgM and the number of neutrophils (r=-0.2363, p<0.001) and the levels of IgG3 (r=-0.3968, p<0.001) and IgG4 (r=-0.3546 p<0.001). CD40 antigen was normally expressed on patient B-cells. This was demonstrated by both the proportion of CD40$^+$ cells within the CD19$^+$ cell fraction (93.37±11.49 in patients versus 94.06±3.81 in controls; p=0.2447) and the mean fluorescence intensity of CD40 expression (9.60±3.06 in patients versus 9.49±3.11 in controls; p=0.7004). CD40L induction on patient T-cells following activation (42.46±40.07%) did not differ significantly from the controls (46.09±38.81%; p=0.558). The above data suggest that the CD40/CD40L system seems unlikely to have a pathogenetic role in the abnormal immunoglobulin levels of CIN patients. Because a number of cytokines, including TGF-,1, have been implicated in the immunoglobulin class/subclass switching we evaluated serum cytokine levels in the patients. TGF-β1 levels were significantly higher in CIN patients (56.68±31.18 ng/mL) compared to controls (19.21±10.69 ng/mL; respectively; p<0.001) and correlated positively with serum IgM (r=0.3610, p<0.001) and inversely with serum IgG3 (r=-0.2544, p=0.008) and IgG4 (r=-0.3664, p<0.001) levels. *Summary and Conclusions.* CIN patients display increased serum IgM and decreased IgG3 and IgG4 levels compared to healthy subjects. These abnormalities have a major clinical significance because they correlate with the severity of neutropenia. Increased serum TGF-β1 levels may have a pathogenetic role for these abnormalities, at least in part.

0604

NOVEL G6PC3 MUTATIONS IDENTIFIED IN AUTOSOMAL RECESSIVE SCN PATIENTS WITH AND WITHOUT CONGENITAL ABNORMALITIES

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Over the past decade there has been considerable progress in elucidating the underlying genetic defects thought to be causative of severe con-

genital neutropenia (SCN). Patients with autosomal dominant (AD) or sporadic disease predominantly have mutations in the ELA2 gene encoding neutrophil elastase, and rare AD cases are attributed to mutations in the transcriptional repressor Gfi1. A few cases of X-linked SCN have mutations in the Wiskott-Aldrich syndrome gene. In autosomal recessive (AR) disease, homozygous mutations in the HAX1 gene have been identified in patients of Middle-Eastern and Jewish origin, as well as the original Kostmann family and two British cases with unrelated parents. More recently, a homozygous missense mutation was identified in the G6PC3 gene encoding glucose-6-phosphatase, catalytic subunit 3 of two consanguineous SCN families of Aramean ethnicity (Boztug *et al.*, NEJM, 360:32-43, 2009). Subsequently, the same group found other biallelic missense and nonsense mutations in unrelated SCN patients. Of note, the G6PC3 mutations were associated not only with neutropenia but also other distinct features such as structural heart defects and urogenital abnormalities. We used denaturing HPLC analysis to determine the frequency of G6PC3 mutations in our cohort of SCN patients, who were negative for ELA2, HAX1 and WAS mutations. All six G6PC3 exons and splice junctions were screened in samples from 36 patients with known or probable AR inheritance using mixtures of patient and control PCR products to detect homozygous mutations. The hotspots in exons 1 and 6 were screened in a further 60 patients with sporadic inheritance. Novel exon 1 mutations were detected in two AR kindreds. Two affected sibs from a consanguineous family of Pakistani origin had a homozygous 21bp deletion (c.190_210del) resulting in an in-frame deletion of seven amino acids (p.Thr64_Ile70del). The parents were heterozygous for the mutation and clinically normal. The index case possessed a complex phenotype characterized by severe neutropenia, an atrial septal defect, splenomegaly, digital clubbing, short stature, growth retardation, granulomatous inflammatory bowel disease and an abnormally low respiratory burst. He has also had intermittent presentations with perianal abcesses, recurrent oral ulceration and bacterial pneumonia in which he responded to standard bacterial therapy and intermittent G-CSF. These features are consistent with those reported for G6PC3-mutated patients. Another male patient of unrelated Pakistani parents had an atypical SCN picture. He presented at 13yrs of age with intermittent mouth ulcers and a neutrophil count of 0.4×10^6cells/mL, was negative for auto-antibodies, and has remained well over the past 5 years with no bacterial infections and no complicated symptoms, receiving only intermittent G-CSF on recurrence of ulcers. He harboured a homozygous missense variant (c.130C>T) resulting in an amino acid change in exon 1 (p.P44S). His mutation may reflect a rare polymorphism but it does cluster with two published exon 1 mutations in more severely affected patients. No mutations have been identified, to date, in sporadic patients. Our results indicate that biallelic G6PC3 mutations are relatively uncommon in our cohort (6% of presumed AR patients) and may be associated with an SCN phenotype without additional congenital abnormalities.

0605

DIFERENTIAL STIMULATION OF MONOCYTIC CELLS RESULTS IN DISTINCT POPULATIONS OF MICROPARTICLES

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Background. Microparticles (MPs) are small vesicles (0.05-1.00 um), shed from the plasma membrane of cells in response to stress, which permit cross talk between cells within a particular environment. Their composition is thought to reflect their cell of origin and differs when MPs are produced by stimulation versus apoptosis. Whether or not MP properties vary according to the stimulus is not currently known. Monocyte-derived MPs are hereby of particular interest, as they are known to promote inflammation and to be highly procoagulant. *Aims*. The present study addresses the hypothesis that different activating stimuli produce MPs that vary in composition and biological function, even when they originate from the same parent cell. *Methods*. We studied the characteristics of MPs, produced from monocytic THP-1 cells upon stimulation with: two pathophysiologic stimuli: (1) lipopolysaccharide and (2) soluble P-selectin chimera (P-sel-Ig) and with: their controls: (3) PBS (to study spontaneously generated MPs) and (4) IgG control, using proteomics, flow cytometry, western blotting, and electron microscopy. *Results*. Utilizing a novel criterion of calcein-AM staining to define MPs, we compared the properties of the four MP populations. We found that MP populations were similar with respect to size, presence and organization of cytoskeleton, expression of certain antigens such as adhesion molecules: as $\beta2$ and αL integrins or presence of tetraspanins: as CD81 and to procoagulant activity. We found that MPs also have distinct characteristics depending on stimuli. These include: (1) differences in outer plasma membrane lipid bilayer composition as assessed by phosphatidylserine (PS) expression, with less PS-positive MPs produced upon P-sel-Ig stimulation, (2) expression of proteins from specific subcellular locations such as the mitochondria, which was much increased in MPs generated through LPS and (3) of presence of unique antigens such as leukocyte-associated immunoglobin-like-receptor (LAIR)-1 which was found only upon stimulation with the soluble P-selectin chimera. *Conclusions*. We found that the properties of MPs depend on the stimulus that produced them. This supports the concept that monocytic MPs differentially modulate thrombosis, inflammation and immune regulation according to stimulus.

M. P. Bernimoulin and E.K.Waters contributed equally

0606

LEUKOPENIA AND WAS GENE MUTATION IN AN INFANT. A CASE REPORT

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Background. Wiskott-Aldrich syndrome is a primary immunodeficiency with a characteristic clinical picture of thrombocytopenia with small platelets, eczema and recurrent infections. X linked neutropenia (XLN) is a rare syndrome of severe congenital neutropenia described in 2001 and with very few cases reported in the literature. The Wiskott-Aldrich syndrome protein (WASP), encoded by the WAS gene, is an important regulator of the actin cytoskeleton polymerization. Mutations in WAS gene causing a reduced WASP activity are responsible for the Wiskott-Aldrich syndrome (WAS) and X linked thrombocytopenia. XLN is caused by gain-of-function type mutations of the WAS gene resulting in a loss of autoinhibition of WASP. *Aims*. To present a peculiar case with severe leukopenia and a novel WAS gene mutation. *Case*. A 6 month old boy was referred to our institution to study severe leukopenia (1.6 leucocytes $\times10^9$/L: 0.3 neutrophils $\times10^9$/L, 0.5 lymphocytes $\times10^9$/L, 0.1 monocytes $\times10^9$/L) demonstrated in a blood count at 5 month old during a febrile episode. Except for a mild eczema his physical examination was normal with no growth impairment. The bone marrow aspirate showed a normal maturation of the myeloid compartment, no dysplasia signs neither other abnormal features. Cytogenetic analyses were consistently normal. Metabolic disorders, viral infections and autoimmune neutropenia were ruled out. ELA 2 and HAX1 gene mutations were negative but interestingly we found a novel mutation in exon 7 of WAS gene (p.R211Q). Immunologic analysis revealed low natural Killer cells (4%), normal CD4/CD8 ratio, normal phytohemaglutinin response test and normal response to vaccination (rubella and measles). He has presented 4 mild infections during all the follow up time. Platelet counts have always been in the normal range and size. He is now 2 years old and maintains normal white blood counts during the last six months. Familiar genetic analysis revealed that his mother is a sane carrier of WAS mutation. She has normal blood cell counts. *Discussion*. A novel mutation in WAS gene is described. This mutation region has been described as a mutational hot spot for Wiscott-Aldrich syndrome with high scores disease. However our patient has a normal phenotype with normal platelet number and size. Surprisingly the patient has recovered completely from his leukopenia. More studies must be done to determinate how WASP is affected in this individual. Meanwhile a diagnosis of mild WAS vs XLN has been done.

0607

GLUCOCORTICOIDS REVERT THE DASATINIB-MEDIATED ENHANCEMENT OF HISTAMINE RELEASE IN IGE-R CROSS-LINKED BASOPHILS

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Background. Dasatinib is a novel multi-kinase targeting drug that blocks several tyrosine kinases including BCR/ABL. Apart from its well-known anti-leukemic activity, the drug produces several side effects including edema formation and pleural effusion. We have recently

shown that low doses of dasatinib (<0.1 μM) promote IgE-dependent secretion of histamine in human blood basophils, especially in allergic individuals, whereas at 1 μM, dasatinib potently blocks IgE-mediated histamine secretion. *Aims*. In the present study, we examined the effects of 3 different glucocorticosteroids (dexamethasone, prednisolone, and hydrocortisone) on histamine release provoked by IgE receptor cross-linking in blood basophils in the absence or presence of low dose dasatinib. *Methods*. Normal blood basophils (n=5) or basophils derived from allergic donors sensitized against Bet v 1 and/or Phl p 5 (n=5) were preincubated with glucocorticosteroids for 24 hours and then exposed to an anti-IgE antibody (0.001-10 μg/mL) or allergen (Bet v 1 or Phl p 5; dose range: 0.0001-1 μg/mL) with or without low dose dasatinib (0.025 μM). After incubation, basophils were examined for histamine release and/or expression of CD63 and CD203c by multicolor flow cytometry. *Results*. All three glucocorticosteroids were found to counteract histamine release provoked by anti-IgE or allergen in the presence or absence of low dose dasatinib in non-allergic or allergic individuals. Even in patients with maximum histamine release, glucocorticoids were found to counteract the dasatinib-induced enhancement of IgE-dependent histamine release in basophils. The inhibitory effects of glucocorticosteroids on basophil histamine release were dose-dependent. In addition, all three glucocorticoids were found to decrease anti-IgE-induced upregulation of CD63 and CD203c in the presence or absence of low dose dasatinib. *Summary*. The addition of glucocorticosteroids may be an effective therapeutic maneuver to counteract the co-inflammatory effects of low dose dasatinib in IgE-receptor cross-linked basophils.

0608

CLINICAL CHARACTERISTICS AND TREATMENT OUTCOME OF LANGERHANS CELL HISTIOCYTOSIS: LARGE STUDY OF SINGLE INSTITUTE

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Background. Langerhans cell histiocytosis (LCH) is a disease with unknown etiology which is characterized by abnormal proliferation and accumulation of antigen presenting dendritic cells in various tissues. Large studies of a single institute were rare because of the rarity of the disease and the diversity of clinical manifestations. *Aims*. In this study, we analyzed the clinical manifestations and prognostic factors of LCH and evaluated the outcome. *Methods*. We retrospectively analyzed the medical records of patients who were diagnosed as LCH in our institute for 22 years from January, 1986 to December, 2007. *Results*. A total of 167 patients were evaluated. There were 99 (59.3%) male and 68 (40.7%) female patients. The median age at diagnosis was 2.7 years (range 0-13.6 years). Sites of involvement at the time of diagnosis are bone in 152 (91.0%), skin in 33 (19.8%), lymph nodes in 26 (15.6%), liver in 24 (14.4%), bone marrow in 20 (12.0%), soft tissue in 18 (10.8%), spleen in 17 (10.2%), pituitary gland in 11 (6.6%) and lung in 7 (4.2%) patients. In 58 (34.7%) patients, more than two organs were involved. Risk organ (bone marrow, liver, spleen and lung) involvements were observed in 31 (18.6%) patients. Patients with single bone lesion received indomethacin for 6-12 months. Other patients received induction regimen (vinblastine, prednisolone, cyclophosphamide and methotrexate) for 12 weeks, and then maintenance therapy (6-mercaptopurine, methotrexate and cyclophasphamide). Thirty patients (18.0%) had relapse or disease progression during treatment. Overall survival and event free survival were 96.5% and 76.2% with median follow-up time of 6.3 years. Event-free survivals were significantly lower in patients younger than 3 years of age, patients with risk organ involvement, and patients with multiple organ involvement (p=0.003, 0.000, 0.000). Endocrine sequelae were observed in 13 patients, and diabetes insipidus was most common. *Conclusions*. LCH showed high survival rate despite of the frequent relapses. Age, numbers of involved organ, and involvement of risk organ were evaluated to be prognostic factors.

Acute lymphoblastic leukemia - Biology

0609

NK-LIKE HOMEODOMAIN PROTEINS ACTIVATE NOTCH3-SIGNALING IN LEUKEMIC T-CELLS

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Background. Three NK-like homeobox genes (NKLs), TLX1/HOX11, TLX3/HOX11L2 and NKX2-5/CSX, are ectopically activated via chromosomal aberrations in subsets of T-cell acute lymphoblastic leukemia (T-ALL). Their close relation suggests shared activities which are involved in leukemogenesis, however, remaining largely unknown. *Aims*. Here we screened additional NKLs in 24 T-ALL cell lines by RT-PCR and identified common expression of MSX2. Analysis of this homeobox gene may contribute to the understanding of the function of its oncogenic relatives. *Results*. Quantification of MSX2 mRNA in primary hematopoietic cells demonstrated higher levels in $CD34^+$ stem cells as compared to peripheral blood cells and mature $CD3^+$ T-cells, suggesting downregulation during T-cell development. Subsequent analysis of MSX2 expression levels in T-ALL cell lines after treatment with core thymic factors confirmed their involvement in regulation. These results indicated that MSX2 represents a physiological NKL family member in T-cells. For functional analysis T-cell-derived JURKAT cells were lentivirally transduced, overexpressing either MSX2 or oncogenic TLX1 and NKX2-5, respectively. These cells displayed transcriptional activation of NOTCH3-signaling, including NOTCH3 and HEY1 as analyzed by gene expression profiling and quantitative RT-PCR, and consistently decreased sensitivity for gamma-secretase inhibitor as analyzed by MTT-assays. Furthermore, in addition to MSX2, both TLX1 and NKX2-5 proteins interacted with NOTCH-pathway repressors, SPEN/MINT/SHARP and TLE1/GRG1, representing a potential mechanism of (de)regulation. In T-ALL cell lines containing the t(5;14)(q35;q32) which activates expression of TLX3 or NKX2-5, respectively, we detected chromosomal deletion of MSX2 (at 5q35), resulting in functional exchange between MSX2 and oncogenic NKLs. Finally, corresponding to the cell line data, elevated expression of NOTCH3 and HEY1 was detected in primary TLX1/3 positive T-ALL cells. *Conclusions*. Identification and functional analysis of MSX2 in hematopoietic cells implicated a modulating role via NOTCH3-signaling in T-cell differentiation and in leukemogenesis if supplemented by oncogenic NKLs.

0610

PROGNOSTIC SUBGROUPS DEFINED IN A NOD/SCID/HUALL XENOGRAFT MODEL FOR PAEDIATRIC B CELL PRECURSOR ALL ARE CHARACTERISED BY A GENE SIGNATURE INVOLVING SURVIVAL PATHWAYS AND IDENTIFY POTENTIAL NEW THERAPEUTIC TARGETS

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Despite increasingly successful treatment of children with acute lymphoblastic leukaemia (ALL), the poor outcome of particularly early relapsed patients remains a challenge to identify factors which reflect characteristics of leukaemia biology and provide therapeutic targets. In a recent study we transplanted primary paediatric B cell precursor (BCP) ALL samples (N=50) into NOD/SCID mice and demonstrated a clearly inferior survival for patients whose cells led to manifestation of leukaemia in the recipients within 10 weeks (TTLshort) in contrast to patient samples with prolonged *in vivo* growth (TTLlong). Short TTL was strongly associated with high risk for early relapse providing a new independent prognostic factor for outcome in paediatric BCP- ALL. In this study we further characterised the biological properties of the two phenotypes TTLshort and TTLlong to identify novel parameters which ideally reflect the functional biology of the disease and identify therapeutic targets. Gene expression profiles were analyzed in NOD/SCID/huALL xenograft

leukaemia samples (N= 14) using a human whole genome array approach (Affymetrix U133 Plus 2.0). 108 probe sets (102 genes) were found to be differentially regulated between TTLshort and TTLlong. Application of this signature (108 probe sets, derived from xenograft samples) on expression profiles obtained from patients (N= 197) who have been treated according to a BFM-based protocol (AIEOP-LLA-2000) resulted in separation into 2 groups of which patients clustering in the TTLlong signature group showed a superior relapse free survival. When applying Significance Analysis of Microarrays (SAM) on the profiles of the 14 xenografts, 2 of the differentially regulated genes identified by T-test were also identified by SAM to be highly discriminatory: DNA-damage-inducible-transcript-4-like (DDTI4L, a negative regulator of the mTOR pathway) and phosphodiesterase-4A (PDE4A, involved in cAMP-mediated apoptosis signalling). These 2 genes also separated the cohort of 197 patients into 2 groups with TTLlong signature patients showing a superior relapse free survival. TTLshort is characterized by DDIT4L^{low}/PDE4A^{high} and TTLlong involves DDIT4L^{high} and PDE4A^{low}. Our findings have important therapeutic implications since both pathways identified provide immediate targets for therapeutic intervention. The lacking mTOR inhibition in TTL^{short}/DDIT4L^{low} leukaemia could be overcome by rapamycin leading to growth inhibition of TTLshort leukaemia. Growth inhibition of TTLshort leukaemia could be also achieved inhibiting PDE4A activity using compounds like rolipram resulting in increased apoptosis sensitivity. In fact, *ex vivo* treatment of primary xenograft leukaemia cells with inhibitors of both pathways, rapamycin and rolipram, showed clearly increased cell death in TTL^{short} (DDIT4L^{low}/PDE4A^{high}) but not in TTLlong (DDIT4L^{high}/PDE4A^{low}) samples. Taken together, the importance of our observations in the xenotransplant model could be translated into an independent group of patients and led to identification of pathways involved in regulation of cell growth and apoptosis which can be targeted by well known drugs such as the mTOR inhibitor rapamycin. Therefore, our model provides a powerful tool to identify prognostic factors in paediatric acute leukaemia leading to rational targets for directed therapy.

0611

APOPTOSIS DEFICIENCY DEFINES PROGNOSTIC SUBGROUPS AND THERAPY RESISTANCE IN A XENOGRAFT MODEL OF PEDIATRIC BCP- ALL

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Deficient activation of apoptosis signaling pathways is known to play a role in leukemogenesis as well as in drug resistance and treatment failure. In two previous retrospective studies we described the importance of intact apoptosis signaling for treatment response in pediatric ALL and AML. A correlation of 2 key apoptogenic events, caspase-3 activation and cytochrome c release, reflecting the functional integrity of this molecular mechanism, was only found in patients with good outcome. Using a NOD/SCID xenotransplantation model for primary pediatric B-cell precursor (BCP)-ALL, we found that rapid engraftment in the mouse (short time to leukemia, TTLshort) determines poor patient outcome while delayed manifestation of leukemia in the recipient (TTLlong) indicates a good prognosis for the patient. In this study, we analyzed the importance of deficient apoptosis signaling for leukemia engraftment and therapy resistance in this xenotransplantation model. 17 BCP-ALL xenograft leukemia samples (5 TTLshort and 12 TTLlong) were analyzed. Correlation of cytochrome c release and activation of caspase-3, indicating proficient apoptosis signaling, was only found in the TTLlong group, whereas it was absent in TTLshort. Likewise, intact connection of both parameters was exclusively found in xenograft leukemia samples of patients with favorable prognostic factors (e.g. absence of hyperleucocytosis at diagnosis, presence of TEL/AML1, no or late relapse). In addition, a significant positive correlation of time from transplantation of the leukemia and clinically ouvert leukemia in the mouse (time to leukemia, TTL) and cytochrome c release (Spearman's, p=0.022) indicated prolonged leukemia engraftment to be due to efficient apoptosis signaling. Mice transplanted with different leukemias (5 TTLshort and 4 TTLlong, all standard and medium risk groups, no prednisone poor responders) were treated according to induction therapy regimens used in pediatric ALL-patients (including vincristine, dexamethasone and asparaginase) for 21 days and the time to reoccurrence (TTR) of leukemia was estimated. Recipients carrying a TTLshort leukemia showed an inferior survival after treatment compared to those transplanted with a TTLlong leukemia (log rank, p<0.001). Furthermore, a significant positive correlation of post-treatment time to reoccurrence (TTR) of leukemia in the mice and cytochrome c release (Spearman's, p=0.001) was found, indicating that proficient apoptosis signaling is necessary for effective therapy. These results demonstrate that deficient apoptosis signaling is an important feature determining not only leukemia growth but most importantly also therapy resistance in the NOD/SCID/huALL model *in vivo*, and accordingly poor prognosis of the patient. The propensity to undergo apoptosis not only serves as a biomarker for prognosis but also as an indicator of treatment response. Thus, the apoptotic machinery represents a promising therapeutic target. In fact, preliminary *in vitro* toxicity experiments with XIAP-antagonists/Smac-mimetics confirm this assumption and will eventually translate into new clinical applications.

0612

METHADONE IMPROVES THERAPEUTIC SUCCESS IN LEUKEMIA CELLS *IN VITRO* AND *EX VIVO*

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Background. Chemoresistance and radioresistance is one of the primary causes for therapeutic failure in radiotherapy and chemotherapy of cancer. Therefore new options are needed to improve therapeutic success in the treatment of leukemias and solid tumors. The therapeutic opioid drug methadone (D,L-methadone), a long-acting μ-opioid receptor agonist, is a highly effective and safe medication for the opioid dependence of outpatients. It suppresses opioid withdrawal, and provides blockade to the effect of illicit opioids. In addition, to treatment of opioid withdrawal, methadone is of significant utility as a long-acting analgesic, particulary for neuropathic pain syndromes. *Aims*. In our studies we analyzed the effect of methadone and methadone in addition of doxorubicin on different B cell precursor (BCP)-ALL cells *in vitro* and *ex vivo*. *Methods*. BCP-ALL cell lines (Nalm6, Reh, Tanoue) and primary BCP-ALL cells isolated from xenografted/transplanted NOD/SCID mice were treated with different therapeutic concentrations of D,L-methadone alone or in addition to therapeutic concentrations of doxorubicin. After different time points, cell death was measured by flowcytometry analyses and activation of apoptosis pathways was analyzed by Western Blot analyses. *Results*. We found that methadone is a potent inducer of cell death and apoptosis in leukemia cells. A strong increase of cell death from 15% up to 90% was measured at low therapeutic concentrations of methadone in addition to doxorubicin in leukemia cell lines *in vitro*. In addition, a high cell kill were also observed on primary BCP-ALL cells *ex vivo*. This indicates that methadone improves therapeutic success of doxorubicin in treatment of leukemia cells. At higher concentrations of methadone leukemia cells were killed without doxorubicin treatment. Methadone alone or in combination with doxorubicin inhibited proliferation in leukemia cells and induced cell death through apoptosis induction. Activation of apoptosis pathways through activation of caspase-9, caspase-2 and caspase-3, downregulation of Bcl-xL and XIAP, and cleavage of poly(ADP-ribose)polymerase were found after treatment with methadone alone or in combination of methadone and doxorubicin in leukemia cells. In addition, methadone reversed deficient induction of cell death by doxorubicin in doxorubicin-resistant and apoptosis-resistant leukemia cells. *Summary and Conclusions*. We found that methadone improves therapeutic success of doxorubicin in treatment of BCP-ALL leukemia cells *in vitro* and *ex vivo* and breaks doxorubicin-resistance. These results demonstrate that methadone provides the foundation for new strategies using methadone as additional anticancer drug in leukemia therapy to improve therapeutic success especially when conventional therapies are less effective.

0613

PERIPHERAL BLOOD INVOLVEMENT PROVIDES AN IMPORTANT PROGNOSTIC INFORMATION DURING EARLY TIME POINTS OF CHILDHOOD ALL TREATMENT

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Background. In the most progressive pediatric acute lymphoblastic leukemia (ALL) treatment protocols, minimal residual disease (MRD) testing is essential for the risk group stratification procedure and is an

important predictor of outcome. While routinely performed on bone marrow (BM) samples, MRD in peripheral blood (PB) is not usually examined although PB sampling could cause less discomfort especially in children. Some studies have shown a good correlation of MRD levels in PB and BM in T-ALL; the data concerning B-cell precursor (BCP)-ALL remain controversial, with most studies lacking sufficient number of samples with quantifiable MRD in both compartments taken during the early phase of treatment. *Aim and Methods.* To simultaneously evaluate MRD in BM and PB using patient-specific immunoglobulin and T-cell receptor gene-based RQ-PCR. *Results*. 221 paired samples from 47 children with BCP-ALL treated according to the Berlin-Frankfurt-Muenster (BFM) ALL IC-BFM 2002 protocol were taken at diagnosis (dg, n=47), day 8 (d8, n=39), day 15 (d15, n=44), day 33 (d33, n=34), week 12 (w12, n=31) and at the end of maintenance therapy (post-MT, n=26). Informed consent was obtained from patients or their guardians. In 125 paired samples MRD was detected in both tissues, in 18 pairs in BM only and in 5 pairs in PB only. MRD levels in PB varied greatly and were a mean of 149-fold lower than in BM (range 0.04-8293). Like in the BM at d15, patients with lower MRD in PB at d15 were more likely to achieve MRD negativity in BM at d33 in the univariate analysis (p=0.01, Mann Whitney). Patients younger than 10 yrs had lower MRD in PB at d8 and at d15 than other patients (p=0.03 and p=0.01, respectively). Unlike in BM, patients with hyperdiploidy had lower MRD in PB at d15 than other patients excluding TEL/AML1 cases (p=0.05). There were no significant associations with diagnostic white blood cell count (WBC), sex, immunophenotype (cALL/prae-B ALL) or presence of TEL/AML1 fusion at any time point. Patients with MRD<1E-04 in PB at d15 had a 5-year relapse-free survival (RFS) of 100% vs. 62.5±9.9% for those with a higher MRD (p=0.0089). No such threshold could be set for dg, d8 and d33 PB MRD level. Low numbers of MRD-positive results at w12 and post-MT samples precluded statistical analysis. We next examined the prognostic impact of BM/PB MRD ratio. Patients having MRD levels in PB similar to those in BM (BM/PB MRD<10) at d8 and d15 were more likely to relapse (d8: RFS 88.1±6.4% vs. 61.5±13.5%, p=0.04; d15: RFS 89.5±5.7% vs. 54.5±15%, p=0.01). No such relationship was observed for dg or d33. *Summary*. Our data show that in childhood BCP-ALL, MRD in PB is not simply proportional to the BM level and provides additional prognostic information. A higher relapse rate in patients with PB MRD level similar to that in BM suggests that leukemic blasts with the propensity for massive escape from BM to PB during the induction treatment have a great potential for giving rise to relapse.

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0614

AID EXPRESSION IN BCR/ABL POSITIVE ALL IS ASSOCIATED WITH A PECULIAR GENE EXPRESSION PROFILE AND DEREGULATION OF DNA REPAIR AND REPLICATION GENES

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Background. Activation-induced cytidine deaminase (AID) initiates somatic hypermutation and class switch recombination of immunoglobulin (Ig) genes by deaminating deoxycytidine residues in mature germinal center B cells. AID can also induce mutations in non-Ig genes. In addition to the full-length isoform, five splice variants have been reported in normal B cells and in mature B-cell disorders. Furthermore, aberrant expression of AID has been detected in B-lineage acute lymphoblastic leukemia (B-ALL) and a strong correlation between AID expression and BCR/ABL-positivity has been found; intriguingly, BCR/ABL$^+$ ALL, the most frequent genetically-defined ALL in adults, is characterized by a high genetic instability. *Aims*. To deepen the role of AID in BCR/ABL$^+$ ALL, AID expression was studied in these patients and then its association with gene expression profiling was evaluated. *Methods*. We analyzed 16 adult BCR/ABL$^+$ ALL patients at the onset of disease for AID expression: after cDNA amplification, PCR products were loaded on the ABI Prism 3730 DNA Analyzer for automated capillary gel electrophoresis and the results were plotted with the AbiPrism GeneMapper v3.5 software (Applied Biosystems). Subsequently, gene expression profiling experiments were performed on the same set of patients using the HGU133 Plus 2.0 arrays (Affymetrix); statistical analysis was carried out using the dChip software. Unsupervised and supervised analyses (Analysis of Variance, ANOVA) were used to evaluate the presence of potential subsets and to compare the BCR/ABL$^+$ ALL subgroup based on AID results. *Results*. AID mRNA was detected in 7 patients: 4 expressed only the full-lenght isoform, 2 displayed the splice variant that retains exons 1, 2 and 5, while 1 patient co-expressed the full-lenght isoform and two splice variants. The remaining cases proved negative for AID expression. By unsupervised clustering, gene expression profiling data analysis revealed that AID-full-lenght and AID-splice variants patients display a similar profile. This finding was confirmed by supervised analysis: in fact, comparison on the 3 AID subsets (i.e. AID-full-lenght, AID-splice variants and AID-negative) grouped AID-full-lenght and AID-splice variants in the same cluster, as opposed to AID-negative cases. Among the 1151 differentially expressed genes, 328 resulted specifically and homogeneously upregulated in AID-full-lenght patients. Of interest, this group of patients was characterized by the overexpression of a large set of genes involved in i) DNA repair (PCNA, RPA3, RAD50, XRCC4, TP53), ii) DNA replication (ORC5L, MCM10, CETN3, ANAPC7), iii) cell cycle regulation (CDK6, MKI67, MYC), iv) nucleotide metabolism (NT5C3, PPAT, PRPS2). Functional annotation analysis, performed using the DAVID software, corroborated these findings. *Conclusions*. These results indicate that AID-full-lenght and splice variants exert a similar genomic profile, distinct from AID-negative patients, suggesting that gene expression profiling is mainly affected by AID positivity or negativity, rather than by the presence of AID-splice variants. Furthermore, AID positive patients are characterized by a peculiar signature in which genes involved in DNA repair and replication are overrepresented, indicating a possible deregulation of these mechanisms: it is intriguing to speculate that this phenomenon might be implicated in the genetic instability observed in these patients. Functional studies are ongoing to confirm this hypothesis.

0615

THE IMMUNOMODULATORY AGENT FTY720 INDUCES APOPTOSIS AND INHIBITS GROWTH OF BCR-ABL POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA CELLS AND OVERCOMES IMATINIB RESISTANCE *IN VITRO*

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Background. Imatinib is highly effective initial therapy for *de novo* Philadelphia chromosome-positive ALL (Ph$^+$ ALL) but resistance to this tyrosine kinase inhibitor (TKI) eventually develops in the majority of patients. Approximately 80% of patients with acquired imatinib resistance harbour mutations in the tyrosine kinase domain (TKD) of Bcr-Abl, whereas primary resistance appears to be largely independent of TKD mutations but the mechanisms have not been elucidated. FTY720 (fingolimod) is a synthetic compound produced by modification of a natural immunosuppressor. This immunomodulator is noted to interfere with T-cell trafficking and is used in organ transplantation. Recent studies indicate that FTY720 induces apoptosis through activation of the serine/threonine phosphatase PP2A in a variety of B-cell malignancies independently of Bcl-2 expression, but precise mechanisms of action are incompletely elucidated. *Aims.*We examined the potential effect of FTY720 in a Ph$^+$ ALL cell line displaying non-mutational imatinib-resistance and in long-term cultured primary human Bcr-Abl positive and negative ALL cells. *Methods.* As a model of imatinib resistance we employed the SupB15RT cell line which was derived from the previously well characterized Bcr-Abl positive B-precursor SupB15W cell line by gradually increasing the exposure to imatinib. SupB15RT cells are cross-resistant to the second generation Abl kinase inhibitors with no evidence of commonly implicated mechanisms of imatinib resistance, e.g. Bcr-Abl gene amplification, point mutations in the TKD mutations, or Bcr-Abl overexpression. In addition we used long-term cultured primary human ALL cells obtained from patients with Bcr-Abl positive (n=2) and Bcr-Abl negative cells (n=3). Cells were treated with 1 to 10 µM FTY720 from a period of 7 days. Cell viability was determined by trypan cell counting. Apoptosis was measured using the Annexin V-FITC apoptosis kit. Abl phosphorylation was analyzed by western blot. *Results.* FTY720 induced apoptosis in a dose-dependant manner in all cases of Bcr-Abl positive ALL cells (n=4) and in none of the three Bcr-Abl negative cells (n=3). The maximum apoptotic effect was observed after 48 hours. In SupB15W, FTY720 has dramatic effect with 75% of apoptosis with principally late apoptosis (53%) after 48 hours and suppresses growth already with low concentration (as of 2.5 µM). In imatinib-resistant SupB15RT, we show the apoptotic and antiproliferative effects after 48 hours. However the apoptotic effect is different with less apoptosis (39%) but mostly early apoptosis (27%).The potential effect of FTY720 on phosphorylation of Bcr-Abl, a known substrate of PP2A, was examined by Western blotting, demonstrating that in SupB15W and in SupB15RT cells, the level of Abl phosphorylation is not modified by FTY720. *Conclusions*. The immunomodulator FTY720 is a

potent inducer of apoptosis and an antiproliferative agent in Ph+ ALL but not in Ph negative ALL cells. It partially abrogates mutational-independent imatinib resistance and may be of potential use as a therapeutic agent in Ph+ ALL. The mechanism by which FTY720 induces cell death appears to differ between imatinib-sensitive and imatinib-resistant Ph+ ALL cells, and is currently being elucidated.

0616

PROGNOSTIC INTEREST OF BIPARAMETRIC CD45-DNA ANALYSIS OF MEDULLAR LYMPHOBLASTS AT DIAGNOSIS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. The increase of survival in childhood acute lymphoblastic leukaemia (ALL) during the last fifty years was induced by the amelioration of antileukemic treatments and by their early adaptation depending on clinical and biological risk factors at diagnosis and on chemotherapy response. DNA index (DI), which expresses the blast cells DNA content measured by flow cytometry (FCM), is a prognostic factor in childhood ALL. When the leukemic clone does not show pejorative structural abnormalities, a better survival is associated with a hyperdiploidy (DI>1.16) and a poor prognosis is induced by a severe hypodiploidy (DI<0.8). Single DNA staining provides accurate DI, but the percentage of blastic S-phase cells (%S), (which can predict chemotherapy sensibility and prognosis) is difficult to determine accurately in DNA-diploid and near-diploid cases, as it reflects the mean of all cells in a bone marrow aspirate. *Aims*. In order to ascertain the prognostic value of specific blast cell DI, CD45-FI and %S, a CD45-DNA double staining was realized in childhood ALL samples and the FCM parameters were studied according to clinical, biological and therapeutic characteristics. *Methods*. One hundred and eighty-five patients younger than 18 years with newly ALL, treated between 1987 and 2006 (CHU Angers, Caen, Nantes, Poitiers and Toulouse), were included in this retrospective study. Patients were treated in different treatment schedules, according to the period of diagnosis (EORTC 58881, 58951, FRALLE 2000-A, FRALLE 2000-BT and INTERFANT). Main characteristics were noted at diagnosis: clinical such as sex, age, extramedullar localization and: biological ones, such as leucocytosis, immunophenotypic, cytogenetic and molecular characteristics. During follow-up, were noted: the steroids sensitivity at day 8 and cytological or molecular remission at day 42, the treatment schedule, the relapse and/or death events. One hundred thirty-nine frozen and 46 fresh diagnostic samples were analyzed by a CD45-DNA double staining and the DI, CD45-FI and %S were determined for all detected subclones. *Results*. The DI and CD45-FI were correlated with validated risk factors and survival. The hyperdiploidy was associated to a longer event-free survival (EFS) (p<10-4, 5-year EFS rate=89.8%). A high CD45-FI was strongly correlated to poor prognostic factors (younger than 1 year, extra-medullar localization, T phenotype, hypodiploidy, (9;22) translocation, and associated to shorter EFS (p=0.017, 5-year rate=61.7%) and OS (p=0.02, 5-year rate=69.3%). The S-phase percentage value was not identified as a prognostic parameter in our study. Supplementary clones were detected by FCM, including 3 severe hypodiploid cases (DI: 0.56, 0.73 and 0.77) and 8 cytogenetic failure cases. *Conclusions*. The accurate, rapid and cheap determination of heterogeneous blastic populations by biparametric CD45-DNA analysis, associated to the prognostic value of DI and CD45-FI, justify the interest of this technique at diagnosis and can be added to monitor residual disease in childhood ALL.

0617

ECTOPIC CDX2 EXPRESSION IS A FREQUENT AND PROGNOSTICALLY RELEVANT EVENT IN HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA

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We previously could show that the homeobox gene CDX2 is aberrantly expressed in 79% of acute myeloid leukemia (AML) patients with especially high expression levels in patients with normal karyotypes (median ΔCT 7.58). In AML, expression levels of CDX2 were highly correlated with deregulated expression of HOX genes. To test if an aberrant expression of CDX2 could be also observed in acute lymphoblastic leukemia (ALL), we analyzed the expression of CDX2 in 57 adult patients with ALL by TaqMan real-time qRT-PCR. Of these patients, the majority of 81% was positive for CDX2 expression. With highest median expression in pre-T ALL (ΔCT 4.1, n=7) followed by cALL (ΔCT 5.4, n=10) and pro-B ALL (ΔCT 5.78, n=9) those groups showed higher expression of CDX2 than AML patients with normal karyotype. The proportion of patients ectopically expressing CDX2 differed between ALL subtypes with 100% positivity in patients with pro-B ALL, c-ALL and Ph+ ALL versus 40% positivity in B-ALL/Burkitt lymphoma, 70% in thymic T-ALL and 71% in pre-T ALL. In contrast to AML, where CDX2 expression correlated with HOX gene deregulation, in ALL we could not detect any correlation between CDX2 and HOXA7 or HOXA9, respectively, and only a weak correlation between CDX2 and HOXB6 (p=0.048, Mann-Whitney U-test). As these results suggest that in ALL CDX2 is exerting its leukemogenic effect not by deregulation of HOX genes, we tested by TaqMan LDA for differential regulation of genes involved in lymphopoiesis. Hereby we found that CDX2 is able to significantly upregulate lymphoid genes such as Lef1 (3.9-fold, p=0.001), Tcf3 (13.3-fold, p=0.0004), and Id3 (10.2-fold, p=0.0001). Promoter hypomethylation could be excluded as a possible cause for the aberrant CDX2 expression as we did not detect any methylation differences between CDX2 positive and negative ALL patients in a methylation region surrounding the transcription start site of CDX2 (n=9). Expression of CDX2 was highest in high risk and very high risk patients. Despite the small number of only 30 patients included in statistical analysis, CDX2 turned out to significantly correlate to poor overall survival (p=0.019, log rank test), and remained a significant risk factor also after adjusting for the other risk factors age or presence of molecular markers by bivariate analysis. Here we show that CDX2 does not perturb HOX expression in the same extent as in AML, but is able to upregulate also lymphopoietic genes. Furthermore, we could demonstrate for the first time that aberrant CDX2 expression is a frequent event in adult ALL, that high expression levels of this protooncogene predict poor treatment outcome in these patients and CDX2 expression therefore has prognostic impact in adult patients with ALL.

0618

MIRNA EXPRESSION PROFILE OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA EXPRESSING OR NOT CD56

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The expression profiles of miRNAs in tumour samples have been used to provide specific phenotypic signatures with better discrimination than mRNA expression profiles. Recently our group has demonstrated that the expression of CD56, a marker of natural killer (NK) cells, in T cell acute lymphoblastic leukaemia (T-ALL) is an independent adverse prognostic factor for disease free survival (DFS) in multivariate analysis (855 vs 2095 days, p=0.002). Also patients tended to be older and to present normal platelet counts in the T-LLA/CD56+ group. Therefore, better characterization of these two subgroups of T-ALL is necessary to understand its diversity as disease entities. Our aim was to determine the miRNA expression profile of T-ALL with or without CD56 expression as well as normal mature T and NK cells. We used 48 T-ALL samples (36 T-ALL/CD56- and 12 T-ALL/CD56+) with more than 80% of blasts from peripheral blood or bone marrow of patients diagnosed at Hospital das Clinicas de Ribeirão Preto - January 2000 to December 2006. Normal CD3+/CD56- and CD3-/CD56+ cells were isolated from peripheral blood of four healthy subjects by immunomagnetic labeling. Total RNA (1ug from each sample) was pooled according to groups described above. We used the Taqman MicroRNA Assay Human Panel (Applied Biosystems) to perform a screening of 164 knowledge mature miRNA sequences. Total RNA (2.5 ng/reaction) from pools was reverse transcribed with specific looped RT primers for each miRNA. In the real-time PCR step, specific primers and probes for each miRNA were used according to manufacturer instructions. Total RNA input was normalized based on the geometric means of Ct values obtained for the endogenous RNAs: RNU6B, RNU19, RNU38B and RNU66. All reactions were run in duplicate and a coefficient of variation greater than 5% was used as an exclusion factor (seven miRNAs were excluded). The fold

change was calculated using comparative 2-ΔΔCt method. We defined a strength cutoff for differential expression as 10 times for upregulation and 0.1 for downregulation. With this strategy we identified nine miRNAs for further validation using real time PCR of the individual 56 samples (Figure). MiR-29b is upregulated in leukemia compared to normal samples (A). This is a new finding in disagree with previous work that described it as a tumor suppressor gene downregulated in tumors of aggressive behavior. It is possible that miR-29b be specifically involved in initial phases of T-cell maturation. MiR-181a (E) and miR-181b (F) are upregulated in T-ALL when compared to normal T cells. Indeed the increase of miR181a was previously described involved with antigenic stimulation in normal T cells. Finally, in the comparison between T-ALL/CD56$^+$ and T-ALL/CD56$^-$ we observed that miR-221 is up regulated in the T-ALL/CD56$^+$ group (G). Indeed, miR-221 is commonly upregulated in aggressive tumors as glioblastoma and melanoma. Interestingly, in a previous work of Lawrie and colleagues, miR-221 was upregulated in the aggressive activated vs the more indolent germinal center form of diffuse large B cell lymphoma. Our findings implicate miR-221 as a marker of T-ALL expressing CD56 and possible as a target for future therapy.

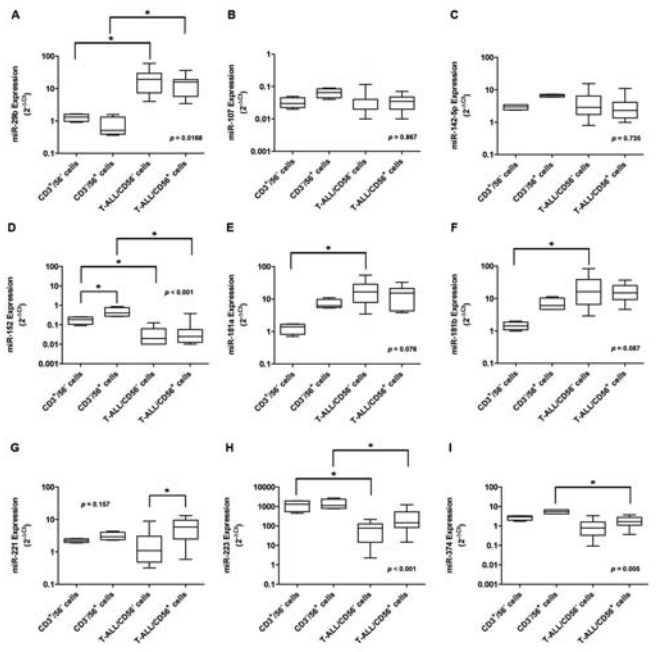

Figure. Quantitative analysis of miR-29b (A), miR-107 (B), miR-142-5p (C), miR-152 (D), miR-181a (E), mir-181b (F), miR-221 (G), miR-223 (H) and miR-374 (I) expression. Leukemic samples from 48 T-ALL patients were obtained from cell bank, and normal cells were isolated from peripheral blood of four healthy subjects. Expression of miRNAs was quantified by quantitative real-time PCR. The horizontal bars represent the mean and 95% confidance interval of miRNAs relative expression calculated by $2^{\Delta CT}$. T-ALL samples were subdivided according to presence or not of CD56 cell marker: 12 samples were positive (T-ALL/CD56$^+$), whereas 36 were negative for CD56 (T-ALL/CD56$^-$). Normal samples represent the population of T (CD3$^+$/56$^-$) and NK (CD$^-$/56$^+$) cells. Asterisks indicate significant difference in comparison with the other group. Non-parametric one-way ANOVA analysis of variance and Fisher's multiple comparison test were performed using the software Minitab 14.

0619

METHYLENETETRAHYDROFOLATE REDUCTASE GENE 677C/T POLYMORPHISM AND GEOGRAPHIC ORIGIN IN ITALIAN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Several factors have been proposed and identified in the past years as prognostic indexes in the treatment of acute lymphoblastic leukaemia (ALL) in childhood. Folate pathway enzymes play a peculiar function in DNA synthesis and methylation. Role of polymorphism 677C/T of Methylenetetrahydrofolate reductase (MTHFR) in paediatric ALL has been investigated with conflicting results. *Aims*. to evaluate the role of MTHFR 677C/T polymorphism and other covariates in determining relapse risk in our Italian population of childhood ALL. *Material and Methods*. we genotyped MTHFR 677C/T (DNA extraction, PCR amplification, digestion by restriction enzymes and agarose gel electrophoresis) in 107 Italian children enrolled in ALL-AIEOP 2000 trial. The allelic frequency obtained was compared with that expected in the Italian population (Bauduer, Mol Genet Metab, 2005). We collected information on age at diagnosis, gender, WBC count at diagnosis, immunophenotype, steroid type used in induction phase (prednisone or dexamethasone), protocol risk class (standard, intermediate, high). We assigned each patient to a geographic origin group (northern, central or southern Italy) on the basis of their parents' birthplace. Time-to-event analysis (single and multiple Cox regression) was performed to model the risk of relapse given by MTHFR 677C/T polymorphism and other covariates. Univariate and multivariate analysis was performed. *Results*. Genotype frequencies: 677 C/C 43% (46/107); C/T 37% (40/107); T/T 20% (21/107). Allele T frequency in ALL (38%) was not different from general population (p<0.05). 20 relapses were registered (median relapse time 62 months from diagnosis, range 5-211; median follow-up time 170 months from diagnosis, range 5-251). None of the variables on univariate analysis resulted to be significantly associated with relapse risk. On multivariate analysis, only geographic origin was significantly related with relapse risk. In particular, relapse risk is 4.5 times higher in patients of southern origin compared to patients of northern origin (p=0.016). *Conclusions*. Our data do not confirm the role of 677C/T MTHFR polymorphism in conferring a higher relapse risk in ALL patients. Interestingly, multivariate analysis shows a higher relapse risk in patient of southern Italian origins, independently from the medical centre in which they were treated. Our results rise questions about the presence of unknown genetic factors that predispose southern Italian patients to a higher relapse risk. Anamnestic data about patients' families should be taken into consideration in wider retrospective or prospective studies on childhood ALL, especially in series from Italy and Southern Europe, in order to clarify the role of geographic origin in ALL relapse risk.

0620

VALIDATION OF MX1 (MXA) AS A GENETIC MARKER FOR GLUCOCORTICOID-INDUCED APOPTOSIS IN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS?

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Background. The response to initial glucocorticoid therapy in childhood acute lymphoblastic leukemia (ALL) reliably predicts the response to multi-agent chemotherapy. Patients resistant to glucocorticoids (prednisone poor responders (PPR)) have a poorer event-free survival compared to glucocorticoid-sensitive patients (prednisone good responders (PGR)). In an expression profiling study to investigate differential gene expression in leukaemic blasts from childhood ALL patients at time of diagnosis, the Mx1 myxovirus (influenza virus) resistance 1 gene was identified as one of the highly expressed genes in standard-risk patients (Cario *et al.* 2005), whereas the expression in the high-risk patient group was decreased 2-fold. *Aims*. The aim of our study was to investigate whether the expression of Mx1 on the transcriptome and on the proteome level differs between prednisone good and prednisone poor responders, respectively, particularly after prednisone treatment. *Methods*. The human B-cell precursor leukemic cell lines with known gc sensitivity, i.e. MHH cALL 2 (PPR) and MHH cALL 3 (PGR), and further ALL cell lines, e.g. REH, SD-1 and SUP-B15, were studied after 24, 48, 72 and 96 hours, untreated and after induction with prednisone (3 µg/mL Solu-DecortinH®) or the proteasome inhibitor bortezomib, respectively. Real-time PCR were performed to quantify the MX1 expression. Western blot analyses were performed on whole cell lysates using a rabbit polyclonal Mx1 (MxA) antibody (sc-50509, Santa Cruz). To quantify viable cells, WST-1 assays were performed. *Results*. The level of Mx1 RNA was 2.4-fold higher in untreated PGR cells compared to the untreated PPR cells. After prednisone induction, the MX1 RNA expression in PGR cells increased 18-fold in comparison to the initial level, whereas it decreased slightly in PPR cells and in the SD1 cell line, whereas it remained unaffected in REH and in SUP-B15 cell lines. In agreement with these findings, Mx1 protein was detectable at low levels in untreated PGR cells, but undetectable in PPR cells. Prednisone treatment led to

a 30-fold rise in Mx1 protein expression in the PGR cell line, but caused no significant changes in the PPR cells, 72 and 96 hours after induction. In contrast, the protein levels of Mx1 decreased in SD-1 and SUB-P15 following prednisone treatment. Bortezomib, particularly in combination with prednisone, alleviated the increase in MX1 RNA expression the PGR cell line, but did not affect Mx1 protein expression. In the PPR cell line, bortezomib treatment caused no significant changes. All pre-B-ALL cell lines, that showed an unaltered viability under treatment with 6.2 µM prednisone, also had an unaltered or slightly decreased MX1 RNA expression. In contrast, the only pre-B ALL cell line with a significant prednisone response, showed an immediate and persistent increase of MX1 RNA expression. *Summary and Conclusions*. These *in vitro* results show that MX1 expression is increased and further induced after prednisone treatment in PGR cells. They are in agreement with previous gene expression profiling data, reporting an increased MX1 gene expression in standard-risk childhood ALL patients. MX1, a GTP-binding interferone-induced gene, is involved in the induction of apoptosis, again linking apoptosis defects with prednisone resistance in childhood ALL.

0621

THE MAMMALIAN TARGET OF RAPAMYCIN INHIBITOR RAD001 INHIBTS PROLIFERATION OF T- AND B-LYMPHOBLASTIC CELLS VIA CELL CYCLE ARREST

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Background. The protein kinase mammalian target of rapamycin (mTOR) regulates cell growth, survival and cell proliferation. Activation of mTOR leads to phosphorylation of downstream targets S6 kinase and 4E-BP thereby influencing translational control. RAD001 is a specific mTOR inhibitor which has been shown to inhibit cell growth of solid tumor cells as well as low grade lymphoma cells. It's efficacy in those diseases is currently investigated in clinical trials. Effects of mTOR inhibition on proliferation and cell function of acute lymphoblastic leukemia (ALL) are unknown. *Aims*. Investigation of the effects of mTOR pathway inhibition on ALL cells using RAD001, in particular in regards to the cell proliferation, apoptosis, necrosis and its influence on the PI3K/Akt pathway. *Methods*. Acute lymphoblastic leukemia cell lines with different cytogenetics and phenotypes were used (SEM, RS4;11, Jurkat and MOLT4). Cells were incubated with different concentrations of RAD001 (1 nM, 10 nM and 100 nM). Cell count, apoptosis, necrosis, cell cycle analysis and metabolic activity were determined at 24 h, 48 h, 72 h and 96 h using microscopy, flow cytometry and WST-1, respectively. Phosphorylationstatus of Akt (Ser473, Thr308) and FoxO3A (Thr32) were determined by western blot. *Results*. Treatment with RAD001 inhibited proliferation in a dose and time dependent manner in all investigated ALL cell lines. Changes in apoptosis and necrosis rates were not detected. Metabolic activity decreased significantly in Jurkat and MOLT4 cells (both T-ALL) using 10 nM and 100 nM RAD001 and decreased not significantly in SEM and RS4;11 (both B-ALL) cell lines. RAD001 induced cell cycle arrest in G0/G1 phase after 48 h and 72 h. In Jurkat cells means of G0/G1 phase were 57% in control cells compared to 68% in treated cells. Similar results were obtained in MOLT4 cells: in control cells means in G0/G1 phase were 65% compared to 74% in treated cells. Western blot results were inconsistent. It seemed that Akt phosphorylation (Ser473 und Thr308) was not influenced early after treatment (0.5-4 h), but subsequently increased after 24-96 h in RAD001 treated cells. In line with this data phosphorylation of FoxO3A (Thr32) seemed to increase in RAD001 treated cells. *Conclusions*. Inhibition of mTOR reduces cell proliferation by cell cycle arrest and reduces metabolic activity in ALL cells with more pronounced effects in the T-ALL cells compared to B-ALL cells. Combination of RAD001 with conventional cytostatic drugs might induce synergistic effects and warrant further investigation.

0622

IMMUNOPHENOTYPIC MODULATION IN ACUTE LYMPHOBLASTIC LEUKEMIA WITH POSTIVE MRD AFTER INDUCTION TREATMENT

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Background. Antigen modulation has been demonstrated in viable acute lymphoblastic leukaemia (ALL) cells resistant to the induction therapy. Up-regulation of CD20 and down-regulation of CD10 have been the phenotypic changes most frequently described. The study of antigen modulation in specific scenarios (such as BCR-ABL1 positive patients or ALL adult patients) requires further investigation. *Aims*. The primary objective of the study was to describe phenotypic, genetic and molecular characteristics of ALL with positive MRD after induction treatment. Secondary objectives were to investigate antigen modulation and to identify common immunophenotypic modulation patterns among molecular and age groups. *Methods*. We studied 45 bone marrow samples from adult (n=32) and pediatric (n=13) patients diagnosed with ALL with MRD >0.1% at day +14 or +35 of induction therapy. Patients were treated according to adult and pediatric standard protocols with induction regimens including daunorubicin, vincristine and prednisone. Multiparapetric flow cytometric analysis was performed at diagnosis, day +14 and day + 35. Mean fluorescence intensity (MFI) was measured for CD10, CD19, CD20 and CD34. Over and underexpression of antigens were considered with a difference greater than 100 MFI units. Cytogenetic and molecular data were also included in the analysis. Pearson correlation coefficient was used to compare MFI values between time points. The Wilcoxon signed-rank test (2-tailed) was used to asses the significance of observed differences between cohorts. *Results*. Median age at diagnosis was 29 years (range 2-79). Positivity for CD10, CD 19, CD 20 and CD 34 was 80%, 96%, 62% and 91% respectively. Thirty and 23 patients had positive MRD at day +14 and at day +35, respectively, and were included in the analysis. Most frequent aberrant phenotypes at diagnosis were underexpression of CD45 (n=13), crosslinage of CD33 (n=11), and overexpression of CD 34 (n=10). Most frequent cytogenetics were t (9;22) (n=8), normal cariotype (n=6) and anomalies involving MLL (n=3). Ten patients (22%) had BCR/ABL1 rearrangement and one patient had TEL/AML1 rearrangement. For the whole group, we observed a significant decrease of CD10 and an increase of CD 19 expressions at day +14 compared to the diagnosis (mean MFI CD10, 140 vs 129 [p=0.02], mean MFI CD19, 145 vs 216 [p=0.04]). Differences persisted at day +35 for CD10 (140 vs 48 [p=0.007]) but not for CD 19. We did not identified differences regarding expression of CD20 and CD34 between diagnosis and time points. In the subgroup analysis, BCR-ABL1 negative patients had an overexpression of CD20 and CD19 at day +14 as compared to underexpression in BCR-ABL1 positive patients (p=0.006 for CD19 and p=0.03 for CD20). We did not find any difference in antigen modulation between pediatric and adult patients, high vs low levels of MRD (more or less than 5%) at day +14, or in normal vs abnormal caryotipe. *Conclusions*. Antigen modulations in ALL occur during induction therapy with down-regulation of CD10 being the most consistent pattern throughout the induction treatment. BCR-ABL1 positive patients seem to have a specific pattern of antigen modulation throughout induction.

0623

ONCOGENIC PIK3CA MUTATIONS ARE ABSENT IN BCR-ABL POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (PH+ ALL)

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Background. Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase. It regulates cellular growth and proliferation. It is an important downstream mediator of BCR-ABL signalling, implicated in leukemogenesis and in clinical resistance to ABL kinase inhibitors such as imatinib. Somatic mutations in the gene encoding the PI3K catalytic subunit p110α (PIK3CA) have been reported in various cancers. These somatic missense mutations were predominantly located at specific *hot spots* at residues E542K and E545K (exon 9) in the helical and H1047R (exon 20) in the kinase domains. Further studies in murine cells have confirmed the leukemogenic potential of these three mutations leading to an elevated activity of the PI3-kinase and the constitutive phosphorylation of the downstream targets. *Aims*. To investigate the possible involvement of the PIK3CA gene in leukemogenesis, we analysed 33 genomic DNA samples from 26 patients with BCR-ABL positive ALL, 2 chronic myeloid leukemia patients with blast crisis and 5 long-term cultured primary human BCR-ABL positive ALL cells for the presence of activating helical and kinase domains mutations. Additionally, AKT1 cDNA from the patients and primary Ph+ ALL long term cultures was analyzed for the shortly described transforming mutation E17K. *Methods*. Bone marrow or peripheral blood samples from newly diagnosed and imatinib naive patients (n=8) and patients after first (n=16) or second (n=4) relapse on imatinib were examined. Genomic *touch down* PCR amplifications of

exons 1,2,6,7,9,14 and 20 of the PIK3CA gene were performed. The PCR products were directly sequenced. The cDNA from these samples was examined for BCR-ABL kinase mutations and the AKT1 mutation E17K by dHPLC and sequencing. *Results*. A single nucleotide alteration in the PIK3CA gene located in exon 6 (A1173G) was detected in 4 of 28 (14%) patient samples and in 2 of the 5 (40%) analysed Ph+ ALL long-term cultures. A search of the SNP database revealed that A1173G is an annotated SNP (rs2230461). None of the 33 analysed samples harboured the known activating mutations in the PIK3CA gene. Mutations of the ABL kinase domain were not detected in newly diagnosed ALL but in 12 of 20 patients (38%) after first or second relapse on imatinib. In three of these patients the SNP in the PIK3CA gene was found. Furthermore, we failed to identify the transforming AKT1 mutation E17K in the Ph+ ALL patients and long-term cultures. *Summary and Conclusions*. The absence of mutations in the PIK3CA gene in a sizeable number of patients with advanced Ph+ALL suggest that such mutations are at best rare events. In conjunction with the absence of activating AKT mutations in this patient samples and long-term cultured primary human Ph+ALL cells, these data suggest that mutations of these members of the PI3K/AKT pathway do not play a significant causal role in resistance or disease progression of BCR-ABL positive ALL. Our data indicate that mechanisms other than mutations are involved in deregulation of PI3K/AKT signalling in BCR-ABL positive ALL, but do not exclude the possibility of functionally relevant mutations of other subclasses of PI3-kinases or other catalytic subunits (e.g. 110δ and 110γ).

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PROLIFERATIVE EFFECT OF HUMAN HEMOKININ-1 ON HUMAN PRE-B LYMPHOCYTE CELL LINE REH

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Background. Hemokinin-1 (HK-1), such as previously known substance P (SP), neurokinin A and neurokinin B, is a member of the tachykinin family. This peptide is encoded by the gene TAC4 which has been identified in lymphoid B hematopoietic cells of mouse bone marrow, where HK-1 promotes survival and expansion of B cell lineage. *Aims.* The aim of our study was to investigate the effects of HK-1 on differentiation, apoptosis and proliferation of the human pre-B lymphocyte cell line REH, in comparison to those of SP. *Methods*. REH cells were cultured in RPMI medium and stimulated by either SP or human HK-1 (1 nM to 100 µM) with or without LPS (5 ng/mL) in the presence of the peptidase inhibitor phosphoramidon (0.1 µM). After 12, 24 or 48 hrs, differentiation was assessed by extensive immunophenotyping, apoptosis quantified with annexin V and propidium iodure staining and proliferation measured with [3H]-thymidine incorporation. mRNA expression was evaluated with custom designed qRT-PCR assay. *Results.* Hemokinin-1 increased REH cell proliferation in a dose-dependent manner after 48 hrs of stimulation. This effect was potentiated in presence of LPS and phosphoramidon (maximal effect for HK-1 (1 µM): 7870 ± 2086 cpm versus 1723±841 cpm for control, n=3, $p<0.01$). Under these conditions, substance P had no effect. TAC4 mRNA was detected in REH cells. NK1 receptor and neutral endopeptidase mRNA were also abundantly expressed, whereas a low expression of TAC3 was observed. Expression of these mRNA was not affected by a 24 hrs stimulation with LPS (5 ng/mL). Effects of HK-1 (1 µM) on proliferation were however not abolished by a mixture of NK receptor antagonists (SR140333, SR48968 and SR142801 0.1 µM). TAC1 (coding for substance P), NK2 receptor and NK3 receptor mRNA were not found. Finally, under our experimental conditions, pre-B cells REH did not differentiate to B stage after stimulation with either hemokinin-1 or substance P. Apoptosis was not affected. *Conclusions*. Our results demonstrate that hemokinin-1 induces proliferation of human leukemic pre-B lymphocytes. TAC4 mRNA was detected in these cells concomitantly with a high expression of NK1 receptor mRNA. However, this receptor does not seem involved in the response to HK-1. In accordance with mouse studies in literature, substance P had no effect in this model. These data, together with previous biochip studies (Campo Dell'Orto *et al.*, 2007) showing TAC4 expression in 22 acute lymphoblastic leukemias, suggest a role for hemokinin-1 in physiological or pathological hematopoiesis.

Chronic myeloid leukemia - Clinical

0625

THERAPY WITH IMATINIB IN ELDERLY CML PATIENTS (≥65 YEARS) IS WELL TOLERATED BUT CYTOGENETIC AND MOLECULAR REMISSIONS SEEM TO BE ACHIEVED LATER COMPARED TO YOUNGER PATIENTS

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Background. Preliminary observations indicate that elderly CML patients are treated with imatinib less frequently and at lower dosage. *Aims*. To verify these observations a subanalysis of the German CML Study IV, a randomised 5 arm study to optimize imatinib therapy by combination, dose escalation and transplantation, has been performed. *Methods*. Patients older and younger than 65 years were compared with regard to time to hematologic, cytogenetic and molecular remissions, imatinib dose and adverse events (AEs). *Results*. From July 2002 to February 2009 1273 patients with Ph+ or BCR-ABL+ CML in chronic phase were randomized, 302 patients for treatment with imatinib 400mg. Of the latter group 223 patients were evaluable, 58 aged ≥65 (26%) and 165 aged <65 years (74%). Median age was 69 and 49 years, respectively; the median daily dose in both groups was 400mg with ranges between 291 to 671mg and 231 to 720mg, respectively. In the older group 7 patients (12%) discontinued the randomized therapy and 5 (8.6%) died.

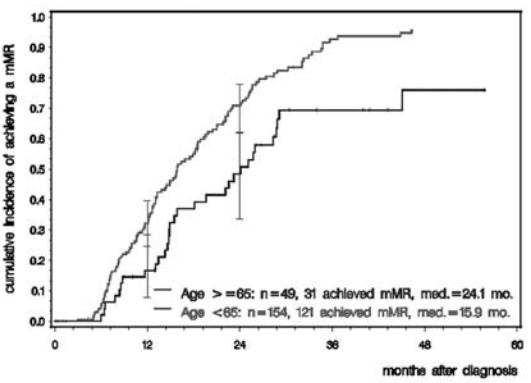

Figure 1. Time to major molecular remission.

In the younger group 7 patients (8.4%) discontinued the randomised therapy, 4 (2.4%) died and 4 (2.4%) received an allogeneic stem cell transplant. Euro risk score was in the elderly low in 13.8%, intermediate in 75.9% and high in 10.3% as compared to 44.2%, 40.6% and 13.3% in younger patients. 223 patients (58 and 165, respectively) were evaluable for hematologic, 198 (48 and 150, respectively) for cytogenetic and 203 (49 and 154, respectively) for molecular responses. Older patients achieved complete cytogenetic and major molecular responses later than younger patients. Median time to complete cytogenetic remission (CCyR) in the older group was 12.3 months versus 10.7 months in the younger group. Median time to major molecular remission (MMR)

was 24.1 months versus 15.9 months, respectively (image 1_102392). Some differences were observed in type, frequency and severity of AEs. 193 patients (50 and 143, respectively) were evaluated on common toxicity criteria (WHO). Hematologic AEs grade III/IV were documented slightly more often in the older than in the younger patients (leukopenia <2000/µL 4.2% vs. 2.8%; thrombocytopenia <50.000/µL: 4.2% vs. 2.8%, respectively). Non hematologic AEs were mainly gastrointestinal symptoms (29% vs. 28%) and myalgia (17% vs. 16%). Edema (17% vs. 23%) and neurologic symptoms (6% vs. 14%) were observed more often in younger patients, dermatologic AEs were seen more often in the elderly (17% vs. 13%). There was no evidence that non hematologic AEs grade III/IV were more frequent in older patients. *Conclusions*. These data show that imatinib 400mg is well tolerated also in the elderly (≥65 years) at similar dosages as in the younger patients, but the responses seem to be achieved later compared to younger patients.

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SOKAL SCORE AND RESPONSE TO IMATINIB IN EARLY CHRONIC PHASE CML: THE GIMEMA CML WORKING PARTY EXPERIENCE ON 559 PATIENTS

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Background. Imatinib (IM) 400 mg daily is the standard treatment for chronic myeloid leukemia in early chronic phase (ECP): the results of the IRIS trial have shown for the IM 400 mg arm a 12-months probability of complete cytogenetic response (CCgR) of 69% but CCgR rates were different according to Sokal risk, being 76%, 67% and 49% in low, intermediate and high Sokal risk categories, respectively (Hughes *et al.*, NEJM 2003). At 84 months, overall survival was 86%, EFS and PFS (no ABP) 81% and 93%, respectively. The outcome was significantly influenced by the Sokal score: at 5 years, the estimated probability of progression to ABP was 17%, 8% and 3% for low, intermediate and high risk Sokal. However, the risk of progression to ABP was not significantly influenced by the Sokal risk in pts who obtained a CCgR (Druker *et al.*, NEJM 2006). *Aims*. To evaluate the long-term outcome, overall and by Sokal risk, of 559 IM treated, early chronic phase patients, treated according to 3 investigator sponsored trials of the GIMEMA CML Working Party.

Table.

	Low	Int	High	Total	P value
Patients, n (%)	219 (39)	216 (39)	124 (22)	559	/
Age, median (range)	44 (18-69)	61 (18-84)	52 (21-79)	52 (18-84)	/
CCgR 12M, %	85	83	65	80	<0.01
MMR 12M, %	62	58	44	56	<0.01
CCgR overall, %	95	94	79	91	0.11
CCgR overall, %	95		79	91	0.04
MMR overall, %	92	89	72	87	<0.01
EFS, %	79	74	50	71	<0.01
FFS, %	89	83	56	81	<0.01
PFS, %	95	89	85	90	0.02
OS, %	96	91	79	91	0.01

Methods. The 559 patients were enrolled in 3 simultaneously running trials of the GIMEMA CML WP: CML/022 (Clin Trials Gov. NCT00510926), phase III, IM 400 vs 800 mg in high Sokal risk; CML/021 (Clin Trials Gov. NCT00514488), phase II, IM 800 mg in intermediate Sokal risk; CML/023, observational, IM 400 mg. Response monitoring was based on conventional cytogenetic examination of bone marrow cells every 6 months and quantitative molecular (Q-PCR) evaluations (PB) after 3, 6 and 12 months on IM (every 6 months thereafter). Definitions: Major Molecular Response (MMR): BCR-ABL/ABL ratio < 0,1%IS. Failures (ELN criteria): no CHR at 6 months, no CgR at 6 months, no PCgR at 1 year, no CCgR at 18 months, loss CHR, loss CCgR, progression to accelerated/blastic phase and death. Events: failures, off-treatment for toxicity, refusal and lost to follow-up. *Results*. (Table): 559 patients were treated with IM 400mg daily (76%) or 800 mg daily (24%). Currently the median follow-up is 42 (1-64) months. Responses and outcome are significantly different by Sokal group; the overall CCgR rate was not different but comparing low and intermediate risk vs high the P resulted significant. The PFS of CCgR patients only, stratified by Sokal risk, is not significantly different but high risk CCgR patients have a significantly greater probability to fail treatment (ELN criteria) vs low and intermediate CCgR ones. *Conclusions*. These data confirms that the Sokal risk influences significantly responses and outcome. Moreover, high risk patients achieving a CCgR still has a significantly greater probability of failing IM with respect to non high risk ones.

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NILOTINIB IN CHRONIC MYELOID LEUKEMIA PATIENTS IN CHRONIC PHASE (CML-CP) WITH IMATINIB RESISTANCE OR INTOLERANCE: 24-MONTH FOLLOW-UP RESULTS OF A PHASE 2 STUDY

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Background. Nilotinib is a potent and highly selective BCR-ABL inhibitor approved for treatment of Ph^+ CML patients in CP or accelerated phase who are resistant or intolerant to prior therapy including imatinib. *Aims*. This study evaluated the efficacy and safety of nilotinib in CML-CP patients resistant or intolerant to imatinib. *Methods*. Intolerant patients must not have had a major cytogenetic response (MCyR) at study entry, since only these patients could be appropriately assessed for the primary study endpoint. Primary endpoint was MCyR. Secondary endpoints included complete cytogenetic response (CCyR), complete hematological response (CHR), MCyR duration, overall survival (OS) and safety. In this analysis, we evaluated the kinetics and duration of CCyR, along with other parameters described below. *Results*. CML-CP patients (n=321) with a minimum follow-up of 19 months were evaluated. Overall, 70% of patients were imatinib-resistant and 30% were imatinib-intolerant. Patients were heavily pretreated, with 72% having received ≥600 mg/day imatinib prior to enrollment. Median duration of CML was 58 (range 5-275) months, and prior imatinib treatment was 32 (range 1-94) months. Median dose intensity of nilotinib was 790 (range 151-1110) mg/day, closely approximating the planned dose of 800 mg/day. Nilotinib led to rapid and durable hematologic and cytogenetic responses. CHR was achieved or maintained in 94% of patients, with 75% of patients with no baseline CHR achieving a new CHR, at a median of 1 month following initiation of nilotinib therapy. MCyR was achieved in 59% of patients at a median of 2.8 months following initiation of nilotinib therapy. In patients with a baseline CHR, 73% achieved MCyR. Overall, 44% of patients achieved a CCyR with a median time to first CCyR of 3.3 (range 0.9-23.5) months. Responses were durable, with 78% of patients maintaining MCyR, and 83% maintaining CCyR at 24 months. Overall, major molecular response (MMR) was achieved in 25% of patients. Estimated time to discontinuation of study drug was 578 (range 1-958) days. Estimated OS rate was 88% at 24 months. Nilotinib was generally well-tolerated and no new safety issues emerged with 24 months of follow-up. The most frequent grade 3/4 biochemical laboratory abnormalities were elevated lipase (17%), hypophosphataemia (16%), hyperglycemia (12%), and total bilirubin (8%). All biochemical abnormalities were transient and clinically asymptomatic. Grade 3/4 non-hematologic adverse events were infrequent,

with rash, headache, and diarrhea occurring in 2% of patients. The most common grade 3/4 hematological laboratory abnormalities were neutropenia (31%), thrombocytopenia (31%), and anemia (10%). Brief dose interruptions were successful in managing most adverse events. Pleural or pericardial effusions occurred in 2% of patients (all grades), and grade 3/4 were uncommon (<1%). *Conclusions*. These results demonstrate that nilotinib was highly effective, inducing rapid and durable cytogenetic responses in CML-CP patients failing prior imatinib therapy due to resistance or intolerance. Nilotinib was well tolerated with a favorable risk/benefit profile in this study. The estimated 88% OS rate at 24 months in this heavily pretreated patient population suggests that nilotinib is effective and can provide favorable long-term outcomes for patients.

0628

SUBOPTIMAL RESPONSE TO IMATINIB 400 MG DAILY FOR CHRONIC MYELOID LEUKEMIA IN EARLY CHRONIC PHASE: A GIMEMA CML WP ANALYSIS OF 423 CONSECUTIVE PATIENTS

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Background. Imatinib (IM) 400 mg daily is the standard treatment for Chronic Myeloid Leukemia (CML) in early chronic phase (ECP). The European LeukemiaNet (ELN) recommendations suggest, at given timepoints, when the treatment strategy should be changed ("failure" patients), or alert physicians "that the long-term outcome of the treatment would not likely be as favourable" ("suboptimal responders"). Marin *et al*. recently analyzed a single center experience based on 224 patients, reporting that suboptimal responders at 6 and 12 months may have a significantly poorer PFS and lower probability of Complete Cytogenetic Response (CCgR) (in this analysis, the 18-months suboptimal response was less useful). *Aims*. To investigate the outcome of suboptimal responders in a multicentric, nationwide experience. *Methods*. We performed an analysis within 2 simultaneously running trials of the GIMEMA CML WP: CML/022 (Clin Trials Gov. NCT00510926), phase III- imatinib 400 vs 800 mg in high Sokal risk; CML/023, observational - imatinib 400 mg. Response monitoring was based on conventional cytogenetic examination of bone marrow cells every 6 months and quantitative molecular (Q-PCR) evaluations (PB) after 3/6/12 months on imatinib (every 6 months thereafter). Definitions: suboptimal response at 6 months: Ph^+ >35%, in absence of failure; suboptimal response at 12 months: Ph^+ ≥1%, in absence of failure. Major Molecular Response (MMR): BCR-ABL/ABL <0,1%IS. Events: no PCgR at 1 year (for suboptimal responders at 6 months), no CCgR at 18 months, loss of CHR, loss of CCgR, progression, death for any reason. *Results*. 423 consecutive CML patients in ECP were treated with imatinib 400mg daily. Sokal score was low/intermediate/high in 52%/32%/16%, respectively. Median follow-up: 41 (1-64) months. 26 patients went off study within the first 6 months: 361 of the remaining 397 patients were evaluable for CgR at 6 months. 45 patients went off study within the first 12 months: 354 of the remaining 378 patients were evaluable for CgR at 12 months. At 6 and 12 months suboptimal responders were 20/361 (6%) and 31/354 (9%), respectively. At 6 months, the probability of CCgR, MMR and EFS for suboptimal/optimal responders was 60/98%, 50/93% and 60/90%, respectively. At 12 months the probability of CCgR, MMR and EFS for suboptimal/optimal responders was 81/100%, 68/96% and 68%/94%, respectively (all the differences observed were statistically significant). We have observed no difference in PFS and OS. This may be due to the still short period of observation and/or to the efficacy of second generation TKIs in this setting of patients. Finally, we observed no differences between 18-months suboptimal/optimal responders in terms of CCgR, MMR and EFS. *Conclusions*. The suboptimal responders within 12 months represent a minority of all the IM-treated patients (6%/9% at 6/12 months, respectively): responses and outcome are significantly worse with respect to the optimal ones. Our results confirm the predictive value on outcome of ELN recommendations, strengthening the need of an early intervention in this setting of patients to flatten the higher rate of negative events observed beyond 12 months on treatment. *Acknowledgements*. European LeukemiaNet, COFIN, University of Bologna and BolognAIL.

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THE ORALLY ACTIVE AURORA KINASE A INHIBITOR MLN8237 HAS POTENT ACTIVITY IN PRECLINICAL MODELS OF RESISTANT CHRONIC MYELOID LEUKEMIA AND SIGNIFICANTLY INCREASES THE EFFICACY OF NILOTINIB

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Background. Novel therapies are urgently needed to prevent and treat tyrosine kinase inhibitor resistance in chronic myeloid leukemia (CML) particularly in cases expressing the gatekeeper T315I BCR-ABL mutation. Aurora A is a serine/threonine kinase that plays a key role in mitosis and is frequently overexpressed in leukemia. MLN8237 is a novel, orally available inhibitor of Aurora A currently under investigation in Phase I and II studies in solid tumors, lymphomas and myeloma. *Aims*. To evaluate the efficacy of MLN8237 alone and in combination with Nilotinib in preclinical models of CML. *Methods*. Ba/F3 cells expressing wild type (p210) with and without stable shRNA p53 knockdown and T315I BCR-ABL, Lama84 and K562 cells and primary human cells from CML patients were used. Apoptosis was evaluated by PI/FACS and cell viability was assessed by MTT assay. The prolonged *in vitro* effects of MLN8237 were assessed by MethoCult colony formation assays. Effects of MLN8237 on Aurora A activity, BCR-ABL activity and caspase activation and were measured by western blot analysis. An *in vivo* model of AML was generated by injecting K562 cells into the flanks of immunodeficient mice. *Results*. Nanomolar concentrations of MLN8237 potently inhibited the *in vitro* growth and survival of K562 and Lama84 cell lines and disrupted cell cycle kinetics as evidenced by the accumulation of G2/M and aneuploid cells prior to the onset of apoptosis. MLN8237 possessed equipotent *in vitro* and *in vivo* anticancer activity against Ba/F3 cells expressing wildtype (p210) and the mutant T315I form of BCR-ABL. Impairment of p53 function did not significantly affect the anticancer activity of MLN8237. MLN8237 equally inhibited survival of primary human CML cells from patients with unmutated and T315I-mutated BCR-ABL. Co-treatment with MLN8237 and nilotinib in K562 and Lama84 cell lines resulted in significantly greater apoptosis and more effective inhibition of clonogenic survival than treatment with either single agent. Similarly, co-administration of MLN8237 and nilotinib to immunodeficient mice bearing K562 xenografts was well-tolerated and resulted in significantly greater tumor growth inhibition than what was achieved by either agent alone. MLN8237 and nilotinib cooperated to reduce BCR-ABL activity as evidenced by a reduction of BCR-ABL autophosphorylation and its downstream target CrkL. The processing of caspases-9 and -3 to active forms was also potentiated by the combination. *Conclusions*. The combination of MLN8237 and nilotinib is very effective and well tolerated in preclinical models of CML and represents a novel therapeutic strategy for refractory CML, that is available orally and has the potential to suppress the emergence of T315I mutated clones. Based on this promising preclinical data, a clinical investigation of the efficacy of this combination in patients with refractory CML is warranted.

0630

FINAL SAFETY ANALYSIS OF 1,793 CML PATIENTS FROM ENACT (EXPANDING NILOTINIB ACCESS IN CLINICAL TRIALS) STUDY IN ADULT PATIENTS WITH IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA

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Background. Nilotinib, a potent and highly selective BCR-ABL kinase

inhibitor, approved for the treatment of Ph+ chronic myeloid leukemia (CML) patients (pts) in chronic phase (CP) and accelerated phase (AP) who are resistant or intolerant to prior therapy, including imatinib. *Aims*. The ENACT study, which is a Phase IIIb, open label, multicenter study, was initiated to obtain additional safety information in pts with imatinib-resistant or -intolerant CML in chronic, accelerated, or blast crisis (BC) phase in a clinical practice setting outside of a registration study. *Methods*. Pts received nilotinib 400 mg twice daily (BID). Dose escalation was not permitted. 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), lack of response, or persistent disease at the investigator's discretion. Efficacy was provided by investigator assessment. Patients who failed dasatinib were also eligible. *Results*. A total of 1,793 pts enrolled in the ENACT study between Jan. 2006 and Oct. 2008, including 1,422 CP pts (79%), 181 AP pts (10%) and 190 BC pts (11%), from 293 study sites worldwide. The median age of all pts was 52 years and 69.2% of patients received prior imatinib ≥600mg/d. At study completion, 941 (53%) pts were continuing on nilotinib. Median (range) duration of nilotinib exposure was 266 (1-807) days for CP pts, 160 (6-736) days for AP pts, and 78 (1-642) days for BC pts; median average dose intensity was 783, 779 and 773 mg/day, respectively. The main reasons for treatment discontinuation were inadequate responses (22%) and AEs (14%). The majority of grade 3/4 AEs were hematologic and the most common hematologic toxicities were thrombocytopenia (24%) and neutropenia (17%). Non-hematologic AEs were mostly grade 1/2. Grade 3/4 non-hematologic AE's were infrequent and included headache, rash and nausea. Death on study was reported for 54 (3%) pts and occurred most frequently among those with BC (n=27; 14%). A low incidence of QT prolongation (defined as absolute QTcF > 500 msec; n=6, 0.3%) was observed overall. Grade 3/4 lipase elevation was observed in 115 patients (6.4%), but only 5 patients (0.3%) had lipase elevation associated with treatment discontinuation. Pancreatitis, including acute, occurred in 27 patients (1.5%) and was associated with discontinuation in 4 patients (0.2%). Only one patient (0.1%) had hyperglycemia associated with treatment discontinuation. Overall major cytogenetic response (MCyR) rates were consistent with those observed in earlier phase II trial at 45.1% in CP (34.2% CCyR,) 19.3% in AP and 19.5% in BC pts. Likewise, complete hematologic response (CHR) rates were 43% in CP, 22.1% in AP and 8.4% in BC pts. *Conclusions*. This final safety analysis of a large expanded access study further demonstrates that nilotinib is generally well tolerated in heavily pretreated pts in all phases of CML with safety profile reported in ENACT being similar to that observed in the pivotal phase II registration study.

0631

NILOTINIB IN CHRONIC MYELOID LEUKEMIA PATIENTS IN ACCELERATED PHASE (CML-AP) WITH IMATINIB RESISTANCE OR INTOLERANCE: 24-MONTH FOLLOW-UP RESULTS OF A PHASE 2 STUDY

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Background. Nilotinib is a potent and highly selective BCR-ABL inhibitor approved for the treatment of Ph+ CML patients in chronic phase (CP) or AP who are resistant or intolerant to prior therapy including imatinib. *Aims*. This study evaluated the efficacy and safety of nilotinib (400 mg bid) in CML-AP patients resistant or intolerant to imatinib. *Methods*. Intolerant patients must not have had a major cytogenetic response (MCyR) at study entry. Primary endpoint was confirmed hematologic response (HR). Secondary endpoints included MCyR, time to progression (TTP), overall survival (OS), and safety. In this analysis, we also evaluated the kinetics and duration of response, as well as response by subgroup analysis based on peripheral blood basophil (< 20% vs. >20%), blast (>15% to <30% vs other values), and platelet count (>100×10^9/L vs. <100×10^9/L) at study entry. Patients with additional chromosomal abnormalities alone were not eligible. *Results*. CML-AP patients (n=137) with a minimum follow-up of 11 months were evaluated. Overall, 80% of patients were imatinib-resistant and 20% were imatinib-intolerant. Patients were heavily pretreated, with 79% having received ≥600 mg/day, and 45% receiving ≥800 mg/day imatinib prior to enrollment. Overall, 62% of patients exhibited BCR-ABL kinase domain mutations, and 31% of all patients exhibited other chromosomal abnormalities. Overall, 62% of patients exhibited BCR-ABL kinase domain mutations at study entry. Median dose intensity of nilotinib was 775 (range 150-1149) mg/day, closely approximating the planned dose of 800 mg/day. Nilotinib led to HR in 56% of patients, with 31% of patients achieving a CHR at a median of 1 month following start of therapy. MCyR was achieved in 32% of patients at a median of 2.8 months; CCyR was reached in 20% of patients and over 70% of these patients remained in CCyR at 24 months. Overall, no significant differences were found in responses based on pre-treatment basophil, blast, and platelet counts, respectively (Table 1). Estimated OS rate was 67% at 24 months. Overall, 6 (4%) patients received stem cell transplant after discontinuation of study drug. The safety profile of nilotinib did not change with longer follow-up, with 9% of patients discontinuing due to adverse events. The most frequent grade 3/4 laboratory abnormalities were neutropenia (42%), thrombocytopenia (41%), anemia (25%), elevated serum lipase (18%), and hypophosphatemia (14%). All biochemical abnormalities were transient and clinically asymptomatic. Grade 3/4 myelosuppression was predictable, and easily managed with a median onset of 14 to 29 days and a median duration of 8 to 26 days. Grade 3/4 non-hematologic adverse events were rare (<1%) and included nausea, fatigue, and diarrhea. *Conclusions*. These results demonstrate that nilotinib was highly effective, inducing rapid and durable hematologic and cytogenetic responses in CML-AP patients failing prior therapy. Nilotinib was well tolerated with a favorable risk/benefit profile in this study. The estimated 67% OS rate at 24 months in this heavily pretreated patient population with advanced disease suggests that nilotinib therapy is effective and can provide favorable long-term outcomes for patients.

Table 1. Response by subgroups.

	HR n (%)	CHR n (%)	MCyR n (%)
Basophil count			
< 20% (n = 116)	62 (53)	34 (29)	35 (30)
≥ 20% (n = 21)	15 (71)	9 (43)	9 (43)
Blast count			
Other values (n = 123)	69 (56)	40 (33)	38 (31)
≥ 15% to < 30% (n = 14)	8 (57)	3 (21)	6 (43)
Platelet count			
≥ 100 x 10 ^{9}/L (n = 89)	53 (60)	31 (35)	30 (34)
< 100 x 10 ^{9}/L (n = 48)	24 (50)	12 (25)	14 (29)

0632

AGE HAS NO IMPACT ON OUTCOME OF EARLY CHRONIC PHASE, PH-POS CML, IMATINIB TREATED PATIENTS: A NATIONWIDE ANALYSIS ON 559 CASES OF THE GIMEMA CML WP

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Background. Age was a consistent poor prognostic factor in chronic myeloid leukaemia (CML) before the introduction of imatinib (IM). The efficacy and tolerability of IM reduced significantly the impact of age on outcome in late chronic phase (CP) patients and in early CP ones. However, as far as early CP, available data are based on limited number of patients and still the allocation to IM of older CML patients is not a

widely accepted practice. *Aim.* To asses the effects on the responses and outcome of IM in early CP, older patients. *Methods*. We performed an analysis by age groups of 3 concurrent clinical trials of the GIMEMA CML Working Party (Clin Trials Gov. NCT00514488, NCT00510926 and the observational trial CML/023). Overall, 559 patients have been enrolled between April 2004 and April 2007 and treated with IM 400 mg or 800 mg daily ((76% and 24%, respectively); the median age was 52 (extr.18 - 84) years, the median follow-up is currently 42 (extr.1-64) months. WHO has set at 65 years the older age: at diagnosis, 444 patients (79%, median age 46 years) were <65 years (group A) and 115 patients (21%, median age 71 years) ≥65 years (group B). The same proportion in both groups (23% and 25%, respectively) received 800 mg daily IM front-line. The Sokal risk distribution, as expected, was different in the 2 groups A and B: low in 47%/9%, intermediate in 30%/72%, high in 23%/19%. *Results*. In group A and B, the cumulative Complete Cytogenetic Response (CCgR) rate and the cumulative rate of Major Molecular Response (MMR) were 88%/84% and 82% / 81%, respectively. Failures (no CHR at 6 months, no CgR at 6 months, no PCgR at 1 year, no CCgR at 18 months, loss CHR, loss CCgR, progression to accelerated/blastic phase and death) were 68/444 (15%) and 23/115 (20%) for group A and B, respectively. Events (failures, off-treatment for toxicity, refusal and lost to follow-up) were 99/444 (22%) and 34/115 (31%) for group A and B, respectively. The rates of progression to accelerated/blastic phase were the same (5%) in both groups. The OS was 95% and 88% (all causes of death) and 97% and 96% (CML related deaths) for group A and B, respectively. All the differences were not statistically significant. *Conclusions*. Our analysis confirms, based on a large patients population and a proper period of observation, that age per se is not a negative prognostic indicator in early CP CML patients IM treated. Not the patient's age, but the presence of relevant comorbidities suggesting in advance a negative cost-to-benefit balance of IM, should be used as treatment choice advise.

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0633

EXPANDING NILOTINIB ACCESS IN CLINICAL TRIALS (ENACT) STUDY IN ADULT PATIENTS (PTS) WITH IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA (CML): SUBGROUP ANALYSIS OF PATIENTS WHO FAILED PRIOR DASATINIB THERAPY

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Background. Patients with Ph+ CML who are resistant or intolerant to both imatinib and dasatinib have limited treatment options. One option is nilotinib, a potent and highly selective BCR-ABL inhibitor approved for the treatment of pts with Ph+ CML in chronic phase (CP) or accelerated phase (AP) who are resistant or intolerant to prior therapy. *Aims*. The ENACT study evaluated the safety and efficacy of nilotinib in patients with CML-CP, -AP, or -blast crisis (BC) who were either resistant or intolerant to both imatinib and dasatinib. *Methods*. Pts received nilotinib 400 mg BID. Dose escalation was not permitted. Pts requiring dose reduction to 400 mg once daily due to toxicity were allowed to dose re-escalate to 400 mg BID after resolution of the adverse events (AEs), lack of response, or persistent disease at the investigator's discretion. Efficacy was provided by investigator assessment. *Results*. A total of 292 pts with CML-CP (n=218; 75%), -AP (n=34; 12%) and -BC (n=40; 14%) were included in the analysis (16.2% of the entire study). Median time since first diagnosis of CML was 80, 75 and 59 months, respectively. Median duration of prior imatinib therapy was 35 months for CP, 27 months for AP and 23 months for BC pts, and the majority (61%, 80% and 73%) were imatinib-resistant with 71.6% of patients receiving prior imatinib ≥600mg/d. Median duration of prior dasatinib therapy was approximately 9.2 months for CP, 7.9 months for AP and 5.1 months for BC pts. Prior best major cytogenetic response (MCyR) was 14.2% CP, 5.8% AP and 10% BC pts. The median (range) duration of nilotinib exposure was 226 (4-758) days for CP, 111 (7-563) days for AP and 59 (3-553) days for BC pts, with 64%, 27% and 18% remaining on nilotinib at study completion, respectively. In total, 26 (12%) CP, 5 (15%) AP and 2 (5%) BC pts discontinued nilotinib due to AEs; 35 (16%) CP, 16 (47%) AP and 17 (43%) BC pts discontinued due to unsatisfactory therapeutic effect. Complete hematologic response (CHR) was achieved with nilotinib treatment in 40% CP, 10% AP and 3% BC pts. MCyR was achieved in 41% CP (CCyR 27.8%), 7% AP and 14% BC pts. These responses are similar to those observed in the total study cohort. A total of 285 pts (98%) experienced AEs and 196 pts (67%) experienced grade 3/4 AEs. The majority of grade 3/4 AEs were hematologic and the most common of these were thrombocytopenia (25%) and neutropenia (21%). On study, myelosuppression was not increased in pts treated with 2 prior TKI's. Non-hematologic AEs were mostly grade 1/2 and included rash, headache, and nausea. A total of 9 (3%) and 1 (0.3%) pts experienced pleural and pericardial effusion, respectively, of which 2 (1%) pleural effusions were grade 3/4. *Conclusions*. Nilotinib is highly active in Ph+ CML pts who have failed both prior imatinib and dasatinib therapy. These results support the significant efficacy and favorable tolerability of nilotinib in the treatment of CML even after 2 prior TKIs.

0634

RELEASE OF INTRACELLULAR CALCIUM PRIMES CHRONIC MYELOID LEUKAEMIA CELLS FOR TYROSINE KINASE INHIBITOR -INDUCED APOPTOSIS

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Background and Aims. The tyrosine kinase inhibitor (TKI) imatinib has become the standard of care in chronic myeloid leukaemia (CML). More recently, second generation TKI have become available, which are effective in imatinib refractory / intolerant patients, and there is considerable current interest in their use in newly diagnosed patients. Each TKI has slightly different activity and toxicity profiles, suggesting that combinations of TKI are worthy of investigation. Currently there are no clinical and very little *in vitro* data on TKI combinations. In initial experiments in CML cell lines, we showed that any pairing of dasatinib, nilotinib and imatinib leads to potentiation of the anti-proliferative and pro-apoptotic effects of TKI. The original aim of the present work was to investigate the mechanism of potentiation between different TKI, with particular reference to whether one TKI affects the cellular transport of another. In the light of our findings, this evolved into an investigation of the effect of TKI on intracellular $Ca2^+$. *Methods and Results*. Imatinib though not dasatinib and nilotinib is transported into cells by the influx transporter hOCT1, and amantadine and prazosin are widely used as blockers of hOCT1 function. In experiments on LAMA84 and 3 other CML cell lines using amantadine to block hOCT1, it was found that pre-treatment with amantadine 500 uM or prazosin 20 uM significantly reduced cell proliferation in response to 0.5 uM imatinib or 0.05 uM nilotinib or 0.02 uM dasatinib, concentrations which are ineffective when used alone (p<0.01 in each case). Similar significant effects on cell viability were also seen. However, this effect was not seen in 3 BCR-ABL negative leukaemia cell lines or in normal blood mononuclear cells. Amantadine alone significantly attenuated CrKL phosphorylation to 64% (p=0.003), suggesting a priming effect. BCR-ABL expressing cells are known to have decreased free releasable $Ca2^+$ in the endoplasmic reticulum (ER). In experiments with the $Ca2^+$ chelator EGTA and the ER $Ca2^+$ releasing agent thapsigargin, here it was found that amantadine caused an increase in intracellular $Ca2^+$, mediated by a combination of release from the ER and increase in capacitative $Ca2^+$ uptake. Addition of TKI to amantadine-primed cells further increased the intracellular $Ca2^+$ concentration. Furthermore, the effect of amantadine priming of TKI apoptosis was attenuated by both BAPTA (a membrane permeable $Ca2^+$ chelator) and Z-VAD-FMK (pan caspase inhibitor). *Summary and Conclusions.* These data suggest that release of free $Ca2^+$ in the cytosol may be an important mechanism of TKI-induced apoptosis. Taken with existing data, our findings suggest that, unlike normal and other leukaemic cells, BCR-ABL+ cells may be particularly vulnerable to rises in intracellular $Ca2^+$, and that $Ca2^+$ mobilisation may be an important component of TKI action. Further study of agents that alter intracellular $Ca2^+$ is warranted in CML.

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A SHORT (12M) IFNA/IMATINIB COMBINED THERAPY CAN SECURE LONG TERM MAINTENANCE OF IMATINIB INDUCED CYTOGENETIC REMISSION AFTER DISCONTINUATION OF IMATINIB IN CML PATIENTS WITHOUT MOLECULAR REMISSION

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Background. Imatinib Mesylate (IMA) is a gold standard of therapy in CML, however it is a crucial question whether this therapy can cure CML, as its discontinuation usually results in an early disease progression. Cessation of IMA therapy is feasible in about half of the patients that achieve complete molecular remission (CMR, the minority of CML patients), mainly those that were also previously exposed to IFN. IFN is active in CML. Responding patients can maintain long term complete cytogenetic remission (CCyR) off therapy, probably due to the effect of IFN on the early/dormant leukemic progenitor stem cells. Recently it was shown that IFN activates dormant haematopoietic stem cells to be exposed to therapy. *Aims*. To facilitate discontinuation of IMA therapy in patients with CML and complete cytogenetic, but not molecular, remission, by the addition of a short course of IFN to the therapy. *Methods*. CML patients with IMA induced CCyR (without molecular remission) of more than 12 month duration were randomized to either continue with IMA therapy and no cessation of therapy, or to add a weekly injection of IFN to the IMA therapy followed by cessation of therapy (study group). The study group patients received Peg- IFN (Pegasys, Roche pharmaceutics) for 52 weeks and stopped IMA therapy after 39 weeks of IFN therapy. *Results*. 12 eligible patients (9M,3F, median age 49y) were enrolled in the study group since January 2006. One patient stopped therapy after two weeks dew to intolerability, and 11 patients completed 52 weeks of Peg-IFN therapy (median dose/w/pt 135ug), stopped IMA therapy after 39 weeks of IFN therapy and are eligible for evaluation > 12 month off all therapy. The median time from diagnosis to enrolment for the 11 patients was 55 month (range 18-84 month). Five of the eleven patients were exposed to IFN at diagnosis, one had a previous allogeneic and one an autologous BMT. After a median follow up of 18 month (range 12-25) from discontinuation of IFN therapy (21 month from cessation of IMA therapy), six of the 11 patients (*responders*) are in a maintained CCyR of a median duration of 24 month (four patients > 24 month, one patient 18 month and one patient 12 month). Five patients (*non responders*) progressed (at 6-7 month after cessation of therapy in the five pt's) and regain complete remission following reintroduction of IMA therapy. The Sokal score of the responders is higher than in the non responders.The degree of molecular response was similar or responders and non responders. Five of the six patients that were exposed to IFN at diagnosis responded compared with two of five patients that were not exposed. *Summary*. The addition of a short (12 month) course of IFN to Imatinib can facilitate discontinuation of Imatinib therapy and secure a long term maintenance of Imatinib induced cytogenetic remission off all therapy in patients with CML and complete cytogenetic, without a molecular, remission. Exposure to IFN at diagnosis may be a positive predictor of success by this strategy.

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RESPONSE TO NILOTINIB IS SIMILAR IN IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS IN ACCELERATED PHASE (CML-AP) WITH AND WITHOUT BCR-ABL MUTATIONS EXCEPT E255K/V, Y253H, AND F359C/V

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Background. CML-AP is an aggressive form of CML characterized by the presence of the Philadelphia chromosome (Ph^+), combined with hematologic progression and often additional cytogenetic abnormalities. Together, these factors contribute to lower response to therapy, an increased likelihood of imatinib resistance and BCR-ABL mutations, and poorer overall survival, than patients with chronic phase (CP) CML. Nilotinib is a selective, and potent BCR-ABL inhibitor, developed through structure-based drug design, with *in vitro* activity against most BCR-ABL mutants (excluding T315I), indicated for the treatment of CML-CP and -AP patients resistant or intolerant to prior therapy including imatinib. In a previous analysis of nilotinib in CML-CP patients with BCR-ABL mutations at baseline, mutations occurring at 3 specific amino acid residues (E255K/V, Y253H, or F359C/V) were shown to be associated with less favorable response to nilotinib. *Aims*. Imatinib resistant CML-AP patients were subdivided into groups who had *no mutation*, *all other mutations* excluding those occurring at these 3 residues, or the *E255K/V, Y253H,* or *F359C/V* mutations. *Methods*. Patients were analyzed for response to nilotinib, as well as time to disease progression (TTP) and overall survival (OS), by mutation group. *Results*. Overall, 62% (54/87) of patients had baseline mutations, including 31 (36%) patients with *all other mutations* and 17 (20%) patients with E255K/V, Y253H, or F359C/V mutations. Patients with T315I mutations (6/87, 7%) were excluded from this analysis. Response and outcomes to nilotinib by mutation type are summarized in Table 1. Hematologic response (HR), major (MCyR), and complete cytogenetic response (CCyR) were achieved within 12 months of therapy in 55%, 30%, and 18% of patients without mutations, 58%, 29%, and 6% of patients with all other mutations, and 24%, 6%, and 0% of patients with E255K/V, Y253H, or F359C/V mutations, respectively. Median time to MCyR was 3.3 months (range, 1.0-13.1) for patients without mutations and 2.5 months (range, 0.9-27.6) for patients with all other mutations. At 24 months, MCyR was maintained in 73% of patients without mutations and in 70% of patients with all other mutations. Median TTP was 9.9 months (range, 0.8 - 29.4) for patients without mutations, 19.5 months (range, 1.5-28.7) for patients with all other mutations, and 3.3 months (range, 0.4-12.2) for patients with E255K/V, Y253H, or F359C/V mutations. Estimated OS rate at 24 months was 50% for patients with no mutations, 70% for patients with all other mutations, and 42% for patients with E255K/V, Y253H, or F359C/V mutations.

Table 1. Nilotinib efficacy by mutation.

	HR n (%)	MCyR n (%)	CCyR n (%)	TTP (months) median (range)	Estimated OS at 24 months, %
No mutation (n=33)	18 (55)	10 (30)	6 (18)	9.9 (0.8-29.4)	50
All other mutations (n=31)	18 (58)	9 (29)	8 (26)	19.5 (1.5-28.7)	70
E255K/V, Y253H, or F359C/V mutations (n=17)	4 (24)	1 (6)	0 (0)	3.3 (0.4-12.2)	42

Conclusions. For CML-AP patients with BCR-ABL mutations (excluding E255K/V, Y253H, or F359C/V) following imatinib therapy, nilotinib

treatment resulted in rapid responses, and estimated TTP and OS, mirroring that of patients with no BCR-ABL mutations. Excluding T315I patients, patients with E255K/V, Y253H, or F359C/V mutations had poorer responses and outcomes than patients with other mutations. Alternative therapies such as transplant or clinical trials may need to be considered for these patients. The results of mutation analysis need to be considered in the clinical context of each individual patient when deciding the best treatment strategy to use.

0637

A STUDY OF PLASMA LEVELS OF IMATINIB AND ITS BIOACTIVE METABOLITE CGP-74588 REVEALS NO CORRELATION WITH SUBSEQUENT CLINICAL OUTCOME IN IMATINIB-TREATED CHRONIC MYELOID LEUKAEMIA

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Background. In patients with chronic myeloid leukaemia (CML) receiving treatment with imatinib, 2 papers have reported that trough (24±3 hours post dose) plasma levels after either 29 days or 12 months of treatment are predictive of the clinical response. If verified in a general CML population, this could be clinically useful for identifying patients who might benefit from an early switch to alternative therapy. However, the value of serial plasma levels in predicting outcome has not been reported, and the interpretation of levels measured at time points earlier than trough has not been investigated. *Aims*. The aim of this work was to investigate whether plasma levels of either imatinib or its main bioactive metabolite CGP-74588 were predictive of subsequent cytogenetic and molecular response. *Methods*. All 60 unselected patients aged 16 or over with chronic phase CML diagnosed between July 2000 and September 2007 receiving 400 mg imatinib daily at our institution were included in the study. Twenty-two were currently excluded from the analysis, pending analysis of an earlier vaccination protocol, and incomplete follow-up data are currently available on a further 8 cases; leaving 30 patients used for this analysis. All gave informed consent. Samples were taken at routine clinic visits in the mornings, together with information on concurrent medications and timing of dose. Steady state plasma levels of imatinib and CGP-74588 were assessed by a novel UV-HPLC technique, that in quality control testing gives high concordance with results from mass spectrometry techniques. Individual patient 24 hour trough plasma levels were estimated from a sparse sampling data set consisting of 155 serial samples collected from May 2006. Responses were monitored at 12, 18 and 24 months by BCR-ABL transcript level using standard real-time quantitative PCR; a major molecular response (MMR) was defined as a BCR-ABL/ABL transcript ratio level below 0.1%. Complete cytogenetic response (CCR) was measured either by conventional cytogenetics or defined as a transcript ratio below 1%. *Results*. Overall, the median 24 hour trough plasma level for imatinib was 875ng/mL (range, 147-1542); and for CGP-74588 was 274ng/mL (range, 61-619). No significant difference was seen between the plasma imatinib levels of patients who did and did not achieve CCR, at either 12, 18 or 24 months of therapy. Similarly, no significant difference of imatinib levels was seen between patients who did and did not achieve MMR, at either 12, 18 or 24 months. Furthermore, no difference in plasma CGP-74588 levels was seen between cytogenetic or molecular responders versus non-responders. Exclusion of patients whose 24 hour levels were estimated from sparse sampling did not alter these findings. Analysis of serial samples taken at the same time of day from the same patient revealed a high degree of variation in the plasma imatinib and CGP 74588 concentrations. *Conclusions*. The present data do not confirm earlier reports of the predictive value of plasma imatinib concentrations. Further work is required to investigate how additional factors such as concomitant medications and diet modify plasma levels, before recommending its routine use in the clinic.

0638

DASATINIB IN THE TREATMENT OF CHRONIC PHASE CML PATIENTS AGED > 60 YEARS RESISTANT/INTOLERANT TO IMATINIB

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Background. Dasatinib is a 2nd generation tyrosine-kinase inhibitor active in CML patients resistant or intolerant to Imatinib; at present there is no data on its toxicity and efficacy in elderly patients. *Aims*. To highlight this issue, 83 patients treated with Dasatinib when aged > 60 years were retrospectively evaluated. Patients There were 50 males and 33 females, median age at Dasatinib start was 69.9 years (IR 65.5 - 73.9), Sokal Risk at diagnosis was low in 19 patients, intermediate in 33, high in 13 and not valuable in 18. Thirty-four patients (40.9%) were primarily resistant, 11 (13.3%) were intolerant and 38 (45.8%) had secondary resistance to Imatinib; all patients were in CP when Dasatinib was started. Median time from diagnosis to Dasatinib treatment was 87.8 months (IR 49.9 - 118.1); 44/83 patients (53.0%) had been pretreated with IFN ± Ara-C before Imatinib, all patients received Imatinib at standard dose (400 mg/day) followed in 48/83 (57.8%) by increased dose (600 - 800 mg/day) with an overall median period of Imatinib treatment of 50.5 months (IR 29.5-66.7). In addition, 21/83 patients (25.3%) received other 2nd line treatment (9 Nilotinib, 7 HU, 2 Imatinib + HU and 3 other drugs) before Dasatinib. *Results*. Starting dose of Dasatinib was 140 mg/day in 38 patients, 100 mg/day in 40 patients and ≥50 mg/day in 5 patients, respectively. After a median period of treatment of 14.3 months (IR 8.6-19.7) all patients were evaluable for toxicity; among 38 patients receiving 140 mg, grade 3 - 4 haematological and extra-haematological toxicities were reported in 19 (50.0%) and 13 (34.2%) patients, respectively; among 40 patients receiving 100 mg, grade 3 - 4 haematological and extra-haematological toxicities were reported in 9 (22.5%) and 7 (17.5%) patients, respectively. Pleuro-pericardial effusions (grade 3-4) occurred in 8 patients (21.0%) treated with 140 mg and in 5 patients (12.5%) treated with 100 mg. Overall, 8/83 patients (5 treated with 140 mg) permanently discontinued Dasatinib due to toxicity; a dose reduction was needed in 53/83 patients [37/38 (97.3%) treated with 140 mg and 13/40 (32.5%) with 100 mg]. As to response, 79 patients were considered evaluable (≥3 months of treatment) and 4 were considered as too early; eight patients (10.2%) did not have any response (including 4 patients with early Dasatinib discontinuation for toxicity), 24 (30.3%) achieved a Haematological Response only,. 26 (32.9%) achieved a Cytogenetic Response (CyR) (Major CyR in 11, Complete CyR in 15) and the remaining 21 (26.6%) also achieved a Molecular Response (MolR) (Major MolR in 11, Complete MolR in 10). *Conclusions*. Present analysis shows that Dasatinib, when employed at the current recommended dose of 100 mg/day, is effective and has a favourable safety profile also in heavily pretreated elderly subjects.

0639

EFFICACY OF NILOTINIB AFTER IMATINIB FAILURE IN PH-POSITIVE CHRONIC MYELOID LEUKEMIA: IMPACT OF ADDITIONAL CHROMOSOMAL ABERRATIONS AND FREQUENCY OF BCR-ABL POINT MUTATIONS

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Background. Additional chromosomal aberrations (ACA) in Philadelphia chromosome-positive (Ph-positive) chronic myeloid leukemia (CML) are nonrandom and strongly associated with disease progression, but their prognostic impact and influence on treatment response is not clear. Point mutations of the BCR-ABL kinase domain are the clinically most relevant mechanisms of imatinib resistance. Nilotinib is a second-generation tyrosine kinase inhibitor with significant clinical activity in imatinib-resistant or -intolerant Ph-positive CML. However, resistance to nilotinib is a major reason for treatment failure and can be attributed to various mechanisms. *Aims*. We retrospectively investigated the influence of ACA on the clinical efficacy of nilotinib. *Methods*. Patients with Ph-positive CML and imatinib-resistance or -intolerance received nilotinib 400 mg bid. Dose was reduced if necessary. Bone marrow samples were analysed for cytogenetic aberrations before initiation of treatment and during follow-up. Sequencing of Abl was performed in a subset of patients. *Results*. 52 patients (median age 62 years, range 25 - 85) with a median follow-up of 16 months (range 1-50) were included in the present study. 37, 5 and 10 patients were in chronic phase (CP), accelerated phase (AP) and blast crisis (BC), respectively. Median duration of previous treatment with imatinib was 31.5 months (range 1-107). 85% had received hydroxyurea and 40% interferon. Median duration of nilotinib was 13.5 months (2-50). Overall, ACA were identified in 21 of 52 patients with 1 -3 aberrations per patient. More than half (62%) were so-called major route aberrations (+8, +Ph, i(17q), +19, -Y, +21, +17, -7). Whereas ACA were found in 24% of patients in CP, patients in AP or BC had ACA in 80% (p=0.001). 16 of 32 patients (50%) had point mutations in the abl kinase domain with no significant differences between patients with or without ACA. 8 of 13 patients carried both point mutations and ACA. There were no significant differences with respect to age, disease duration, duration of imatinib or nilotinib treatment between patients with or without ACA. Best response as assessed by cytogenetics was comparable with a major cytogenetic response in 62 and 68%, respectively. However, more patients with ACA failed nilotinib (38%) than patients without ACA (6%, p<0.005) and survival was significantly worse for patients with ACA. Overall survival at 36 months was 53 and 93% (p<0.005) for the whole cohort and 62 and 100% (p<0.005) for patients in CP, respectively. *Conclusions*. ACA impact negatively on the efficacy of nilotinib in patients with Ph-positive CML. Whether the decreased efficacy reflects instrinsic aggressiveness of the disease or rather specific mechanisms of resistance remains to be determined.

0640

THE ONCOGENIC KINASE BCR-ABL DIRECTLY REGULATES SPLICING EVENTS OF BCLX THROUGH A QUATERNARY COMPLEX COORDINATED BY NCK-BETA AND SAM-68 ADAPTER PROTEINS

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Background. Philadelphia positive (Ph+) disorders, such as Chronic Myelogenous Leukemia (CML) are characterized by the presence of abnormal chromosome rising from translocation between chromosome 9 and 22 thus giving birth to a chimeric oncogenic protein named Bcr-Abl. This oncogenic kinase displays constitutive tyrosine kinase activity which leads to tyrosine residues autophosphorylation, in turn recruiting SH2 and/or PTB containing proteins. In the last decade Bcr-Abl targeted therapy has been successfully employed and, among currently available drugs inhibiting Bcr-Abl activity, Imatinb mesylate represents the most efficient. Despite the huge amount of data reporting the effects of Imatinib on signal transduction pathways (for example ERK1/2 and PI3K activation, CrkL phosphorylation etc[3DOTS]) in Ph+ leukemic cells rather few experimental evidences are available on the effects of Imatinib on adapter molecules. Therefore we are currently attempting to investigate such field. *Aims and Methods*. The significance of interactions occurring between Bcr-Abl and adapter molecules is still matter of debate. Most of the interactions so far described (CrkL, Grb2, PI3K p85 regulatory subunit etc[3DOTS]) appear to play a role in coordinating and integrating a plethora of signals which in turn lead to proliferation, cell survival and/or cytoskeletal organization. In the last years few, but very interestingly, data supporting the hypothesis that adapter molecules might also act as c-Abl catalytic regulators have been presented. By means of an interactomic approach, based on proteomic strategy using GST-Pull Down assay with an array of SH2 containing proteins, we attempted to gain insight into the role played by adapter molecules and Bcr-Abl interactions. *Results and Conclusions*. The data herein presented aims to demonstrate the presence of quaternary complex involving the SH2-SH3 containing adapter protein Nck-β, the oncogenic tyrosine kinase Bcr-Abl, the RNA binding protein Sam68 and the spliceosome ribonucleprotein hnRNPA1. The experimental evidences we have collected support the hypothesis of an Imatinib-dependent interaction occurring between Nck-β and Bcr-Abl. Furthermore, Pull Down experiments indicate an intermolecular interaction between Nck-β, Sam68, and hnRNPA1 supporting the idea of a novel complex Bcr-Abl/Nck-β/Sam68/hnRNPA1. Biochemical analysis carried-out by Pull-Down experiments has been further corroborated by immunofluorescence staining. RNA Pull Down assay suggest that the quaternary complex Nck-β/Sam68/hnRNPA1/Bcr-Abl might modulates splicing process of a gene encoding for a protein capable of regulating apoptosis events, such as Bcl-X whose function has been recently described as crucial in myeloproliferative disorders. Astoningshly, the data collected so far indicates that other mRNAs can be sequestered by the complex and among them Bcr-Abl mRNA itself. Taken together these results represent the first experimental evidences showing an interaction between the oncogene Bcr-Abl and Sam-68 leading to speculate a novel putative role played by Bcr-Abl in the intriguing and complex mRNA splicing scenario.

0641

COMPARATIVE *IN VITRO* CELLULAR DATA ALONE IS INSUFFICIENT TO GUIDE CHOICE OF BCR-ABL INHIBITOR TO TREAT IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA

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Background. Following the paradigm of imatinib as a BCR-Abl kinase inhibitor for the treatment of chronic myeloid leukemia (CML) and an understanding of the molecular mechanisms that can lead to drug resistance, new targeted therapies have been developed to treat relapsed patients. Relapse is often caused by the emergence of clones that express mutant forms of BCR-Abl having single amino-acid substitutions in the kinase domain, which interfere with imatinib binding and reduce the sensitivity of the enzyme to inhibition by the drug. *Aims*. The potency of kinase inhibitors against wild-type and mutant forms of BCR-Abl is assessed by *in vitro* assays to quantify their effects on BCR-Abl-mediated protein phosphorylation and/or cell proliferation in murine haematopoietic cells expressing the BCR-Abl oncoprotein. It has been proposed (J Clin Oncol 2009) that such *in vitro* data could serve as a tool to help clinicians in their choice of drug to combat particular BCR-Abl mutants in CML patients. However, because this approach neglects other important variables, caution should be exerted when attempting to predict comparative clinical efficacies from *in vitro* data. To translate *in vitro* potency into *in vivo* efficacy, a drug must be delivered at adequate concentrations to its target in the relevant tissue compartment which, in the case of CML is the BCR-Abl kinase in the cytoplasm of leukemic cells located within the patient's bone marrow and/or peripheral blood. This process requires satisfactory absorption, metabolism, distribution and excretion properties, which are independent variables directly related to molecular structure. The purpose of this analysis was to understand these dynamics for nilotinib and dasatinib. *Methods*. A comparison of nilotinib and dasatinib was performed by analyzing *in vitro* and pharmacokinetic data. *Results*. In Ba/F3 cells, transfected to express 210 kDa wild-type, M315T or E255K BCR-Abl, nilotinib and dasatinib have been shown to have anti-proliferative mean GI50 values of 25 / 38 / 548 and 6.4 / 8 / 83 nM, respectively (Biochim Biophys Acta 2005). In patients, steady-state peak and trough plasma levels of nilotinib following oral administration of 400 mg q12h are 3600 and 1700 nM (NEJM 2006). For comparative purposes, following an oral 70 mg q12h regimen of dasatinib, day 8 peak and trough plasma levels are approximately 90 and 10 nM (J Clin Pharmacol

2008). Consequently at peak levels, nilotinib is present at concentrations 144-, 94- and 6.5-fold greater than its GI50 value for the inhibition of wild-type, M351T and E255K respectively, compared to ratios of 14-, 11- and 1.1-fold for dasatinib. At trough levels, the drug-concentration: GI50 ratios for nilotinib are also greater than those for dasatinib for all three forms of BCR-Abl (Table 1). *Conclusions*. Although dasatinib is more potent *in vitro*, under current dosing regimens nilotinib delivers higher plasma drug concentrations to CML cells, predicting it would be more effective against any of the three forms of BCR-Abl considered. Although this analysis does not account for parameters such as protein binding and cell influx/efflux, it illustrates the inadequate predictability of *in vitro* data alone as a tool for choosing second generation agents.

Table 1. Plasma levels and GI50 values.

BCR-Abl	Proliferation GI_{50} nM		Peak level:GI_{50} ratio		Trough level:GI_{50} ratio	
	Nilotinib	Dasatinib	Nilotinib	Dasatinib	Nilotinib	Dasatinib
p210 *wt*	25	6.4	144	14	68	1.6
M315T	38	8.0	94	11	44	1.3
E255K	548	83	6.5	1.1	3.1	0.12

0642

DYNAMICS OF CYTOGENETIC AND MOLECULAR RESPONSE TO NILOTINIB IS SIMILAR IN IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS IN CHRONIC PHASE (CML-CP) WITH AND WITHOUT BCR-ABL MUTATIONS EXCEPT E255K/V, Y253H, AND F359C/V

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Background. Nilotinib is a selective and potent BCR-ABL inhibitor, developed through structure-based drug design, with *in vitro* activity against most BCR-ABL mutants (excluding T315I) indicated for the treatment of Philadelphia chromosome positive (Ph^+) CML patients in CP or accelerated phase (AP) resistant or intolerant to prior therapy including imatinib. In a previous analysis of nilotinib in patients with BCR-ABL mutations, mutations occurring at 3 specific amino acid residues (E255K/V, Y253H, and F359C/V) were shown to be associated with less favorable response to nilotinib. *Aims*. Imatinib resistant patients were subdivided into groups who had "no mutation", "all other mutations" excluding those occurring at these 3 residues, or the "E255K/V, Y253H, or F359C/V" mutations, at baseline. Patients with T315I mutations were excluded from this analysis. *Methods*. Patient groups were analyzed for kinetics and durability of cytogenetic and molecular response to nilotinib, as well as time to disease progression (TTP) and overall survival (OS). *Results*. In patients with no mutation, all other mutations, or E255K/V, Y253H, F359C/V mutations, complete cytogenetic response (CCyR) was achieved in 24% (22/91), 17% (13/79), and 0% (0/27) of patients by 3 months, 31% (28/91), 32% (25/79), and 0% (0/27) by 6 months, 34% (31/91), 42% (33/79), and 0% (0/27) by 9 months, and 40% (36/91), 43% (34/79), and 0% (0/27) by 12 months, respectively. Median time to CCyR was 3.2 months (range, 1.0-23.5) for patients with no mutations, and 5.5 months (range, 0.9-22.1) for patients with all other mutations. No patients with E255K/V, Y253H, or F359C/V mutations achieved CCyR with a median follow-up of 6.5 months. At 24 months, CCyR was maintained in 73% of patients with no mutation and in 88% of patients with all other mutations. Major molecular response (MMR) was achieved within 12 months of treatment in 28% (23/82) of patients with no mutation, 28% (19/69) of patients with all other mutations, and 4% (1/25) of patients with E255K/V, Y253H, or F359C/V mutations. The dynamics of BCR-ABL transcript level reductions for each group are shown in Table 1. Median TTP was not reached for patients with no mutations, was 28.6 months (range, 0.3-29.9) for patients with all other mutations, and 7.6 months (range, 0.5 -29.9) for patients with E255K/V, Y253H, or F359C/V mutations. Estimated OS rate at 24 months was 88% for patients with no mutations, 83% for patients with all other mutations, and 92% for patients with E255K/V, Y253H, or F359C/V mutations. *Conclusions*. For patients with BCR-ABL mutations (excluding E255K/V, Y253H, or F359C/V) resistant to imatinib therapy, nilotinib treatment resulted in rapid and durable cytogenetic responses, and estimated TTP and OS, mirroring that of patients with no mutations. Patients with E255K/V, Y253H, or F359C/V mutations had lower and less durable responses, and shorter TTP (but comparable OS) than patients with other mutations. Alternative therapies may be considered for patients with these uncommon mutations. However, the results of mutational screening should only be considered within the greater clinical context for each patient when deciding the best treatment strategy to use.

Table 1. BCR-ABL transcripts by mutation type

		3 month	6 month	9 month	12 month
No mutation (n=82)	≤0.1%	12	21	26	28
	>0.1% - ≤1%	8.5	15	14.5	13
	>1% - ≤10%	19.5	16	19.5	21
	>10%	52.5	46	39	37
	Not evaluated	7.5	2	1	1
E255K/V, Y253H, F259C/V mutations (n=25)	≤0.1%	0	0	4	4
	>0.1% - ≤1%	0	0	0	0
	>1% - ≤10%	16	16	20	20
	>10%	80	80	72	72
	Not evaluated	4	4	4	4
All other mutations (n=69)	≤0.1%	4.5	22	26	27.5
	>0.1% - ≤1%	10	6	7	7
	>1% - ≤10%	20	24	25	25
	>10%	61	48	42	40.5
	Not evaluated	4.5	0	0	0

0643

LONG-TERM SURVIVAL OF PATIENTS WITH LATE CHRONIC PHASE PH-POSITIVE CHRONIC MYELOID LEUKEMIA RECEIVING TYROSINE KINASE INHIBITOR THERAPY AFTER INTERFERON FAILURE

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Background. Imatinib, a potent selective bcr-abl inhibitor, introduced into clinical practice in 2001, provides an effective treatment for chronic myelogenous leukemia (CML). Data from three-years follow-up of patients receiving imatinib after interferon (IFN) failure, demonstrated that Major Cytogenetic Response (MCyR) and Complete Cytogenetic Response (CCyR) were the most important prognostic factors for survival. The aim of this study was to evaluate long-term survival and stability of hematological response (HR) and MCyR in chronic phase (CP) CML patients treated with imatinib after IFN-α failure with 7-years follow-up in one-center (Hematology Research Center, Moscow, Russia). *Patients and treatment*. This non-randomized, open-label trial recruited patients from July 2000 through September 2001. In total, 79 patients with CP CML resistant/intolerant to IFN-α were enrolled. Median age was 39 (15-64) years old, male:female ratio 41:38. Median time from diagnosis to treatment was 36,1 (3-160) months: <1 year - 10 patients (12,8%), 1-5 years - 51 patients (64,5%), >5 years - 18 patients (22,7%). Initial imatinib dose was 400 mg/day. Median duration of imatinib treatment at the time of analysis (June 2008) is 58,1 (2-87) months. Patients characteristics are listed in Table 1. *Results*. 62 of 79 patients (78,4%) were alive on June 2008. Complete hematological response (CHR) was achieved in 74 (93%) patients with a median time to response of 3 (1-21) months. Failure to achieve HR after 3 moths of therapy was determined to be a strong negative prognostic factor associated with CML progression ($p<0.05$). MCyR was achieved in 51 (65%) pts, including Complete Cytogenetic Response (CCyR) in 44 (56%) pts. Minor CyR was achieved in 3 (4%), minimal in 10 (12%) pts and 15 patients failed to achieve CyR. Median time to CCyR was 9 (5-53) months. Of 74 CHR patients 32 (43%) lost CHR. In those patients median duration of response was 38 (2-82) months. Thus, primary and secondary hemato-

logical resistance was diagnosed in 7% and 43% of patients, with primary and secondary cytogenetic resistance in 35% and 31%, respectively. Clonal evolution in Ph^+ clones was observed in 8% of patients and found to have no prognostic importance for survival. Imatinib therapy was discontinued in 44 (56%) and continuing in 35 (44%) patients with stable CCyR (21 patients on dose 400 mg/day, 9 on 600 mg/day, and 3 on 800 mg/day; 2 on 300mg/day). 27 (34%) patients were switched to second line tyrosine kinase inhibitors (TKI) (10 patients to nilotinib with median duration of treatment 10 months, 12 patients to dasatinib with median duration of treatment 26 months, 2 patients to bosutinib); 2 patients were subsequently switched to third line TKIs. In total, 17 patients died (15 patients with progression to Accelerated Phase/Blast Crisis, 1 patient with brain hemorrhage, 1 patient with hepatocellular carcinoma). It is noteworthy that only one patients died after second line TKIs initiation. In June 2008 median duration of the disease from diagnosis was 105,4 (14-231) months; 22 patients are alive for more than 10 years after diagnosis. Estimated 8-year survival of patients receiving TKIs after IFN-αH failure is 78%. *Conclusions*. Imatinib resulted in significant improvement of survival in CML patients resistant or intolerant to IFN. In majority of patients achieving MCyR on imatinib therapy it was sustained longterm with stable MCyR and CCyR associated with significantly increased survival. Second line of TKIs will further improve prognosis of CML patients. Long-term follow-up of CML patients receiving TKI therapy demonstrated preservation of residual clones of Ph-negative cells and a capacity of normal hematopoesis recovery on TKI treatment.

Table 1.

		3 month	6 month	9 month	12 month
No mutation (n=82)	≤0.1%	12	21	26	28
	>0.1% - ≤1%	8.5	15	14.5	13
	>1% - ≤10%	19.5	16	19.5	21
	>10%	52.5	46	39	37
	Not evaluated	7.5	2	1	1
E255K/V, Y253H, F259C/V mutations (n=25)	≤0.1%	0	0	4	4
	>0.1% - ≤1%	0	0	0	0
	>1% - ≤10%	16	16	20	20
	>10%	80	80	72	72
	Not evaluated	4	4	4	4
All other mutations (n=69)	≤0.1%	4.5	22	26	27.5
	>0.1% - ≤1%	10	6	7	7
	>1% - ≤10%	20	24	25	25
	>10%	61	48	42	40.5
	Not evaluated	4.5	0	0	0

0644

PREGNANCY AMONG THE PATIENTS WITH CHRONIC MYELOID LEUKEMIA ON IMATINIB THERAPY

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Background. The imatinib therapy has improved a survival rate and a quality of life for the majority of CML patients. Therefore the ability to become parents is one of the crucial issues for the patients of childbearing potential. Current view is to avoid pregnancy because of a possible fetus injury. Furthermore, imatinib discontinuation at pregnancy period can be critical for possible disease relapse. Clinicians are forced to find individual decisions for the patients wishing to maintain pregnancy despite of all the risks. We report about the 6 cases of successful pregnancy of the females and the 6 cases of pregnancy in the partners of the males on imatinib therapy. *Results*. We observed 6 females with CML, 5 in chronic phase (CP), 1 in accelerated phase (AP) at diagnosis, age 27-36 years (median 31.8), disease duration 30-112 months (median 86.6), imatinib treatment period 25.7-84.2 months (median 44.4). Imatinib dose was 400 mg for 3 of 6, 600 mg for 2 of 6 and 1 of 6 had a temporal 3 weeks treatment interruption at the impregnation. 5 of 6 females had been exposed to imatinib for the first 4-8 weeks of pregnancy (except 1 of 6 mentioned above). After pregnancy identifying the patients expressed their strong wish to keep the pregnancy and the imatinib treatment was stopped. At that moment their CML status was the following: complete molecular response (CMR) -3 of 6, major molecular response (MMR)- 1 of 6, only partial cytogenetic response (PCyR) - 2 of 6. During the pregnancy period 2 patients remained in CMR but after the delivery both of them had only MMR. 1 patient lost CMR and kept the complete hematologic remission (CHR), cytogenetic examination wasn't done during the pregnancy. 1 patient lost MMR and had a cytogenetic relapse (CyR) (Ph^+ cells 59%). 2 patients lost PyCR and 1 of them had a partial hematologic response (PHR). For those 3 patients with CyR and PHR a maintenance treatment by interferon α 3 ME daily was applied during pregnancy. All 6 females delivered healthy infants at term, restarted imatinib immediately after delivery and didn't breastfeed. At present time the infants are from 3 months to 2.7 years old with no abnormalities in their development. There were also 6 pregnancy cases in the partners of the CML CP males receiving imatinib therapy. The patients age was 26-36 years (median 31.5), disease duration 21-93 months (median 42.8), imatinib treatment period 13-78 months (median 26.2). Imatinib dose was 400 mg for 4 of 6 patients, 600 mg for 2 of 6 at impregnation moment. In all 6 cases the healthy infants were born at term, now the children are from 6 months to 5.5 years old with no abnormalities in their development. *Summary*. We demonstrated that despite of all risks the successful outcomes of pregnancy in CML patients are possible. Pregnancy management is still an individual decision for every patient. An experience exchange and careful study of every case is significant for developing the further strategy for this actual issue.

Myeloproliferative disorders - Clinical

0645

INCREASED RISK OF LYMPHOID MALIGNANCIES IN PATIENTS WITH PHILADELPHIA CHROMOSOME-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

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Backgroud. Relatives of patients with myeloproliferative neoplasms (MPN) have a 5- to 7-fold increased risk of developing polycythemia vera (PV), essential thrombocythemia (ET) and MPN-NOS (Landgren, Blood 2008), suggesting susceptibility predisposing genes. Cases of association of MPN with lymphoproliferative disorder (LPD) have been reported. *Aims*. The aim of this study was to assess whether presence of a MPN also carried an increased risk of developing a LPD. *Methods*. Clinical records of 820 MPN patients referred to the Hematology Department of Florence from 1980 to 2008 were examined. Diagnosis was reviewed according to WHO criteria for both MPN and LPD. For each subject, the period at risk was calculated since date of diagnosis of MPN to diagnosis of LPD, death, or last follow-up. Expected numbers of LPD incident cases were calculated based on 5-year age groups, gender and calendar time specific cancer incidence rates in the general population of the same area, provided by the Tuscany Cancer Registry covering an area of approx 1,160,000 inhabitants. Standardized Incidence Ratios (SIR), i.e. the ratio of Observed to Expected cases in MPN, were computed to estimate the relative risk of developing LPD compared to the general population. Analyses were carried out for the whole series and then separately for ET and PV, gender and JAK2V617F genotype. *Results*. We considered 353 patients with PV and 467 with ET, median age at diagnosis 59 yrs. Median follow-up was 3.3 yrs, corresponding to 4,421 person years (PY), 1,882 and 2,539 in PV and ET, respectively. JAK2V617F mutation was found in 70%. We identified 37 patients who developed a LPD (4.5%), 12 with ET (1.4%) and 25 with PV (3.0%). Twenty-six patients had a MGUS, but they were not analyzed because of wide occurrence of this abnormality in the general population. Of the 11 LPD considered there were 4 CLL, 5 NHL and 2 plasma cell disorders (PCD); 7 cases occurred in PV and 4 in ET patients. The median interval time from diagnosis of MPN to LPD was 68 months. A LPD was more common in PV than ET (2% vs <1%, p=0.02). A three-fold increased risk of LPD emerged in the whole series (SIR 3.44; 95% CI 1.90-6.20), ranging from 2.86 (95% CI, 0.72-11.43) for PCD to 12.42 (95% CI 4.66-33.09) for CLL. The risk of developing LPD was increased five-fold among JAK2V617F mutated patients (5.46, 95%CI 2.45-12.15) while there was only one case among wild-type patients (SYR 2.60, 95% CI 0.37-18.43). According to gender, males had significantly increased risk (4.52, 95%CI 2.26-9.03). Sorted $CD19^+/CD20^+/CD23^+$ cells from one CLL patient harbored JAK2V617F mutation with allelic burden comparable to granulocytes; similarly, the V617F allele was found in cells of one mediastinal lymphoma unlike one case of ileal lymphoma where cancer cells were JAK2 wild-type. *Conclusions*. These data indicate that risk of developing a LPD is significantly increased in PV and ET patients compared to the general population, which might be ascribed to the genetic instability that characterizes MPN hematopoietic cells, particularly in association with JAK2V617F mutation.

0646

THE LONG-TERM DURABILITY OF MOLECULAR RESPONSES IN PATIENTS WITH FIP1L1-PDGFRA CHRONIC HEOSINOPHILIC LEUKEMIA TREATED WITH IMATINIB: THE ITALIAN HES0203 EXPERIENCE AFTER A 4-YEAR FOLLOW-UP

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Background. Imatinib mesylate is the first line treatment for hypereosinophilic syndrome with FIP1L1-PDGFRα (F/P) fusion gene. The use of imatinib modified the natural history of the disease, independently from the organ damage present at diagnosis time or from the type of transcript detected with molecular sequencing. However, few clinical and molecular data on the outcome of these patients based on prospective and controlled trials are available. *Aims*. to evaluate the long term follow up of patients with FIP1L1-PDGFRA positive CEL treated with imatinib. *Design and Methods*. A prospective phase 2 multicenter study of the use of imatinib 400 mg/daily in patients with hypereosinophilic syndrome, irrespectably of F/P status was established in 2001. Hypereosinophilic syndrome was defined according to Chusid criteria. The presence of F/P transcript was investigated on bone marrow cells using a nested reverse transcriptase polymerase chain reaction (RT-PCR). Patients at diagnosis were systematically screened for organ damage with instrumental evaluation (chest radiography, echocardiogram, abdomen ultrasonography) and for the presence of symptoms. 72 patients were treated with IM 100 to 400 mg daily; the 33 F/P positive patients (F/P+) were monitored every three months for two years then every six months using nested RT-PCR. The observation period of F/P^+ patients ranges between 23 and 85 months (median 48 months). *Results*. There were 32 males and one female patients. Organ involvement was recorded in 42% of F/P^+. After imatinib therapy all patients achieved a complete hematologic response (CHR) in less than one month, and PCR negativity in a median time of 3 months (range 1-9). They became negative for organ localizations and free of symptoms. All patients who continue imatinib therapy remain in CHR and RT-PCR negative, with a dose of 100-400 mg daily. From September 2007 all patients except one (late responder) were treated with 100 mg daily. In six patients IM treatment was discontinued for variable period for different reasons, and in 5 cases the fusion transcript became rapidly detectable. CHR was maintained, other than in one case. The transcript was again undetectable upon treatment resumption, other than in one case. All samples were valuable for molecular analysis. Fusion gene sequencing demonstrate an extreme variability of FIP1L1-PDGFRA junction sequences, but no evidence of correlation was noted with kinetic of molecular response or with the presence at diagnosis of peculiar organ involvement. More complexity of transcript is noted in patients with longer history of disease prior to imatinib therapy. Transcript of the only female patient is the same of one of the males. *Interpretation and conclusions*. With this large series of patients we can confirm the extremely sensitivity of F/P^+ CEL to imatinib therapy, without any significant toxicity after protracted therapy and without acquisition of resistance. The complexity and variability in FIP1L1-PDGFRα transcripts seems to no correlate with phenotype of disease, even though different kinetic of response have been observed. Prolonged clinical and molecular follow-up of these patient is essential to understand the CEL disease.

0647

REDUCED SURVIVAL IN PATIENTS WITH PRIMARY MYELOFIBROSIS IS PREDICTED BY A LOW BURDEN OF JAK2V617 MUTATED ALLELE

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Backgroud. Sixty-percent of patients with primary myelofibrosis (PMF) harbor a JAK2V617F mutation. A mutated genotype has been variably associated with hematological and clinical characteristics, including reduced overall survival (OS) (Campbell P; Blood 2005) and increased risk of leukemia (Barosi G, Blood 2007). Recently, an inferior survival was found in mutated patients with low burden of mutated JAK2 allele (Tefferi A, Leukemia 2008). *Aims*. The aim of the study was to evaluate the impact of V617F allele burden on disease progression and outcome in patients with PMF. *Methods*. Presence and burden of JAK2V617F allele was evaluated by real-time quantitative PCR in DNA obtained from gradient-purified granulocytes of 186 PMF patients who were evaluated at the time of diagnosis, in the absence of any treatment. Mutational status and mutated allele burden were correlated with baseline hematological and clinical characteristics and with variables associated with disease progression, including time to development of anemia, leukocytosis or leukopenia, thrombocytopenia, massive splenomegaly, transformation to acute leukemia and OS. *Results*. JAK2V617F mutation was found in 68.2% of the patients; median allele burden was 54% (range 5-100%). Frequency of patient according to V617F burden was 13.3% in the first quartile, 33.8% in the second, 24.4% in the third, and 28.3% in the fourth. JAK2V617F-mutated patients had significantly higher leukocyte (p=0.009) or platelet (p=0.02) count and hemoglobin level (p<0.0001) compared to wild-type; anemic patients (hemoglobin <10g/dL) were significantly less among JAK2V617-mutated (12% versus 26%, p<0.007). JAK2V617F mutated patients were preferentially found in the Dupriez low risk category (p=0.017), likely as a result of mutated status association with higher hemoglobin level, while there was no difference according to risk categories of Cervantes score. Time to anemia and leukopenia was significantly longer in JAK2V617F-mutated patients compared to wild-type. Converesely, time to both anemia and leukopenia was significantly shorter in mutated patients belonging to the lower quartile as compared to patients in upper quartiles (p<0.01) as well as to wild-type patients (p<0.01). After a median follow-up of 17.2 months, 23 patients (12.3%) had died, 15 of whom because of leukemia. A JAK2V617F mutated genotype did not impact on leukemia transformation or OS. In multivariate analysis OS was predicted only by age (p<0.0001) and leukocytosis (p=0.001). However, OS was significantly reduced in patients in the lower V617F quartile as compared to either mutated patients with >25% allele or wild-type ones. In multivariate analysis that included patient stratification according to V617F allele burden, factors significantly associated with reduced survival were age (p=0.002), leukocytosis (p=0.02), a blast count >1% (p=0.003) and a JAK2V617F burden <25% (p=0.002). Causes of death in the 1-25% quartile were represented by thrombosis, infections and multi-organ failure, and no case of leukemia was recorded. Conversely, leukemia transformation occurred at similar rate in mutated patients with >25% allele and wild-type ones. *Conclusions*. These data indicate that a low JAK2V617F allele burden in PMF is associated with a mainly myelodepletive rather than myeloproliferative hematological phenotype and represents an independent factor associated with shorter survival due to causes other than leukemia.

0648

POMALIDOMIDE IN MYELOFIBROSIS: RESULTS OF A PHASE-1 DOSE-SEEKING TRIAL

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Backgroud. Pomalidomide (POM) is an immune-modulatory drug (IMiD) recently tested in persons with myelofibrosis (MF). POM appears safer and more effective than other IMiDs in MF including thalidomide (Br J Haematol 2002;117:288) and lenalidomide (Blood 2006;108:1158). An adaptive phase-2 trial of POM (0.5 mg or 2.0 mg/d for 28 d (with or without prednisone; Blood 2008; 112[11]:a663) showed efficacy in MF-associated anemia with no dose- limiting toxicities (DLTs). We performed a subsequent dose-escalation trial to determine whether higher doses of POM can be given safely. *Methods.* A classic phase-I 3 x 3 trial was done in persons with symptomatic MF (anemia and/or symptomatic splenomegaly). The starting dose was 2.5 mg/d d 1-21 every 28 d. Dose-escalation at increments of 0.5 mg/d was done if no subject had a DLT (≥grade-4 hematologic toxicity, ≥grade-3 febrile neutropenia or ≥grade-3 non-hematologic toxicity) in cycle-1. Subsequent cohorts were treated until the maximum tolerated dose (MTD) was reached (dose level before that resulting in DLT in >1 of 6 subjects). *Results*. Subjects: 12 subjects with MF were enrolled 06/08-12/08. 8 had primary MF, 3, post-polycythemia vera MF and 1, post-essential thrombocythemia MF. Median age was 67 years (range, 51-83), 7 were female. 9 had a JAK2-V617F mutation. 11 were RBC-transfusion-dependent and 1 had a hemoglobin <10 g/dL. Median WBC was 4.5×10^9/L (range, $2\text{-}64\times10^9$/L). Median platelets were 111×10^9/L (range, $52\text{-}538\times10^9$/L). 10 subjects had splenomegaly, median size of 12 cm below the LCM (range, 4-26 cm). MTD: 3 subjects were enrolled in each of the 2.5, 3.0, and 3.5 mg dose cohorts. DLTs were observed at the 3.5 mg level: 2 of 3 subjects had grade-4 neutropenia and 1, grade-3 thrombocytopenia. 3 more subjects were enrolled at the 3.0 mg level cohort confirmed this dose as the MTD. 4 of 9 subjects receiving ≥3.0 mg/d had grade-3 neutropenia and 1 had grade-3 thrombocytopenia. Other toxicities were <grade-3 including fatigue, dyspnea, rash, lymphadenopathy and headaches. Efficacy: Subjects received a median of 3 cycles (range, 2-6 cycles). 8 subjects remain on-study; reasons for discontinuation were progression (N=2) and no response (N=2). There is a decrease in RBC-transfusion levels in 2 subjects. *Conclusions*. POM, 0.5-2 mg/d for 28 d (with or without prednisone) reverses anemia in persons with MF. Our data indicate the dose of POM can be increased to 3.0 mg/d for 21 d of a 28 d cycle. The DLT at higher doses is neutropenia. It is unclear as yet whether higher doses alter the anemia response rate seen at lower doses. An expanded phase-2 trial of 3.0 mg/d is ongoing; results will be presented.

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PRIMARY AND SECONDARY RESISTANCE TO IMATINIB IS VERY RARE IN CHRONIC AND BLAST PHASE OF MYELOPROLIFERATIVE NEOPLASMS WITH REARRANGEMENTS OF PDGFRA AND PDGFRB

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Constitutively activated fusion genes with involvement of the receptor tyrosine kinases (TK) PDGFRA and PDGFRB play a central role in the pathogenesis of eosinophilia-associated myeloproliferative neoplasms (Eos-MPN). The diverse fusion genes fully retain the TK domain which is either activated by dimerization through the partner gene or loss of the TK autoinhibitory domain. Similar to chronic myeloid leukemia (CML), Eos-MPN may can evolve from chronic phase (CP) to blast phase (BP). While primary response rates to imatinib are even higher than those seen in CML, data on secondary resistance are still limited. Here, we report the follow-up of 48 imatinib-treated patients (100-400 mg/d) with a FIP1L1-PDGFRA fusion gene (CP, n=29; BP, n=9) or diverse PDGFRB fusion genes (CP, n=9; BP, n=1). Median age was 51 years (range 20-67) with a remarkable male predominance (46/48, 96%). Overall, all but one patient are currently still on imatinib for a median of 43 months (range 14-76). Complete molecular remissions (CMR) were seen after 6, 12 and 24 months in 55% (16/29), 76% (22/29) and 96% (26/27) of FIP1L1-PDGFRA positive patients in CP. Molecular relapse was only seen intermittently in one patient after imatinib was stopped but CMR was rapidly reinduced. The kinetics of responses was similar in Eos-MPN in CP with PDGFRB fusion genes. Rapid complete hematologic remission (CHR) in all patients and complete cytogenetic remissions in every tested patient within 6 (n=2), 12 (n=4) and 24 (n=6) months. CMR was reached in three of seven patients (43%). No relapse has yet been observed. In patients with diagnosis of BP (n=10), imatinib was used as monotherapy (n=7) or after intensive chemotherapy (n=3). All patients achieved rapid CHR and all nine FIP1L1-PDGFRA positive patients also achieved CMR. One FIP1L1-PDGFRA positive patient died while in ongoing CMR 24 months after diagnosis due to a cerebral bleeding. The median duration of CMR is 24 months (range 8-53) and no relapse has yet occurred in median 47 months (range 19-70) after diagnosis of BP. Hematological toxicity grade III-IV only occurred in one patient with

thrombocytopenia below 30/nL which was successfully managed by temporary discontinuation of imatinib. In summary, the clinical course and the sensitivity to imatinib of Eos-MPN with rearrangements of PDGFRA and PDGFRB clearly resemble CML. However, the high response rates are usually achieved with lower doses which are associated with less toxicity. In addition, primary and secondary resistance seem to be very rare irrespective of disease stage.

0650

WITHDRAWN

0651

THE EVALUATION OF XAGRID EFFICACY AND LONG-TERM SAFETY STUDY: MONITORING SAFETY OUTCOMES IN AT-RISK ESSENTIAL THROMBOCYTHAEMIA PATIENTS TREATED WITH ANAGRELIDE VS OTHER CYTOREDUCTIVE THERAPIES

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Background. Long-term follow-up studies of cytoreductive treatments in essential thrombocythemia (ET) are needed. The EXELS study is a large-scale, observational safety study that will enrol >3000 at-risk patients with ET. *Aims*. To continuously monitor safety and pregnancy outcomes in at-risk ET patients treated with anagrelide compared with other cytoreductive therapies. Assessments of efficacy (platelet reduction, incidence of thrombohaemorrhagic events) and drug utilisation are secondary objectives. *Methods*. At-risk ET patients (with at least one of the following: aged ≥60 years, platelet count >1000×10^9/L or previous thrombohaemorrhagic events) receiving cytoreductive treatment have been recruited since 2005. Patients are managed according to local practices and are recorded in the anagrelide group or 'other cytoreductive treatment' group at registration. Data on predefined events and suspected serious adverse reactions are collected every 6 months over the 5-year follow-up period. Predefined events include thromboembolic and major haemorrhagic events, disease transformation, heart failure, pulmonary hypertension, mucocutaneous lesions, or non-haematologic malignancy, pregnancy and death. *Results*. Approximately 140 sites across Europe are participating in the study. Of the 2602 patients registered in September 2008, >50% had been diagnosed for >2 years before entry, and about 10% for >10 years. Of the total cohort, 93% received either hydroxyurea (HU) or anagrelide treatment: 592 patients (22.8%) were receiving anagrelide, 1922 were receiving other cytoreductive treatments (HU in 1832 patients [70.4%], interferon-α in 129 patients [5.0%] and pipobroman in 102 patients [3.9%]), and 88 patients were receiving combination therapy. Subjects receiving anagrelide were generally younger than those receiving other cytoreductive therapies (55.3 years vs 67.2 years, respectively): 852 patients were aged <60 years, and 349 of these (41.0%) were receiving anagrelide. Of the 1750 patients aged >60 years, 243 (13.9%) were receiving anagrelide. In the anagrelide group, 11.7% of patients have stopped or changed therapy, compared with 7.4% in the HU group. Overall, 1725 patients (66.3%) were receiving concomitant anti-aggregation therapy (54% in the anagrelide group, 70% in the other treatment group). By September 2008, over 1200 patients had completed 12 months in the study. The change in haemoglobin level at 12 months was -0.1 g/dL in the anagrelide group (n=15) and -0.2 g/dL in the HU group (n= 184). Major thromboembolic and haemorrhagic events were still too few for statistical evaluation (n=67). As of September 2008, 183 subjects have reported 234 predefined events, with the most frequent being 'other cardiovascular symptoms' (n=37). *Conclusions*. Platelet reduction treatment patterns in Europe seem to be concordant with existing guidelines, with HU being the most frequently used agent, followed by anagrelide. In patients aged <50 years, anagrelide was used as often as HU. No new safety concerns have emerged. This large study is expected to provide useful efficacy data for the main ET treatments, with regard to thromboembolic events, platelet control and transformation. For the first time, a fairly large cohort of patients with combination therapy will be followed. Recruitment will end in April 2009, with >3000 patients in the total cohort and >800 patients in the anagrelide group. For this presentation, data will be updated from September 2008 to March 2009.

0652

LIVING WITH ESSENTIAL THROMBOCYTHEMIA IN FRANCE: PROSPECTIVE SURVEY ASSESSING DAILY LIFE DISEASE IMPACT IN 245 PATIENTS

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Background. Essential thrombocythemia (ET) is the chronic myeloproliferative disease (MPD) with the better prognosis and life expectancy. Morbidity is based on the risk of thrombotic complications and possibility of evolution to myelofibrosis and acute leukaemia. ET more frequently affects patients around 60 years old or younger. Its impact on personal, social and professional life has not been fully studied, and until now, only one US internet-based study evaluated the burden of MPD on quality of life from the patient's perspective. *Aims*. We report a survey in France that evaluated the impact of ET on daily life from patient's perspective compared with data collected from their haematologists. *Methods*. Multi-centre, retrospective and prospective study. Each physician had to include 3 ET patients types (T): T1, <60 years old not receiving cytoreductive (CR) drugs; T2, <60 years old receiving CR drugs; T3, ≥60 years' old receiving CR drugs. Retrospective data about demographics, key diagnosis, treatment and complications were collected from haematologist's patient's files. Prospective data were obtained by the completion of an auto questionnaire by the patients, regarding items about feelings on their disease and their treatment, life style changes, potential complications and quality of information they received about their disease and their expectations. *Results*. 85 French haematologists included 245 patients (T1 (19%), T2 (38%), and T3 (43%)). The characteristics of patients were: median age at diagnosis (53 years), 61% of female, 69% of patients diagnosed on routine blood counts, 16% of patients experienced vascular complications since diagnosis. Although only 30% of patients felt *sick*, 60% considered ET as a *serious* disease. 23% of patients were asymptomatic. Fatigue was the most frequently reported symptom (58% of patients) largely contrasting with fatigue frequency reported by their haematologists (17%). Although the majority of the patients (96%) believed that taking drugs for ET was essential, 32% had *no or little motivation* to do so. The first criterion for treatment efficacy from patient's perspective was diminution of platelet count (89%), when it was primary goal of therapy for only 47% of haematologists. The Majority of patients (85%) didn't feel constrained or felt only slightly constrained by the treatment. Negative impact of ET on professional life was reported by 37%, on hobbies by 24%, on *emotional life* by 24% of patients. ET affected the *mood* in 74% of patients. Although the majority of the patients were aware of ET symptoms, like *burning of hands and feet* or *vascular occlusions*, 17% of patients felt *not informed at all* about their disease, possible disease evolution being the main topic of concern (87%). Doctors remained the major information source for patients (86%), then the internet (40%). *Conclusions*. This survey showed that ET had an important impact globally on patient's life. Discrepancies between patient's and doctor's consideration of symptoms and consequences of ET were highlighted, suggesting that efforts should be made to educate physicians about a global management of ET patients, in particular on patient's information.

0653

APLIDIN IMPROVES MEGAKARYOCYTOPOIESIS AND HALTS NEO-ANGIOGENESIS IN THE GATA1(LOW) MURINE MODEL OF MYELOFIBROSIS

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Background. Primary myelofibrosis (PMF) is a myeloproliferative disorder associated with abnormalities of megakaryocytic (MK) proliferation and maturation ascribed to reduced levels of GATA1 expression. By releasing high levels of cytokines(VEGF and TGF-β) in the marrow, the high numbers of immature MK are considered to be responsible for fibrosis, increased angiogenesis and bone formation observed in PMF. Mice carrying the hypomorphic Gata1(low) mutation express MK abnormalities similar to those observed in PMF patients and develop myelofibrosis, a phenotype resembling PMF and are considered an animal model for the disease. *Aims*. To perform a pre-clinical assessment for the use of Aplidin, a marine-derived depsipeptide currently in several phase II clinical trials that inhibits angiogenesis in *in vivo* and *in vitro* experimental model, as targeted therapeutic agent for myelofibrosis in Gata1(low) mice. *Methods*. Gata1(low) mice (9-months age; n= 18) received Aplidin ip at 60 μg/kg/daily/9 days for two cycles 38 days a part; a second group (aged >12 months; n= 18) received only one course of treatment while a third group (9-months age; n= 36) received four cycles 21 days apart of 100 μg /kg/daily per 5 days. Equivalent numbers of age-matched Gata1(low) mice received saline only and were used as control. At sequential time points, blood was drawn and mice sacrificed for immunohistochemistry, PCR and FACS analyses of bone marrow, spleen and liver. *Results*. A significant increase of platelet count from $<200\times10^6$ to $>600\times10^6$/μL ($p<0.05$) was observed in Gata1(low) mice at day 16 after the first treatment. The increment was sustained for all the duration of the treatment and was associated with increased levels of Gata1 expression (16.6±3.3 vs 34.2±2.6 pixel unit/MK, before and after treatment, $p<0.01$) and reduced numbers of MK (636±37 vs 440±47 MK/mm^2, before and after treatment $p<0.05$) in the marrow. In addition Aplidin normalized all the parameters of the bone marrow under study: the femur cellularity increased from a median of 6×10^6 in untreated Gata1(low) mice to normal values (15×10^6) 53 days after Aplidin treatment, fibrosis was minimal (6.5±2 vs. 3.2±1 reticulin fibers/mm^2 before and after treatment, $p<0.05$), microvessel density (8±1.5 vs 2.6±1.6 pixel/mm^2, $p<0.01$) and bone formation (56.5±8.9 vs. 21.4±7.4 pixel/mm^2, $p<0.05$) greatly reduced. Moreover, mRNA levels for both TGF-β and VEGF were significantly reduced in the marrow of Aplidin-treated mice ($p<0.01$ for both). Of note, Aplidin-treated Gata1(low) mice did not develop extramedullary hematopoiesis in liver, as detectable by CD45-immunostaining or FACS analyses for CD61 and Ter119 expression, in spite of the great numbers of colony forming cells detected in this organ (10^3 and 0.6×10^3 vs 25 colonies/liver in Aplidin-treated and untreated Gata1(low) mice and wild-type controls). *Summary and conclusions*. Aplidin treatment improves most of the myelofibrotic trait expressed by Gata1(low) mice, including thrombocytopenia, decreased marrow cellularity, fibrosis, increased angiogenesis, bone deposition and, most importantly, prevents development of extramedullary hematopoiesis. These data strongly suggest a beneficial effect of Aplidin in the treatment of a murine model of PMF. Based on these results, Aplidin merits to be tested clinically in symptomatic patients with PMF.

0654

EFFICACY OF LENALIDOMIDE IN PATIENTS WITH MYELOFIBROSIS: SPANISH COMPASSIONATE USE PROGRAM. PRELIMINARY ANALYSIS

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Aims. To describe clinical and epidemiological characteristics and preliminary efficacy and safety of patients with myelofibrosis treated with Lenalidomide (LEN). *Patients and Methods*. We designed a transverse and retrospective, multicenter descriptive analysis of cases from which compassionate use was requested. Treatment decision was previous and independent from the decision to conduct the present analysis and depended only on the clinical criterion of the responsible doctors. At least, one response assessment was needed for efficacy analysis. *Results*. From July to December 2008 33 patients have been included of which 26 were eligible for efficacy. Median age was 68.5 years (49-82) and 18 (69.2%) were males. Median interval from diagnosis was 24 months (4-134). Eighteen (72.0%) patients had primary myelofibrosis and 7 (28.0%) secondary. Eleven patients (42.3%) had intermediate Dupriez score at inclusion, 9 (34.6%) high and 6 (26.1%) low. Nine (42.9%) patients had cytogenetic abnormalities and JAK2 mutation was present in 12 (54.5%). Prior median lines of therapy were 3 (0-6). Most patients received blood transfusion [22 (84.6%)], 13 (54.2%) hidroxyurea; 1 (4.2%) interferon; 10 (40.0%) androgens; 14 (56.0%) erythropoyetin; 14 (53.8%) corticosteroids; 6 (25.0%) G-CSF; 6 (24.0%) thalidomide; 1 (4.2%); radiotherapy; 1 (4.2%) splenectomy and 9 (39.1%) "Others". Median LEN dose was 10 mg (5-10) per day. Fourteen (53.8%) patients received the standard dose and schedule (10 mg daily for 3 weeks every 4 weeks) and 12 (46.2%) received less dose and/or different schedule. Median duration of treatment was 3 months (0-17). Dose interruption or reduction was needed in 17 (65.4%) patients. Objective response was obtained in 16 (61.5%) patients. Most of them had moderate (7) or minor response (5) and 2 had complete response. Median Time to progression was 4.0 months (2.2-5.8). At the time of performing this analysis, 14 (53.8%) patients were still on LEN therapy and 21 (80.8%) were alive. Most frequent grade III-IV toxicity were anemia [10 (31.3%)], neutropenia [10 (31.3%)] and thrombocytopenia [7 (21.9%)]. Other important adverse effects (GIII/IV) were asthenia [6 (18.8%)], dyspnoea/hypoxia [2 (6.3%)]; venous thromboembolic disease (VTD) [1 (3.1%)]. The patients with VTD were on low dose aspirin prophylaxis. VTD prophylaxis was done in 26 (81.3%) patients, most of them received low dose aspirin 15 (46.9%), 10 (31.3%) low weight molecular heparin, and 1 oral anticoagulation. *Conclusions*. Compassionate use of Lenalidomide was prescribed for a heavily pre-treated myelofibrosis population. Our results suggest that LEN can be an effective and safe treatment for these patients. Toxicity, mainly myelosuppression was predictable and manageable with dose adjustments. These results should be confirmed in further control clinical trials.

0655

JAK2 V617F-POSITIVE CHRONIC EOSINOPHILIC LEUKEMIA: CLINICAL, LABORATORY AND MOLECULAR DEFINITION OF A DISTINCT DISEASE SUBTYPE

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Hypereosinophilic syndrome (HES) and chronic eosinophilic leukemia (CEL) comprise a spectrum of indolent to aggressive diseases characterized by persistent eosinophilia and target-organ damage. The discovery of the FIP1L1-PDGFRA fusion gene in imatinib-responsive patients, who would otherwise have met the diagnostic criteria for HES has provided substantial insight into the pathogenesis of eosinophilic disorders. The JAK2 V617F point mutation, a molecular hallmark of the typical myeloproliferative neoplasms (MPNs), can be occasionally detected in other myeloid hematological malignancies, such as atypical MPNs and HES. In this study, we assembled a cohort of patients positive for the JAK2 V617F mutation with eosinophilia and signs and symptoms consistent with a diagnosis of HES. We provide evidence that JAK2 V617F positive eosinophilia is associated with myocardial involvement, with clinical and laboratory features that distinguish it from other primary eosinophilias.We conducted a retrospective multicenter study of patients with primary eosinophilia followed-up by the hematology units of six tertiary referral hospitals in Greece and seven in Poland. Patient diagnosis was based on the WHO 2001 criteria. For each patient, clinical files were reviewed and the following data were retrieved using a standardized data collection form: demographic information, clinical examination findings, imaging and biopsy results, and results of laboratory evaluations, target-organ involvement, treatment and response information. Patient management, including treatment and follow-up was based on physician and patient preference and was mandated by local institution policies. The JAK2 V617F mutation was detected using a tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) assay with a sensitivity of 1% and the allele burden was estimated with a semi-quantitative method. Of 230 patients evaluated for moerate to severe eosinophilia, 7 (6 female/1 male; mean age 62.1 years) were positive for the JAK2 V617F mutation, fulfilling WHO criteria for CEL. JAK2 V617F-positive patients were compared to a cohort of FIP1L1-PDGFRA (n=18) and HES (n=61) patients. JAK2 V617F-positive CEL appears to be a relatively homogeneous clinicobiological entity characterized by female preponderance, peripheral eosinophilia, leukocytosis, normal hemoglobin, bone marrow infiltration by dysplastic eosinophils and eosinophil-induced tissue damage, predominantly of the heart. Compared to FIP1L1-PDGFRA-positive patients, JAK2 V617F-positive patients were older (p=0.06) showed a female preponderance (p=0.001), had lower white blood cell (p=0.007) and eosinophil counts (p=0.002), and similar hemoglobin values (p>0.9). JAK2 V617F-positivity was associated with cardiac involvement in multivariable analysis (p=0.002). Five JAK2 V617F patients were treated with a combination of steroids and hydroxyurea; four achieved a complete clinical and hematologic response; one patient evolved into acute myeloid leukemia (AML M6). In conclusion, JAK2 V617F-positive eosinophilia (6.2% of those with primary eosinophilia) is a rare but distinct myeloproliferative neoplasm characterized by eosinophilic marrow infiltration, target-organ damage and the potential of evolution to AML.

0656

UK SYSTEMIC MASTOCYTOSIS REGISTRY: ANALYSIS OF DATA ON 71 CASES

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Background. Systemic mastocytosis (SM) is a rare, multisystem disorder, characterised by clonal expansion of mutated mast cells. It may be indolent or more rarely aggressive. Diagnostic criteria include multifocal infiltrates (major), and one of the minor criteria: >25% abnormal mast cells, D816V(c-kit) mutation, CD2/CD25 positivity and serum tryptase > 20ng/mL. Symptoms are due to mediator-release or mast-cell infiltration. *Aim and Methods*. To improve UK epidemiological data for this condition. Anonymous data was collected noting: age at diagnosis, gender, serum tryptase level, clinical & mediator findings, and treatment. In this study those with cutaneous disease only are excluded. *Results*. We present results on 71 patients. There was an even sex distribution (33F; 31M; 6 unknown) and a wide age-range (17-82), with the majority being middle-aged, mean 51.6 years. Symptoms at diagnosis varied from none in 4 cases to ≥3 in 54%; they included skin involvement, bone pain, gastrointestinal, vascular instability and headache. The full blood-count at diagnosis was normal in 52% (37 cases); and showed pancytopenia (4), thrombocytopenia (4), thrombocytosis (2), eosinophilia (12) and anaemia in 13 cases. Serum tryptase was >20 in 92% cases, range 6 - >200. Most centres did not quantify above this level. WHO criteria were fulfilled in all cases; interestingly one diagnosis was made after splenectomy and one at post-mortem, following missed splenic rupture. 48 had indolent SM (ISM), 19 aggressive disease and 4, SM with associated non-mast cell haematological disorder (ANMHD). There were no mast-cell leukaemia cases. The serum tryptase was >200 in 42% patients with aggressive disease; in the others there was a complete range of tryptase results from 10.5-169 (mean 83). The associated non-mast cell disorders were - myeloproliferative disorder in 3 cases and myelodysplasia (transformed to acute myeloid leukaemia) in one case. *Treatments*. In 18 patients, no treatments were necessary; in a further 26 patients with indolent disease, mediator-release medications and mast-cell stabilisers were given. Two patients with borderline aggressive disease, were treated with hydroxycarbamide Of the remaining 21 patients with aggressive disease, 10 received one treatment regime, 9 had two, and two had tried 3 treatment options. A total of 13 patients received α-interferon. This was generally effective but frequently poorly tolerated. Cladribine was given to four patients. This produced temporary response in three. Six patients had tyrosine-kinase inhibitors: imatinib (3) with partial responses; nilotinib (2) with no response, and dasatinib (1) with good response. One patient had a splenectomy as a third line treatment without response, and is now awaiting allo-BMT. PKC-412 (novartis) was given to six patients with very aggressive disease with good responses in three, and too early to assess in one patient. *Conclusions*. As expected, the majority of cases have ISM (67%). Aggressive disease, (29%) however, may be over-represented in our registry, as being more 'worthy' of reporting. A range of treatments appear to have at least partial response in aggressive disease, and PKC-412 shows particular promise. Patients with'indolent' disease were very heterogeneous in symptomology, with some having none while others suffered multiple symptoms, including severe fatigue.

0657

POLYCYTHEMIA VERA AS A PREDISPOSING FACTOR FOR AORTIC STENOSIS: INCIDENCE AND CORRELATION WITH BLOOD CELLS COUNT AND MUTATIONAL STATUS

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Background. The association between polycythemia vera (PV) and thrombosis is multi-factorial involving the complex interaction between activated leukocytes, platelets and endothelium. Recent reports have postulated that PV patients may over express adhesive molecules on red cell surface, likely by JAK2V617F mutation (Wautier M *et al.* Blood 2007;110:894-901). This process activates endothelium with production of vascular growth factors and other mechanisms leading to atherosclerosis. Aortic Stenosis (AS) is the commonest valvular heart disease in western countries; its pathogenesis is mainly related to a degenerative process sharing many characteristics with atherosclerosis. At the present is not known whether patients with PV are at high risk of developing AS. Objective of the study. We perform a prospective study for evaluating rate of AS and its correlation with blood cells count and mutational status in patients with PV. *Materials and methods*. Incidence rate of AS among PV patients have been compared with control patients matched for age, cardiovascular risk factors (hypertension, hyperlipidemia, diabetes, smoke and alcohol abuse) and coexisting cardiac diseases (i.e. heart failure). Diagnosis of PV has been posted accordingly to PVSG criteria. Diagnosis and severity of AS has been posted by echocardiography: stenosis with a valve area <1.0 cm^2 has been considered severe. *Results*. Over a period of 18 months we recruited 43 PV patients (28 males and 15 females) and 74 controls. No differences were found in regard of the above cited characteristics; mean age was 66.7 among PV patients and 68.2 among controls. The average duration of PV was 5.7 years with an average follow-up of 2.5 years. Most of the PV patients were on antiplatelet/anticoagulant therapy (27/43, 62.7%) and have been treated with cytoreductive therapy. Twelve (27.9%) had a thrombotic event before PV diagnosis; 4 (9.3%) developed thrombosis during the follow-up (median 1.3 years). A moderate/severe AS was found in 11 PV patients (25.6%) in comparison to 4 (5.4%) in control group (p=0.004), thus giving a Relative Risk of 4.7 (CI 95%: 1.61-13.95). Among PV patients, the multivariate analysis did not show any correlation regarding JAK2V617F mutational status, duration of disease, previous thrombosis, cytoreductive therapy and other common cardiovascular factors. A significant trend was demonstrated in favor of patients with elevated haematocrit (>55%) (p=0.001). *Conclusions*. Our study clearly shows that PV patients carry a fourfold risk of developing AS, mainly related to the presence of high haematocrit level. No clear association were found regarding white blood cell or platelets count, effect of cytoreductive therapy or previous thrombosis. Whether high incidence of AS may be related to expression of adhesive molecules on red cells or altered share stress is currently under investigation.

0658

DASATINIB FOR THE TREATMENT OF TREATMENT-REFRACTORY HYPEREOSINOPHILIC SYNDROME AND CHRONIC EOSINOPHILIC LEUKEMIA

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In the absence of imatinib-responsive markers, chronic eosinophilic leukemia (CEL) and hypereosinophilic syndrome (HES) pose a therapeutic challenge. We hypothesized that dasatinib (BMS-354825, Bristol-Myers Squibb) could be effective for the management of treatment-refractory or intolerant HES/CEL. Four female HES and 2 male CEL patients, all of whom were negative for imatinib responsive markers, were enrolled in this exploratory study. Prior treatment with imatinib and at least one other therapy had failed for 5 of them; one was considered intolerant to previous treatments. All patients had elevated bone marrow eosinophil counts. Patients received dasatinib at 70 mg bid; toxicity and safety were evaluated weekly for 4 weeks, every two weeks for 2 months and monthly thereafter. Bone marrow examination performed at 2 and 6 months after treatment onset. Complete remission (CR) was defined as eosinophils <5% with WBC<10,000 cells/mm^3 and complete disappearance of all reversible signs and symptoms of disease. All other changes were scored as no response. Therapy continued for at least 8 weeks unless patients experienced unmanageable toxicity. Pulmonary toxicities were managed according to a protocol used for CML patients receiving dasatinib. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, v 3.0. Western-blot analysis was used to assess response at protein level: phospho-SRC (pSRC), phospho-STAT5 (pSTAT5), STAT5, and phospho-ERK (pERK) were evaluated in pre-treatment and post-treatment specimens. After a mean follow-up of 12.7 months (range: 5-19) after study enrollment, four patients (66%; 95% CI: 0.22-0.96) achieved CR, which was confirmed by BM biopsy (2 male and 2 female). Three of these responded within 2 months, while the fourth achieved CR within 5 months. The mean response duration was 7.3 months (range: 2-12). A CEL patient with a complex cytogenetic abnormality [del(X)(q24), t(2;9)(q12;q32), del(20)(q12)], achieved complete cytogenetic remission in addition to hematological and clinical CR. Four patients developed pleural effusions (grade 3, n=3; grade 2, n=1), which were bilateral in 2 cases. In all patients developing pleural effusions, dasatinib had to be discontinued for 2-4 weeks and diuretics were administered. Treatment was restarted at 100mg daily in three of them; one refused re-treatment. One patient developed new onset hypothyroidism (grade 2 autoimmune reaction). Two patients experienced grade 2 anemia and leucopenia. Responsive patients showed a remarkable reduction in pSRC and pSTAT5 levels in peripheral blood taken one month post-treatment compared to pre-treatment samples. We conclude that dasatinib may be useful for the treatment of treatment-refractory/ intolerant HES/CEL with a severe but manageable toxicity profile. The occurrence of complete cytogenetic response in an imatinib-refractory CEL patient indicates that dasatinib-responsive molecular aberrations are present in a subset of patients with primary eosinophilia.

0659

HIGH RESOLUTION MELTING CURVE ANALYSIS: A SIMPLE, RELIABLE AND SENSITIVE METHOD OF DETECTION OF JAK2 EXON 12 MUTATIONS

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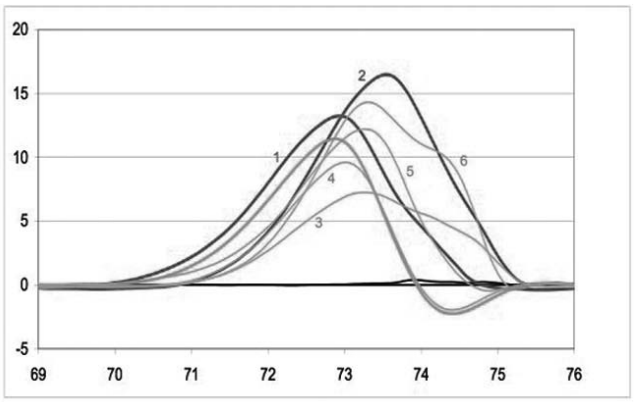
Background. The description of an acquired Jak2 V617F mutation represents a major improvement in the understanding and management of myeloproliferative disorders. Investigation of V617F negative polycythemic cases led to the characterization of new mutations in Jak2 exon12. These exon12 mutations are associated with pure erythrocytosis. About 19 different Jak2 exon12 mutants have now been reported spotting a region from AA 536 to 547. SNPs, deletions, insertions and duplications have been described, all respecting the ORF, that means a huge number of predictable mutational types. So, techniques like AS-PCR are inappropriate and sequencing or pyrosequencing lack sensitivity to screen them. There is therefore obviously a need for a rapid and sensitive assay for Jak2 exon12 mutation screening. We test HRM because it can be used by any medical laboratory and requires just a real time thermocycler, opposed to more expensive technologies like DHPLC. *Aims*. 1) confirm the efficiency of HRM for screening Jak2 exon12 screening; 2) characterize positive HRM signals; 3) analyse Jak2 exon12 biological data. *Methods*. Granulocytes DNA samples from 6 Secondary Erythrocytosis, 15 Idiopathic Erythrocytosis (IE) and 34 Polycythemia Vera V617F negative or with low allelic burden (<25%) patients, diagnosed according to 2001 WHO criteria, were collected by 5 centers (Bordeaux, Clermont-Ferrand, Dijon, Nantes and Nîmes). Nested HRM was performed in Nîmes on LightCycler 480 and confirmed on Rotorgene in Clermont-Ferrand. It consists of a first PCR of 496 bp long, diluted 1:100 for HRM reaction (118

bp). Mutations were characterized by sequencing genomic DNA or cloned plasmids, depending of the allelic burden. *Results*. 1) HRM allows detection of exon12 mutations with high reliability and sensitivity. Use of a nested protocol enhances sensitivity by lowering wild type controls HRM signals compared to genomic DNA. Titrating experiments shows a detection treshold ranking from 1 to 3%. It depends on the mutant and is more or less as sensitive as AS-PCR. Running wild type and mutant plasmids, we did not get false negative and only a small false positive (1 over 30 replicates) which is normally discarded in routine triplicates. Nested HRM detects 13 mutants (24%): 2 IE (13.3%), 11 PV (32.3%), no SE. Among 13 V617F positive PV, 3 harbor an exon12 mutation (23%), adding to the only one double mutant already reported. 2) Sequencing identifies mutations in all the 13 cases. We describe 4 new mutations: H538Q K539L, F537I K539I, K539E and I540T. One of the double mutant presents an exon12 allelic burden higher than the V617F one, as opposed to the others, questioning appraisal pattern. 3) Biological data confirm previous reports: pure erythrocytosis associated with low serum EPO, but the 4 double mutants seems to present a specific profile. *Conclusions*. Nested HRM is a rapid and simple technique particularly appropriate to screen Jak2 exon12 mutations. It allowed description of 4 new mutations. Double mutants are not rare. Exon12 mutations should be looked for in in patients with unexplained pure erythrocytosis and low V617F burden.



HRM differential plot profiles of some Jak2 Exon12 mutations
Wild type controls, black : NiWT1 and NiWT2
New mutants, red : 1, H538G K539L; 2, F537I K539I
Duplication, blue
Already reported mutants, green : 3, K539L; 4, R541-E543del ins K ; 5, N542 E543del; 6, H538Q K539L

Figure 1.

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EXPRESSION OF A SET OF CELL-STROMA INTERACTING GENES IN PATIENTS WITH PRIMARY MYELOFIBROSIS

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Background. Primary myelofibrosis (PMF) is stem cell derived Philadelphia chromosome-negative chronic myeloproliferative neoplasia characterized by marrow fibrosis, leukoerythroblastosis, extramedullary hematopoiesis and splenomegaly. Etiology is still unknown, but mutations in several genes are found at increased frequency (e.g. JAK2, MPL, TET2, IKAROS) in this disease. The therapy for PMF is unsatisfactory and median survival is poor. Different medications have, therefore, been evaluated in clinical trials, including novel JAK2 inhibitors, and immunomodulatory inhibitory drugs (IMiDs) like thalidomide and lenalidomide. *Aims*. To assess genes that impact PMF, we analyzed expression of genes that are involved in cell-stroma adhesion (SPARC, CXCR4), metabolism (COX-2, HIF1), differentiation and signaling (Pax5 and Socs3) in mononuclear cells (MNC) from bone marrow (BM) or peripheral blood (PB) of 32 PMF patients before and after treatment with the combination of lenalidomide and prednisone. *Methods*. PB and BM aspirate samples from healthy individuals and PMF patients were ficolled and RNA was isolated from MNCs with Trizol reagent.Q-RT-PCR was performed to measure the expression levels of SPARC, COX-2, CXCR4, Pax5, Socs3 and HIF1 transcripts with β-actin as internal control. *Results*. Pre-therapy, SPARC and COX-2 expression were decreased in BM MNC, while CXCR4, Pax5 and HIF1 were increased, compared to controls. At the same time, COX-2, CXCR4 and HIF1 expression were decreased in PB MNC. No correlations between gene expression and JAK2 mutational status or cytogenetic abnormalities were found. Six months after treatment with lenalidomide/prednisone further decrease in SPARC, Socs3 and HIF1 in BM MNC was recorded, and COX-2 and CXCR4 further decreased in PB MNC. No correlation with response was found. *Summary and Conclusions.* Pre-therapy down regulation of SPARC may indicate its defect in tumor suppressing activity in PMF. Interestingly, lenalidomide treatment known for the dramatic up-regulation of SPARC expression in patients with 5q- myelodysplastic syndrome, did not show the same pattern in PMF. Further investigation of the SPARC regulation in stromal and other elements of hematopoietic niche of PMF is underway.

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THE IMPACT OF AZACITIDINE AND DECITABINE (HYPOMETHYLATING-AGENTS) IN MYELODYSPLASTIC SYNDROMES: A SYSTEMATIC REVIEW AND META-ANALYSIS

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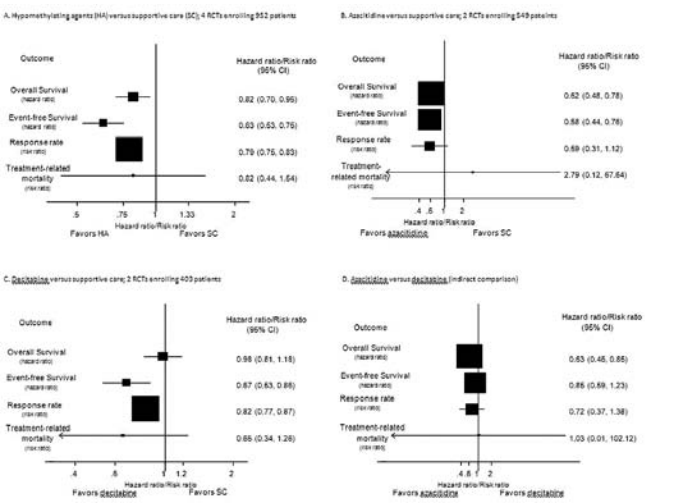
Background. Treatment of patients with myelodysplastic syndrome (MDS) with the hypomethylating agents (HA), 5-azacytidine (AZA-C) and decitabine have been a major focus of clinical research that lead to their FDA approval. Use of these agents has improved hematopoiesis and may impact natural history of disease. However, results for patient oriented outcomes of overall survival (OS), and event-free survival (EFS) have been inconsistent across trials. Furthermore, a direct comparative trial of of AZA-C versus decitabine is lacking. *Aims*. To perform a systematic review of randomized controlled trials (RCTs) to assess the efficacy of AZA-C and decitabine compared to best supportive care (SC) which includes conventional care regimens or SC alone, and AZA-C versus decitabine for the treatment of MDS. *Methods*. A comprehensive literature search of MEDLINE, EMBASE and Cochrane database of RCTs was undertaken to identify all phase III randomized controlled trials (RCT) published until July 2008. Meetings abstracts from ASCO, ASH and European Hematology Association were searched for the years 2006-2008. Data extraction and meta-analysis on benefits and adverse effects of HA for MDS was performed as per the methods recommended by the Cochrane Collaboration. Indirect comparison of AZA-C versus decitabine was conducted according to the methods developed by Bucher, Lumley and Glenny *et al.* and were extended to calculate hazard ratios (HR). We created the following chain of inference: we first pooled RCTs that compared AZA-C with SC, and decitabine versus SC. We then compared the pooled estimates to obtain the unbiased estimate in treatment differences between decitabine and AZA-C.



Figures A through D.

Results. We found 4 RCTs assessing the efficacy of HA for the treatment of MDS. Two RCTs compared AZA-C versus SC, and 2 compared decitabine versus SC. The results have been summarized in Figures A through D. Meta-analysis of RCTs comparing HA versus SC showed significantly better OS, EFS, and response rate in favor of HA without a significant increase in treatment-related mortality (TRM). Comparison of AZA-C versus SC also showed a significant advantage in OS, and EFS favoring AZA-C without significant risk of TRM. The pooled results for the comparison of decitabine versus SC showed significantly better EFS and response rate with decitabine. However, there was no difference in OS and TRM between decitabine and SC. Evaluation of AZA-C versus decitabine showed significantly better OS favoring AZA-C. Nevertheless, EFS, response rate and TRM was similar between AZA-C and decitabine. *Conclusions*. This first systematic review on the efficacy of HA versus SC shows that OS, EFS and RR are significantly better with HA without significant TRM. Use of AZA-C is associated with significantly better OS without a significant increase in TRM compared to decitabine. A RCT comparing AZA-C and decitabine is warranted.

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PRESENCE OF JAK2 V617F MUTATION IS A SINGLE STRONG PREDICTOR OF VASCULAR COMPLICATIONS IN PATIENTS WITH MPD

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Background. Since the discovery of the JAK2 V617F mutation, the clinical and pathological consequences of this acquired defect have been extensively investigated to determine whether its presence characterizes a distinct subgroup of myeloproliferative disorders (MPD). There is increasing clinical evidence to suggest that the mutation may be associated with increased activation of leukocytes and platelets in MPD and may lead to an increased risk of thrombosis. *Aims*. The aim of our study was to investigate the frequency of JAK2 V617F mutation in patients with different subgroups of MPD. Statistical correlation between the presence of JAK2 mutation and clinical and laboratory outcome was performed. *Patients and methods*. We conducted a retrospective study of 233 patients who were referred to our hospital for investigation of JAK2 V617F mutation between January 2006 and July 2008. Only patients with clinically and laboratory confirmed BCR-ABL negative MPD were included. *Results*. There were 99 (42.5%) males and 134 (57.5%) females in our study, with median age of 66 years(21-91). 92/233 (39.5%) patients had polycythemia vera (PV), 107 (45.95%) essential thrombocythemia (ET), 12 (5.2%) idiopathic myelofibrosis (IMF) and 20 (5.2%) unclassified myeloproliferative disorder (CMPD-U). 177/233 (76%) patients were asymptomatic and were referred due to high Hb level, thrombocytosis or leukocytosis. 45/233 (19.3%) patients presented with clinical symptoms, the majority with headache and erythromelalgia. 11/233 (4.72%) patients had a vascular event at the time of diagnosis; 8 presented with an arterial event and 4 with VTE. During the follow up 68/233 (29.2%) patients suffered from arterial or venous thrombotic complications of the underlying MPD; the incidence of arterial or venous thrombosis was similar in PV and ET patients (20/23 and 10/9 respectively). JAK2 V617F mutation was present in 192/233 (83.35%) patients; 82/92 (89.1%) patients with PV, 81/107(74.2%) with ET, 10/12(83.3%) with IMF and 19/20 (95%) with CMPD-U. We found that the majority of thrombotic events- 39/56 arterial and 20/23 venous thromboses- occurred in JAK2 V617F positive patients. Of 80 patients who presented with leukocytosis, 24 suffered from thrombotic complications. They were equally distributed between patients with PV and ET, and 19 of them had positive JAK2 V617F mutation. *Conclusions*. In our retrospective study of 233 patients with MPD we found that our patients with PV had a high incidence of JAK2 V617F mutation (89.1%). Our cohort of patients with ET had a higher incidence of JAK2 V617F mutation than reported in the literature (74.2%). This may be explained by the fact that many of our patients had a well established diagnosis of MPD several years before the test for JAK2 mutation was included in our routine investigations of MPD. We found that 57/79 (72.2%) patients with vascular events were positive for JAK2 V617F mutation. Our findings further confirm the previous reports that presence of JAK2 V617F mutation is a single strong predictor of vascular complications in patients with MPD.

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JAK2 V617F MUTATION IN ACUTE MYELOID LEUKEMIA SECONDARY TO PH NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Transformation to acute myeloid leukemia (AML) is a known complication of myeloproliferative disorders (MPDs). Recently, Theocharides *et al.* have published an interesting report about the incidence of JAK2 negative blasts from patients affected by secondary AML derived from JAK2 mutated MPDs. *Methods*. We collected, by cell sorting, blast cells and mature cells from total bone marrow of 17 patients newly diagnosed of secondary AML [9 derived from primary myelofibrosis (PMF); 4 from polycythemia vera (PV) and 4 from essential thrombocythemia (ET)]. All the samples were genotyped for JAK2-V617F mutation by ASO PCR and the measurement of the allele burden was performed in Real Time PCR. At MPD diagnosis, JAK2-V617F was detectable in 9 of 17 patients (4 of 9 PMF; 4 of 4 PV and 1 of 4 ET). All patients had received cytoreductive treatment with HuOH. *Results*. In our cohort of patients we found that JAK2-V617F mutation was still present at the blast transformation in both compartments (blasts and mature cells) in 8 of 9 JAK2 mutated MPDs. Only 1 of 9 patients developed JAK2-V617F negative AML starting from a mutated MPD. Interestingly, the negativity for the mutation was confirmed in blast cells but also in the rest of mature-myeloproliferative bone marrow tissue. Surprisingly we also described a case of JAK2-V617F mutated AML from a wild type MPD but even in this case the positivity occurred in mature and blast compartments. The remaining 7 wild type JAK2 MPDs maintained the same JAK2 status during blast crisis. No differences in the allele burden were found before and after leukemic transformations comparing the two groups of patients. Two JAK2 positive AML from JAK2 positive MPD (1 ET and 1 PV) achieved CR after induction treatment while the others did not respond to the therapy. Discussion. According to our preliminary results, in contrast to the previous study, we conclude that JAK2-V617F positive MPD yields rarely a JAK2-V617F negative AML and at the same frequency of a JAK2-V617F negative MPD transforming in JAK2 mutated AML. Furthermore we wanted to underline how any modifications in the JAK2 integrity or the persistence of the previous status involved the entire bone marrow during leukemic transformation suggesting that the leukemic hit could take place in a common ancestor precursor able to modify entirely the genomic signature of the disease.

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STUDY OF THE RELATION BETWEEN THE TIME COURSE OF THE JAK2V617F ALLELE BURDEN AND THE CLINICAL OUTCOME IN PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS (MPNS); A SINGLE CENTER STUDY

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Background. The JAK2V617F is a main molecular marker of MPNs. There are few studies that analyze the time course of the JAK2V617F allele burden. *Aims*. To study the clinical outcome and the time course of the JAK2 allele burden in MPNs. *Methods*. We performed a single centre study on 95 patients: 31 polycythemia vera (PV), 60 essential thrombocythemia (ET) and 4 primary myelofibrosis (PMF), all of them with at least 12 months of follow-up and at least two JAK2 determinations. The JAK2V617F mutation was analyzed in DNA from peripheral blood granulocytes by MutaScreenTMKit (IPSOGEN). *Results*: The median follow-up period was 25 months. As previously reported, none of 26/95 JAK2 negative patients became positive for the mutation. 69/95 patients were positive for JAK2 and in 35/69 (50.7%) JAK2V617F allele burden changed (% last JAK2V617F - % first JAK2V617F > 15%) during the follow-up. The JAK2 allele burden increased in 23/35 patients and interestingly, eight of them (34.8%) presented transformation (Table1).

JAK2V617F allele burden		Clinical outcome (n=69)		
Decrease	12/69 (17.4%)	No progresion (n=57)	Progresion (n=12)	
No change	34/69 (49.3%)	11/12 (91.7%)	1/12 (8.3%)	PV-AML (n=1)*
Increase	23/69 (33.3%)	32/34 (94.1%)	2/34 (5.9%)	ET-PV (n=1) PV-MF (n=1)
Decrease	12/69 (17.4%)	15/23 (65.2%)	8/23 (34.8%)	ET-MF (n=4)*** PMF-AML (n=1)** ET-PV (n=3)

Table 1. Clinical outcome and JAK2V617F allele burden.

Two patients progressed to acute myeloid leukemia (AML): first patient showed decrease (transformation PV to AML)* and second (MFI) presented initial increase with subsequent decrease of the JAK2 allele burden once AML was diagnosed**. 54/69 of JAK2 positive patients were on Hydroxyurea (HDU): 22/54 newly (≤12 months at the time of the first JAK2 study) and 32/54 already treated (>12 months). The JAK2V617F allele burden was stable in 29/54 (53.7%) of patients on HDU (newly and already treated). There were no differences between changes in the JAK2V617F allele burden and a period of HDU administration (>12 vs. ≤12 months). There were 10/69 patients who did not receive cytoreductive treatment and 7/10 showed an increase of JAK2 allele burden. Moreover, an increase in JAK2V617F allele burden was showed in two patients after discontinuation of HDU treatment. Conversion to JAK2V617F negativity (n=1) was observed after allogeneic hematopoietic stem cell transplantation (HSCT)***. *Conclusions*. The JAK2V617F allele burden may change during the follow-up and this can be related to clinical outcome of MPNs. We confirm that, JAK2V617F allele burden decrease with transformation to AML. In case of transformation to PV o MF the JAK2V617F allele burden usually increases or is stable. In the majority of patients on HDU treatment the JAK2V617F allele burden seems to be stable. HDU can change the JAK2V617F allele burden only in individual cases and according to our results, there are no difference between newly and already treated patients. The JAK2V617F allele burden can increase without HDU. HSCT can induce conversion to JAK2V617F negativity, as previously reported. Further prospective studies are needed to conclude whether the follow-up of the JAK2V617F allele burden may be used to predict transformation of MPNs patients or to monitor the response to the treatment.

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Non-Hodgkin lymphoma - Biology

0665

CRYPTIC 9P24 MICRODELETIONS COVERING HSA-MIR-101-2 ARE RECURRENT IN HEMATOLOGICAL MALIGNANCIES

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Background. The 9p terminal (9pter) region is recurrently involved in genomic aberrations in hematological malignancies. These aberrations, frequently targeting JAK2 (9p24), include chromosomal translocations, amplifications and mutations. Recently, we have collected a series of leukemia and lymphoma cases with 9p24 deletions. Molecular analysis of these cases is presented. *Aims.* The aim of this study is to identify and molecularly characterize recurrent 9p24 deletions in hematological malignancies. *Methods.* 84 leukemia/lymphoma cases with 9pter aberrations were analyzed by FISH using BAC and fosmid probes. The Agilent oligonucleotide 244k array platform was used to map the identified 9pter deletions. *Results*. FISH analysis with a panel of 9p BACs identified 18 cases with genomic 9pter losses, including 4 cases with cryptic microdeletions. The latter aberrations were mapped by high resolution arrayCGH. The smallest deleted 9p24 region (SDR) established in these cases covers 1.9 Mb and harbours 18 genes including JAK2. The candidate tumor suppressor genes (TSGs) encompassed by SDR include PPAPDC2, CDC37L1 and the microRNA gene, hsa-mir-101-2. The role of hsa-mir-101-2 in tumorigenesis, and particularly in the development/progression of hepatocellular carcinoma has recently been documented. This microRNA targets at least 3 genes, FOS, MCL1 and EZH2. FOS codes for v-fos FBJ murine osteosarcoma viral oncogene homolog, a key component of the activator protein-1 (AP-1) transcription factor and important in regulating the development of cells destined to form and maintain the skeleton. Myeloid cell leukemia sequence 1 (MCL1) is an antiapoptotic member of the BCL2 family, and EZH2 encodes for enhancer of zeste homolog 2, a mammalian histone methyltransferase that contributes to the epigenetic silencing of target genes. It has been shown that deletion of hsa-mir-101-2 in cancer cells correlates with overexpression of EZH2 and results in the increased survival and metastatic properties of cancer cells. In hepatocellular carcinoma, downregulation of has-mir-101 has been observed that may contribute to high expression levels of FOS protein and MCL1. The role of hsa-mir-101-2 in human leukemia and lymphoma has not been documented yet, but Mcl-1 transgenic mice exhibit a high probability of developing B-cell lymphoma. *Conclusions*. The common region of 9pter deletions in hematological neoplasms harbours numerous coding and non-coding genes, including hsa-mir-101-2. Given its postulated role of tumour suppressor, hsa-mir-101-2 is a strong candidate gene affected by the del(9)(p24). Further studies of the candidate TSGs including hsa-mir-101-2 in cases with a del(9)(p24) are ongoing.

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NOVEL T(X;14)(P11.4;Q32.33) RESULTING IN UPREGULATION OF GPR34 AND ACTIVATION OF THE NFKB PATHWAY IS RECURRENT IN MALT LYMPHOMAS

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Background. MALT lymphoma is a low grade B cell neoplasm which commonly develops from a background of chronic inflammatory or auto-immune disorder. Approximately 25% of MALT lymphoma cases is hallmarked by characteristic and mutually exclusive chromosomal translocations including, t(11;18)(q21;q21)/ API2-MALT1, t(1;14)(p22;q32)/IGH-BCL10, t(14;18)(q32;q21)/IGH-MALT1 and t(3;14)(p14;q32)/IGH-FOXP1. The oncogenic products of the first three translocations target the NFkB pathway which plays an important role

in cell survival and proliferation. *Aims*. This study aimed at molecular characterization of a novel t(X;14)(p11;q32) identified in four B-NHL cases. *Methods*. The reported cases were analyzed using FISH, oligo aCGH (Agilent 244K), qRT-PCR, Western blotting and immunohistochemistry. *Results*. Histology, immunophenotype and clinical features of all four cases with t(X;14) were reviewed. MALT type lymphoma was diagnosed in three cases and *de novo* gastric DLBCL in one case. Patients were 3 females and one male with an average age of 75 years (66-82). Two of them had a previous history of Sjögren's syndrome, one of leukocytoclastic vasculitis, and the patient with gastric DLBCL had a chronic gastritis. All patients received an initial therapy and three are alive after 7-86 months from diagnosis. Extensive FISH analysis of t(X;14) showed IGH involvement at 14q32.33 and mapped the Xp11.4 breakpoint in the 3' end of the CASK gene. Of note, this gene houses GPR34 and GPR82 coding for orphan G-protein coupled receptors, located in the intron 5 of CASK. To identify the gene targeted by t(X;14), we performed qRT-PCR analysis of five genes flanking the Xp11.4 breakpoint (tel->USP9X, DDX3X /bkpt/CASK, GPR34, GPR82->cenX) in two available cases. Only one of these genes, GPR34, showed to be highly upregulated (>50 fold) in both analyzed cases. IHC analysis of the GPR34 protein expression is ongoing. So far, involvement of GPR34 in tumorigenesis has not been reported. To examine molecular consequences of an aberrant expression of GPR34 in lymphoma, we analyzed the status of NFkB and MAP kinase pathways by Western blotting with antibodies against phosphorylated Ik-Ba and ERK1. In both analyzed cases a phosphorylated Ik-Ba protein was detected. Further studies to evaluate the NFkB target genes expected to be activated in lymphomas with t(X;14) are ongoing. High resolution aCGH did not identify any recurrent genomic imbalances in these cases. Interphase FISH analysis of 13 MALT lymphomas with unknown cytogenetics showed a normal pattern of the IGH and CASK break apart probes applied in these cases. *Conclusions*. The novel t(X;14)(p11.4;q32.33) is predominantly associated with MALT lymphoma and an underlying autoimmune/inflammatory disorder. The translocation targets GPR34 which is upregulated due to its juxtaposition with strong regulatory elements of IGH. The finding of NFkB activation by t(X;14)/IGH-GPR34 further strengthens the important role of this pathway in the development of MALT lymphoma.

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DIFFERENTIAL EXPRESSION OF MICRORNAS IN PRIMARY CENTRAL NERVOUS (CNS) AND NODAL DIFFUSE LARGE B-CELL LYMPHOMAS

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Background. Primary CNS lymphoma (PCNSL) is a rare subtype of diffuse large B-cell lymphoma (DLBCL) confined exclusively to the CNS. Mechanisms leading to lymphoma development in the immunoprivileged CNS are still not fully understood. MicroRNAs play key regulatory roles in eukaryotic gene expression and recently aberrant microRNA expression has been demonstrated in several lymphoma entities. Thus far, no data are available for PCNSL. *Aims*. The aim of the study was to determine the expression pattern of microRNAs in PCNSL and to test whether microRNA profiling can distinguish PCNSL from nodal DLBCL. *Methods*. Total RNA was extracted from formalin-fixed and paraffin-embedded (FFPE) tissue sections of diagnostic biopsy samples derived from newly diagnosed PCNSL and DLBCL. FFPE blocks used had been stored for up to six years. RNA was reverse transcribed using eight predefined primer pools containing up to 48 multiplex RT primers each. Gene expression was measured by quantitative real-time (RT) PCR using commercially available TaqMan low density arrays (TLDA). Each TLDA contained specific primer-probe combinations for 365 microRNA species, three small nuclear RNAs functioning as endogenous controls and two negative controls. Each RT-PCR was run in duplicate. Normalized expression levels were calculated using the 2-dCT method. Mean expression levels in PCNSL and nodal DLBCL of each microRNA were compared using the Mann-Whitney-U test. Biopsy samples were also subtyped immunohistologically into a GCB- and non-GCB type according to the Hans classifier (CD10, BCL6, MUM1). *Results*. Eleven PCNSL and eight nodal DLBCL samples were analyzed: four non-GCB and four GCB nodal DLBCL; and five non-GCB, three GCB, and three non-classifiable PCNSL. Of 365 microRNA species, 40% showed significant expression levels by means of RT-PCR (Ct value of <38). We detected 20 microRNAs with a significantly different expression in PCNSL and nodal DLBCL. Expression of 12 microRNAs was significantly higher in PCNSL, and eight microRNAs showed reduced expression compared to DLBCL (Table). The most significant differences were detected for miR-204, miR-27b, miR-155, miR-214 and miR-432. Expression levels of specific microRNAs differed from 60-fold upregulation to 12-fold downregulation. *Conclusions*. This is the first report demonstrating a differential expression of specific microRNAs in PCNSL and nodal DLBCL. Several of the differentially expressed microRNAs have been implicated in pathways promoting tumor cell survival, such as miR-214 (PTEN/AKT in ovarian cancer), angiogenesis (miR-27b), or regulation of drug metabolizing enzymes or P-glycoprotein expression (miR-27b, miR-415). MiR-204 is highly expressed in acute and chronic B cell leukemias, and miR-155 is known to be associated with NF-kappaB activity and upregulated in non-GCB DLBCL. Thus, some of the differentially expressed microRNAs identified in this study might be involved in the development of PCNSL and warrant further studies.

Table.

microRNA	PCNSL/DLBCL -fold expression	*p*
hsa-miR-204	60,8	0.006
hsa-miR-219	19,5	0.028
hsa-miR-451	7,6	0.030
hsa-miR-17-5p	5,4	0.042
hsa-miR-27b	4,1	0.005
hsa-miR-155	3,8	0.005
hsa-miR-30b	2,5	0.015
hsa-miR-9	2,5	0.014
hsa-miR-146b	2,4	0.026
hsa-miR-146a	2,3	0.026
hsa-miR-30c	1,9	0.033
let-7g	1,7	0.026
hsa-miR-594	1 : 2,1	0.016
hsa-miR-432	1 : 2,5	0.009
hsa-miR-193b	1 : 2,7	0.049
hsa-miR-296	1 : 2,9	0.018
hsa-miR-145	1 : 3,0	0.012
hsa-miR-199a	1 : 4,4	0.028
hsa-miR-214	1 : 4,9	0.007
hsa-miR-139	1 : 12,2	0.024

0668

CAL-101, A SPECIFIC INHIBITOR OF THE P110DELTA ISOFORM OF PHOSPHATIDYLINOSITIDE 3-KINASE, FOR THE TREATMENT OF NON-HODGKINS LYMPHOMAS

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Background. Phosphatidylinositide 3-kinases (PI3Ks) are a family of lipid kinases that are involved in signaling events which control a diverse number of cellular processes. The PI3Ks are divided into three classes; I, II, and III. The class I kinases contain four isoforms designated p110alpha, beta, delta, gamma, and are activated by cell surface receptors. Aberrant regulation of the PI3K signaling pathway is frequently observed in human malignancies including those of hematological origin. Giving its role in tumorgenesis, PI3K is an attractive target in cancer therapy. Unlike the other class IA isoforms p110delta expression is largely restricted to hematopoietic cells whereas the α and β isoforms are ubiquitously expressed. CAL-101 is an oral p110delta specific inhibitor which is currently being evaluated in a phase I clinical trial for the treatment of non-Hodgkins lymphomas (NHL) and chronic lymphocytic leukemia (CLL). This compound is a novel potent p110delta inhibitor with an IC50 of 2.5 nM against purified p110delta and EC50 of 65 nM in p110delta-mediated basophil activation in whole blood. CAL-101 demonstrates 300-, 200-, and 40-fold selectivity over the other class I family members (alpha, beta, and, gamma respectively) and no activity against class II and III PI3K family members or other PI3K-related proteins including mTOR and DNA-PK. Furthermore, a kinome-wide

screen failed to detect activity any additional kinases. Previous studies have provided preclinical proof of concept through the analysis of cell lines and primary tumor cells from patients with acute myeloid leukemia, chronic lymphocytic leukemia, acute lymphoblastic leukemia and multiple myeloma. For each of these indications, all cell lines and patient samples expressed a high level of the p110delta isoform and treatment with CAL-101 resulted in PI3K pathway inhibition that correlated with the induction of cellular apoptosis or cell cycle arrest. *Results*. In the present study, we show that treatment of NHL cell lines with CAL-101 resulted in a significant decrease in cellular proliferation. High levels of p110delta were observed in all NHL cell lines while expression levels of the other three isoforms varied. The results showed that cell growth was inhibited in a dose-dependent manner in follicular lymphoma (RL, Karpas-422, WSU-FSCCL, and WSU-NHL), diffuse large B-cell lymphoma (SU-DLH4, SU-DLH5, and HS602) and mantle cell lymphoma (Mino and NCEB) cell lines. Growth suppression observed in both the diffuse large B-cell and follicular lymphoma cell lines correlated with an increase in the percentage of apoptotic cells. Furthermore, CAL-101 induced apoptosis was accompanied by PARP and caspase-3 cleavage. Additional mechanistic studies show that inhibition of p110delta abrogated or reduced the phosphorylation of Akt, GSK-3B, and S6. *Conclusions*. Our results demonstrate that specific inhibition of p110delta and its downstream signaling pathways with CAL-101 inhibits malignant cell growth and survival, thereby providing the preclinical rationale for its clinical testing as a novel therapeutic approach in NHL.

0669

AMPLIFICATION AT 11Q23 TARGETS PROTEIN KINASE SIK2 IN DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Subclassification of hematologic malignancies according to chromosomal aberrations and/or gene expression signatures is very important for the judgement and development of therapeutic protocols. This has been done with great success for diffuse large B-cell lymphomas (DLBCLs), identifying three major subtypes: germinal center B-cell-like (GCB), activated B-cell (ABC) and primary mediastinal B-cell lymphoma (PMBL). Nevertheless, in DLBCL several recurrent chromosomal aberrations have been described albeit without knowledge of their effects on gene expressions, including gain/amplification at 11q23-24. *Aims*. Here, we characterized an amplification at 11q23 in the DLBCL-derived cell line KARPAS-422 to identify target genes of this aberration. *Results*. By quantitative genomic PCR and fluorescence *in situ* hybridization analysis we mapped the amplicon to 85-111 Mbp, containing a high peak at 111 Mbp. Quantitative RT-PCR, western blot and immuno-staining identified overexpression of SIK2/SNF1LK2, while neighboring candidates at 111 Mbp were inconspicuously expressed, highlighting this gene as the major target of this amplification. Quantification of SIK2 mRNA in diverse lymphoma cell lines, including anaplastic large cell lymphoma, Hodgkin lymphoma, Burkitt lymphoma and mantle cell lymphoma demonstrated higher average expression levels in DLBCL, indicating a general role of SIK2 in this lymphoma type. SIK2 codes for a protein kinase which has been shown to inhibit cAMP-regulated transcription factor CREB via phosphorylation of its cofactor TORC. Accordingly, siRNA-mediated downregulation of SIK2 expression resulted in elevated expression of the CREB target gene BIM. Functional analysis by treatments of KARPAS-422 cells in comparison to a DLBCL control cell line with SIK2 upstream regulator cAMP or downstream effector 2-deoxy-D-glucose indicated a regulating role for SIK2 in survival and glucose metabolism, respectively. Finally, gene expression profiling of KARPAS-422, analyzing B-cell signature genes suggested that this cell line belongs to the GCB-subtype of DLBCL. *Conclusions*. We have identified SIK2 as a novel potential oncogene in DLBCL which may represent a suitable therapeutic target. The cell line KARPAS-422 may serve as a useful model for further investigations of amp(11)(q23) positive GCB-DLBCLs, overexpressing SIK2.

0670

IMPROVED DIFFERENTIAL DIAGNOSIS BETWEEN WHO-DEFINED MATURE B-CELL MALIGNANCIES USING INTEGRATED 8-COLOR FLOW CYTOMETRY AND NOVEL SOFTWARE FOR MULTIVARIATE ANALYSIS OF IMMUNOPHENOTYPIC DATA

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Background. Mature B-cell malignancies are currently diagnosed according to the WHO classification using a combination of pathological, cytogenetic, clinical, and immunophenotypic data. With the advent of novel disease specific treatment options reproducible, fast, cost-effective and broadly available methods for these differential diagnoses are becoming increasingly important. *Aims*. We investigated the utility of 8-color flow cytometry to exactly diagnose B-NHL entities. *Methods*. We tested panels that included markers for lineage assignment of malignant B cells in heterogeneous samples (CD19, CD20, CD22, CD37) plus markers for differential diagnosis between WHO disease entities. The differential diagnosis marker set initially included antigens associated with B cell maturation (e.g. bcl-2, CD38, CD81, CD43, CD10), associated with particular B-cell malignancies (e.g. CD5, CD103, CD23), integrins (e.g. CD11a, CD11c), and chemokine receptors (CXCR5). Testing in a total of 144 B-cell malignancies aimed to improve the power for differential diagnosis by adding novel markers for difficult to classify entities and to reduce the number of necessary markers by omitting antigens with redundant information. The final 4 tube 8-color panel version was designed using the INFINICYT software based on those 20 markers that in multivariate analyses contributed the most to differential diagnoses between any combination of two B-cell malignancies. The panel was integrated into a diagnostic work flow for mature B-cell malignancies and therefore contains a tube to orientate towards reactive conditions, and towards mature T/NK malignancies, respectively. The final panel was tested in a total of 42 mature B-cell malignancies with an established diagnosis according to current WHO standard criteria (3 hairy cell leukemia, HCL, 2 follicular lymphoma, FL, 14 typical CLL, 9 mantle cell lymphoma, MCL, 3 lymphoplasmacytic lymphoma, LPL, 5 marginal zone lymphoma, MZL, 1 atypical CLL, 5 Diffuse large B cell lymphoma DLBCL). *Results*. The combination of CD19 and CD20 are necessary and sufficient for lineage assignment in B-cell malignancies, whereas CD22 and CD37 did not additionally contribute. Except for some overlap between FL and DLBCL cases, all the remaining entities could be clearly differentiated in 1 x 1 comparisons using the fluorescence intensities of the 20 markers and multivariate analyses algorithms of INFINICYT software. Furthermore, we developed a diagnostic algorithm that allowed to classify all CLL (14), HCL (3), and MCL (9) cases. With two exceptions (1 MZL, 1 DLBCL) all remaining 14 DLBCL, FL, MZL, atypical CLL and LPL cases formed discrete clusters thus predicting that they will be classifiable based on immunophenotypic information only. Current data therefore predict an overall power of 8-color flow cytometry to diagnose B-NHL entities in 95.2% of cases (40/42). *Summary and Conclusions*: We conclude that our extensively tested 4-tube 8-color panel in combination with INFINICYT software algorithms is likely to greatly improve the diagnostic precision of flow cytometry to classify B-NHL entities solely based on immunophenotype.

0671

ALTERATIONS OF CHEK2 GENE IN NON-HODGKIN LYMPHOMA PATIENTS

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Background. The risk of non-Hodgkin lymphoma (NHL) development is modified by genetic background. Numerous studies have evaluated the

risk of NHL in carriers of alterations in genes coding for proteins implemented in various cellular functions (e.g. immunomodulation, detoxification, oxidative stress response, DNA repair). Checkpoint kinase 2 (CHK2) participates on regulation of DNA double-strand break (DSB) repair. The CHK2 protein is responsible for activation of several key effector proteins involved in DSB repair, cell cycle arrest, or apoptosis. These processes could be negatively influenced by inactivating mutations in CHK2 gene (CHEK2). Carriers of CHEK2 mutations are at increased risk of several cancer types development (e.g. colorectal, breast, and prostate cancer), but the relation to the NHL remains unclear. *Aims*. We performed mutation analysis in order to evaluate the risk of NHL development in CHEK2 mutation carriers. *Methods*. Mutation analysis of whole coding sequence of CHEK2 gene was performed in 185 patients with NHL, mainly with diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). Genomic DNA was isolated from peripheral blood of patients that signed approved informed consent prior genetic testing. Individual exons were PCR-amplified and analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic). Samples with aberrant elution profiles were sequenced from independent amplification (ABI 3100; Applied Biosystems). The large rearrangements of CHEK2 gene were analyzed by multiplex ligation-dependent probe amplification (MLPA) method (MRC Holland). The population frequency of new alterations was estimated in population of non cancer controls. *Results*. The CHEK2 region (exons 2 a 3) coding for highly conservative fork head associated (FHA) domain was shown to contain several gene alterations [c.470T>C (I157T), c.542G>A (R181H), IVS1-5T>A, IVS2+1G>T]. Frequency of alterations in this region was significantly higher in NHL patients (5.95%; 11/185) than in controls (2.78%; 19/683) with OR = 2.2 (95% CI 1.03-4.73; p=0.04). Even more significant result was found in subpopulation of FL patients (8.6%; 5/58; OR = 3.3; 95% CI 1.18-9.18; p=0.02). Frequency of alterations in exon 1 c.122C>T (S41F), c.252A>G (E84E), IVS1+39dupA was the same in control population as in lymphoma cases. Two new alterations were characterized in NHL patients (IVS4-78_-100dup23 and IVS10+28A>G). We have analyzed 160 samples from lymphoma patients by MLPA, but no large rearrangement was found. *Conclusions*. Inherited CHEK2 alterations in FHA-coding region could increase the risk of NHL development, especially of FL. Large genomic rearrangements are rare and, therefore, do not play significant role in NHL pathogenesis. Clinical and pathological characteristics will be correlated with individual genotypes.

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0672

DISTINGUISHING OF PRIMARY MEDIASTINAL B-CELL LYMPHOMA AND DIFFUSE LARGE B-CELL LYMPHOMA USING REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

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Background. Primary mediastinal B-cell lymphoma (PMBL) is a subset of diffuse large B-cell lymphoma (DLBCL) with better clinical outcome. These two units can be reliably distinguished only with microarray technology. 24% of patients with clinically expected PMBL diagnosis did not carry the PMBL gene signature when microarrays were used. These patients fell into the DLBCL group and tended to have adverse prognosis. 5% of patients with expected DLBCL were genetically assigned as PMBL ones (Rosenwald, J Exp Med, 2003). *Aims*. Our aim was to help to distinguish PMBL and DLBCL using real-time quantitative polymerase chain reaction (RTqPCR) and routinely available formalin fixed, paraffin embedded material (FFPE). *Methods*. RNA was successfully isolated from 99% (84/85) of originally obtained paraffin blocks. Finally, 82 patients with available clinical data were included in the study. For 39 of them the diagnosis of PMBL was expected (mediastinal bulk > 7cm, histopathology). All the cases were reviewed and the clinical-pathological diagnosis was verified. Based on the published microarray data, CD23, Pdl2 and Blk genes were chosen as potential discriminators between PMBL and DLBCL. Expression of these genes was measured using RTqPCR and ΔΔCT method. *Results*. Testing set included 32 patients. For 11 and 21 of them PMBL and DLBCL diagnosis, respectively, was expected clinical-pathologically. Expression of CD23, Pdl2 and Blk genes was measured and a mathematical formula was established to distinguish both the entities. The formula was verified on a validation set consisting of 50 patients including 28 patients for whom PMBL diagnosis was clinical-pathologically expected. In the validation set, 9 out of 28 of expected PMBL patients were classified as DLBCLs and all 22 expected DLBCLs were confirmed using the formula. When the approach was applied on the whole group of patients the following results were obtained. For 2 out of 43 patients (5%) with clinical-pathologically expected DLBCL the diagnosis was not genetically confirmed. 10 out of 39 patients (26%) with clinical-pathologically expected PMBLs fell into the DLBCL group. Compared to genetically confirmed PMBLs, these discrepant PMBL ones showed some clinical features which were more similar to the DLBCL diagnosis, e.g. more frequent spleen infiltration (p=0.028) and decreased local invasiveness in pericardium (p=0.045). They tended to have worse clinical outcome, more common infradiafragmatic involvement, they had less often tumor sclerosis, fluidothorax, or chest wall involvement and none of them had pleura involvement. *Summary and Conclusions*. A new approach of distinguishing of PMBL and DLBCL patients is presented. It is based on expression of CD23, Pdl2 and Blk genes. Moreover, RTqPCR method and routinely available FFPE material were successfully used.

0673

BIOLOGIC EFFECTS OF P53 ACTIVATION BY NUTLIN-3A IN ALK+ALCL WITH UNMUTATED, MUTATED/PARTIALLY FUNCTIONAL AND MUTATED/NONFUNCTIONAL P53 GENE

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Background. p53 is frequently expressed, but infrequently (<10%) in anaplastic kinase-positive (ALK+) anaplastic large cell lymphoma (ALCL) tumors. Nutlin-3a is a recently developed small molecule that targets Mdm2, a critical negative regulator of p53, and disrupts the p53-Mdm2 interaction resulting in p53 stabilization and activation. *Aims.* To investigate the effects of nutlin-3a on p53-dependent apoptosis and the cell cycle in ALK+ ALCL cells with wild-type (wt), mutated / partially functional (mt-pf) or mutated / nonfunctional (mt-nf) p53 gene. *Methods*. The mutation status of the p53 gene was investigated in 4 ALK+ALCL cell lines by direct sequencing of the entire open reading frame of p53 gene following cDNA amplification. ALK+ALCL cell lines carrying wt, mt-pf and mt-nf p53 gene were treated with increasing concentrations of nutlin-3a,TRAIL, CH11, pifithrin-α, pifithrin-μ, and the chemotherapeutic agent doxorubicin. The biologic effects of these treatments were evaluated using standard cell viability, apoptosis and proliferation assays. Western blot analysis was used to assess the levels of apoptosis and cell cycle-regulating proteins before and after nutlin-3a treatment. *Results*. Nutlin-3a activated p53 in ALK+ALCL cells carrying wt or mt-pf p53 gene resulting in p53-dependent cell cycle arrest and apoptosis. Cell cycle arrest was associated with up-regulation of the cyclin-dependent kinase inhibitor p21. Nutlin-3a-induced apoptotic cell death was accompanied by Bax and Puma up-regulation, down-regulation of Bcl-xL and survivin, and caspase-3 cleavage. Nutlin-3a-induced apoptosis was reduced significantly when p53-dependent transactivation activity was inhibited by pifithrin-α, or inhibition of direct p53 targeting of mitochondria by pifithrin-μ was employed. Nutlin-3a sensitized the activation of the extrinsic apoptotic pathway in wt-p53 ALK+ALCL cells, in part through up-regulation of DR-5 and downregulation of cFLIPS/L, and synergized in apoptotic induction with TRAIL. Also, nutlin-3a treatment enhanced the cytotoxicity of doxorubicin against ALK+ALCL cells harboring mt-nf p53 gene, which was associated with upregulation of p73. *Conclusions*. These data suggest that non-genotoxic agents like nutlin-3a offer a novel therapeutic approach for AKL+ALCL patients harboring wt- or mt-p53 gene.

0674

THE HISTONE DEACETYLASE INHIBITOR ITF2357 (GIVINOSTAT) PROMOTES BURKITT'S LYMPHOMA CELL LINE DEATH MODULATING MICRO-RNA AND TISSUE TRANSGLUTAMINASE 2 EXPRESSION

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Background and Aims. The significant toxicities associated to the conventional treatments of Burkitt's lymphoma (BL) are leading to the identification of novel targets for effective but less toxic therapies. While c-myc overexpression and Epstein-Barr virus (EBV) infection are the hallmarks of BL, how they complement one another and what other factors intervene in oncogenic process is yet to be determined. Recent studies support the existence of a c-myc-microRNA (miRNA) interaction within the genesis and the maintenance of the lymphoma phenotype. Furthermore, myc oncoproteins have been found to inhibit the transcription of tumor suppressor genes, including tissue transglutaminase 2 (TG2), a multifunctional protein that promotes apoptosis and differentiation in normal tissues. This can occur by recruiting histone deacetylase (HDAC) 1 proteins to target genes. Therefore, we tested ITF2357 (Givinostat), a new hydroxamate inhibitor of HDAC, on BL cell lines with respect to its effects on cell viability and on c-myc mRNA, miRNAs and TG2 expression modulation. *Methods and Results*. ITF2357 induced late and early apoptosis respectively on Namalwa and Raji BL cell lines, exhibiting an IC50 of 200nM after 48h from drug administration. Accordingly, ITF2357 induced subG1 peak formation in Namalwa and G1 arrest in Raji cells. Notably, c-myc mRNA decreased only in Namalwa cells after treatment. ITF2357 treated Raji cells instead showed a gradual increase of c-myc mRNA, however paralleled by a reduction of c-myc protein. The profound miRNA modulations, particularly evident in treated Raji cells as assessed by array analyses and quantitative real-time PCR, could explain these apparently inconsistent observations. MiR-155 and miR-98, known for their oncogenic activity in myc-associated tumors, were significantly down regulated after treatment. Conversely, ITF2357 induced the expression of Let-7a, which has been shown to negatively affect c-myc at posttranscriptional level. Finally, immunohistochemical analysis revealed an increased cytoplasmatic expression of the tumor suppressor TG2 in ITF2357-treated Raji cells, compared to their untreated counterparts. *Conclusions*. ITF2357 demonstrated potent cytotoxic and growth inhibitory activities on BL cell lines. These effects might be related to the restoration of the expression of different c-myc targets, including oncogenic and tumor-suppressing miRNAs and the tumor suppressor TG2. Accordingly, the reversion of c-myc abnormal expression was achieved in both BL cell lines studied. The potential candidacy of ITF2357 as a therapeutic agent for BL awaits further evidences from ongoing analyses in BL primary tumors and in animal models.

0675

DIFFERENTIAL EXPRESSION OF SIN1 GENE PRODUCT IN NON-HODGKIN AND HODGKIN LYMPHOMA CELL LINES AND TUMORS

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Background. The novel mammalian stress activated protein kinase (SAPK) interacting protein 1 (Sin1) gene give rise to multiple isoforms through alternative splicing. Recent studies provide evidence that Sin1 is a key component of the mammalian target of rapamycin (mTOR)-Rictor complex (mTORC2), thus critically regulating the Ser473-phosphorylation / activation of AKT kinase that results in tumor cell survival through multiple downstream targets. Moreover, in the absence of Sin1 gene, mTOR-Raptor (mTORC1) activity is upregulated upon stimulation of the PI3K/AKT/mTOR pathway. Previous studies have shown that the AKT/mTOR oncogenic pathway is activated in many cancers including aggressive non-Hodgkin lymphomas (NHL) and Hodgkin lymphomas (HL). However, the potential role of Sin1 protein in the pathogenesis of NHL and HL is yet unknown. *Aims*. To investigate the expression patterns of Sin1 protein and its isoforms in various NHL and HL cell lines and tumors. *Methods*. The expression of Sin1 gene products was assessed by Western Blot analysis in 13 NHL and HL cell lines and 10 fresh-frozen specimens of NHL of various histologic types. In addition, expression and subcellular localization of Sin1 protein was evaluated using immunohistochemical methods, a tissue microarray (TMA) and duplicate tumor cores from 35 specimens obtained prior to treatment, which included reactive lymph nodes, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), marginal zone lymphoma (MZL), diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL), peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS), anaplastic large cell lymphoma (ALCL) and classical HL. The monoclonal antibody used was specific for Sin1 and capable of detecting Sin1 isoforms 80, 76 and 55 kDa. *Results*. Sin1 isoforms 80, 76 and 55 kDa were detected in immunoblots at a variable level in NHL and HL cell lines tested. A reverse correlation between Sin1 protein levels and activation status of downstream targets of the mTORC1, such as phosphorylation of 4EBP1 and rp-S6 as well as total eIF4E was observed with the exception of Granta 519 (MCL) cells. This association was also seen in primary NHL tumor specimens analyzed by Western blot. Immunohistochemical analysis of Sin1 protein revealed that Sin1 was localized in both cytoplasm and nucleus of tumor cells and was expressed at a high level in many NHL types including CLL/SLL, MCL, FL, MZL and a subset of DLBCL. By contrast, A subset of DLBCL, BL and HL expressed relatively lower levels of Sin1 (p=0.007, chi-square test). The expression levels of Sin1 inversely correlated with the levels of 4EBP1 phosphorylation confirming the western blot data. *Conclusions*. Sin1, a critical regulator of mTORC2 integrity and activity, is differentially expressed among NHL and HL tumors and seems to be inversely associated with mTORC1 activation. Additional mechanistic studies are required to shed light on the relative biologic significance of Sin1/ mTORC2 activation in various NHL and HL types, which would provide novel targets for investigational therapies.

0676

SELECTION OF MARKERS FOR IDENTIFICATION OF DISEASE-SPECIFIC PHENOTYPES FOR DIAGNOSIS AND MONITORING OF B-LYMPHOPROLIFERATIVE DISORDERS BY FLOW CYTOMETRY

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Background. The identification of a disease-specific phenotype for CLL has greatly improved the rapidity and simplicity of diagnosis and monitoring. Most other B-lymphoproliferative disorders (B-LPD) do not have a disease-specific phenotype. *Aims*. To perform a comprehensive assessment of the cell surface phenotype in B-LPD and identify suitable markers for each disease category. *Methods*. 66 surface markers were assessed on 12 B-LPD cases. Expression levels were compared using dChip software (http://biosun1.harvard.edu/complab/dchip) and 22 markers with ≤1.5-fold difference between WHO disease categories were excluded from further analysis. The selected markers were then tested on 48 cases and 12 markers were excluded as before, with two (CD43 and CD200) added. The 34 selected markers were further tested on 101 cases and 4 markers were excluded. The remaining 30 antigens were tested on a large series of 1415 cases using 6-colour cytometry. A training set was identified comprising 300 representative cases with a complete diagnosis available from the sample. Three classification approaches were tested: linear discriminant analysis, decision tree analysis, and an algorithm approach, similar to the "CLL score" but based on expression levels derived machine learning vector analyses rather than ± discrimination. Results were defined as an exact match where there was complete concordance between the immunophenotypic classification and WHO classification, a partial match if the phenotype correctly indicated a germinal centre (GC) or non-GC origin of the neoplastic B-cells and a mismatch if GC status was incorrect or if normal B-cells were classified as having a B-LPD phenotype or vice-versa. *Results*. Highly specific and sensitive phenotypes were identified for Burkitt Lymphoma, CLL and Hairy Cell Leukaemia. Differential diagnosis between CLL and Mantle Cell Lymphoma was greatly improved. It was also possible to accurately classify over 70% of Follicular Lymphoma and 60% of Marginal Zone Lymphoma cases with <5% incorrect classification. In addition to standard markers such as CD5, CD10, CD79b and CD103, infrequently used markers such as CD200, LAIR-1, CD31 and CD39 were extremely powerful for classification. Linear discriminant analysis gave an exact match in 80% of cases, and a mismatch in 15%; decision tree analysis an exact match in 79% and a mismatch in 16%; and the algorithm approach an exact match in 66% with a mismatch in 3%of cases. In 204 bone mar-

row samples with no morphological evidence of disease, the B-cell phenotype either confirmed no evidence of infiltration or correctly identified the diagnosis made on subsequent tissue biopsies in 20% of cases using the algorithm, 47% with the decision tree and 65% using linear discriminant analysis. *Summary and Conclusions*. Specific immunophenotypes are identifiable for most B-LPD but this requires ~20 markers and is best suited to 6/8-colour flow cytometry. Statistical approaches to classification are more effective than human-designed algorithms, particularly in samples with low-level infiltration, and ideally should be incorporated within the flow cytometry analysis software. These markers have now been extensively tested by the EuroFlow Consortium using Infinicyt software that incorporates multivariate analysis of data. Such approaches will potentially allow significant improvements in the diagnosis of B-lymphoproliferative disorders.

0677

MUTATION AND SINGLE NUCLEOTID POLYMORPHISM ANALYSIS IN HUMAN HERPESVIRUS 8-ASSOCIATED PRIMARY EFFUSION LYMPHOMA

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Background. Human Herpesvirus 8 (HHV-8)-associated primary effusion lymphoma (PEL) is a rare non-Hodgkin lymphoma often associated with Epstein-Barr virus (EBV) infection. Somatic mutations of tumour suppressor genes and oncogenes are among the most common genetic alterations found in human cancers. Moreover, single nucleotide polymorphisms (SNP) in genes involved in apoptosis or cell cycle regulation have been shown to correlate with an increased risk of cancer development, an accelerated cancer onset, a poor response to treatment or a shorter survival. *Aims*. As mutations of PTEN, PIK3CA, CTNNB1/β-catenin genes, deletion of CDKN2A-ARF (p14ARF-p16INK4a) locus and SNP had never been investigated in primary PEL tumours, we performed an extensive molecular analysis of mutations and SNP in a large series of PEL. *Methods*. Mutations in TP53, PTEN, PIK3CA, CTNNB1/β-catenin genes, deletion of CDKN2A-ARF locus, SNP72 and ins16bp in TP53, SNP309 in MDM2, S31R and 3'UTR (c70t) in CDKN1A/p21Cip1, V109G and 5'UTR (c79t) in CDKN1B/p27Kip1, g870a in CCND1/cyclin D1, A259S in CCND3/cyclin D3, F31I (t91a) in STK15/aurora A, R70C in CDC25C, A655V in CDC2L1 and I441V in CDC6 genes, were investigated in seventeen primary PEL tumours collected from sixteen patients and seven PEL cell lines, using PCR and sequencing. *Results*. TP53 gene mutations were detected in two primary PEL samples (11.8%) and two PEL cell lines (28.6%). BCBL-1 was found to harbor a heterozygous M246I mutation of TP53. BCP-1 contained two missense mutations leading to single nucleotide changes (M246V and D259N) in both alleles of TP53. PTEN gene alterations were identified in two PEL cell lines, associated with a loss of PTEN protein expression in both cases (Figure).

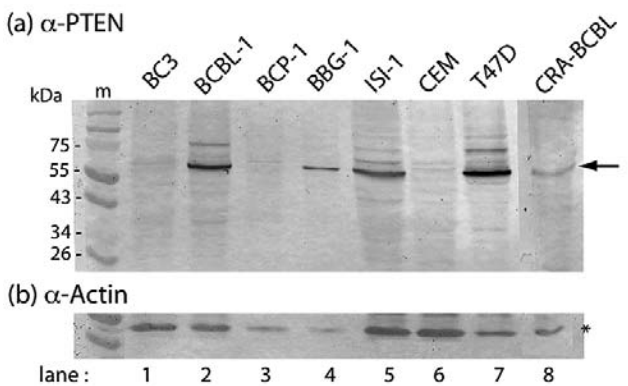

Figure.

BC-3 carried a monoallelic 2 bp-deletion in PTEN exon 7 leading to a non-sense mutation at codon 250. BCP-1 harbored a homozygous deletion of PTEN exons 6 through 9. No mutations were detected in PIK3CA and CTNNB1/β-catenin hotspot sequences. Only BC-3 contained a homozygous deletion of CDKN2A-ARF locus. The mutation rate was found to be significantly higher in EBV-negative PEL (5/12) compared to EBV-positive PEL (0/12, p=0.037). Considering the sixteen patients with PEL, the CDKN1A/p21Cip1 S31R, CDKN1B/p27Kip1 V109G and CDC2L1 A655V polymorphims were found to be significantly associated with an African origin (p=0.033, 0.002 and 0.007, respectively), the CDKN1A/p21Cip1 c70t polymorphism with the presence of a HHV-8-associated multicentric Castleman disease (p=0.015) and the CCND3 A259S polymorphism with Human Immunodeficiency Virus type-1 infection and EBV status of PEL (p=0.001 and 0.015, respectively). No correlation could be found between these SNP, the age of patients at the time of PEL diagnosis and their survival from the date of PEL diagnosis. *Conclusions*. Although detected at a higher frequency in PEL cell lines (3/7) than in primary PEL tumours (2/17), TP53 and/or PTEN gene mutations as well as deletion of CDKN2A-ARF locus are uncommon in PEL, and are found to be restricted to the EBV-negative PEL tumours. No mutations were detected in PIK3CA and CTNNB1/β-catenin hotspot sequences, suggesting that other mechanisms are involved in the pathogenesis of HHV-8-associated PEL.

0678

FOCUSED MICROARRAY FOR LYMPHOMA DIAGNOSTICS

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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, especially in Burkitt's lymphoma. Neither prognostic markers such as the International Prognostic Index (IPI), although highly useful, do not capture all the variability that affects clinical behaviour of lymphomas. The genome-wide transcriptional profiling was reported to accurately define the biological phenotype of the tumor. *Aims*. The aim of the project was to design novel focused oligonucleotide microarray directed at molecular diagnostics and prognostication of lymphoproliferative disorders, particularly non-Hodgkin lymphomas, and to test its reliability on a cohort of newly diagnosed lymphoma samples. *Methods*. We designed custom oligonucleotide microarray (Agilent 8x15K custom array) carrying specific probes for approximately 4000 genes. The genes represented on the microarray were selected on the basis of previously published lymphoma/leukemia gene expression profiling studies. In addition, probes for the genes implicated in crucial cellular processes such as apoptosis or cell cycle control were added. To provide more information, the genes for the majority of CD antigens and'-h*ousekeeping* genes were also included in the microarray design. 67 histologically characterised samples were analysed - 18 Diffuse Large B-cell Lymphomas (DLBCL), 34 Follicular Lymphomas (FL), 3 Burkitt's Lymphomas (BL), 1 MALT Lymphoma, 6 non-malignant lymph-nodes and 3 lymphoma cell lines (SU-DHL-4, WSU-NHL, RAMOS). RNA was obtained either from fresh-frozen lymph-node resections, or from RNAlater preserved needle biopsy samples. *Results*. Cluster analysis revealed following major clusters: a) BL and cell lines, b) DLBCL and c) FL and non-malignant lymph-nodes. The BL branch was clearly distinct and contained only BL and cell line samples, whereas the DLBCL and FL clusters show higher similarity with one another. Statistical analysis of differentially expressed genes among the NHL subtypes revealed tens of significant genes. The indolent subtype (FL) shows higher expression of genes specific for infiltrating cells (e.g. CD3D, CD28, CD7) in comparison to aggressive subtypes (DLBCL and BL). On the other hand, the genes implicated in cell cycle progression and cell proliferation (e.g. cyclins A and E, CDC6, CHEK1) were highly expressed in aggressive subtypes. In the DLBCL sample group, the Activated B-cell like (ABC) and Germinal Centre like (GC) molecular subtypes can be distinguished with our focused microarrays. As the most important finding, we confirmed the characteristic gene expression signature of Burkitt's lymphoma that may help in the diagnosis of histopatologically disputable cases. *Summary*. We demonstrated the benefit of gene expression profiling using novel designed focused microarray for non-Hodgkin lymphoma characterisation. The technology is robust, less expensive compared to whole-genome approach and still capable to retain important information.

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0679

DETECTION OF HYPERMETHYLATION OF TUMOR SUPPRESSOR GENES IN OCULAR ADNEXAL LYMPHOMA USING MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

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Background. Ocular adnexal lymphomas (OAL) occur in the orbit, lacrimal drainage system, conjunctiva and eyelid. They comprise 8% of all extranodal non-Hodgkin lymphomas (NHL) with the extranodal marginal zone B-cell lymphoma (EMZL) being the most common, accounting for 2/3 of them. Hypermethylation in CpG sites in promoter regions is an epigenetic change that could lead to silencing of a gene. Hypermethylation in tumor suppressor genes (TSGs) occurs in various tumors, including NHL. *Aims*. We examined the methylation status of multiple TSGs in OAL, comparing EMZL with non-EMZL. *Materials and Methods*. Formalin-fixed paraffin-embedded (FFPE) OAL, including both EMZL and non-EMZL OALs, were examined. DNA was extracted and purified from FFPE, and only those with DNA of sufficient quantity and quality were selected for subsequent analysis using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). 33 EMZL and 27 non-EMZL were examined with two MS-MLPA kits, in which methylation status of CpG sites in a total of 35 candidate TSGs were analyzed. Reactive lymphoid hyperplasia cases were used as controls. MLPA PCR products are separated by capillary electrophoresis, and sequencing data were statistically analyzed with specifically designed Excel spreadsheets. CpG hypermethylation in patient samples was determined when statistical significance of standard error > 0.1 as compared to the reference samples. *Results*. From MLPA, specific loci in eight TSGs including CDH13, WT1, MSH6, IGSF4, DAPK1, ESR1, p14-ARF and RAR-β showed hypermethylation in at least 65% of the 33 EMZL OALs, as well as in majority of the non-EMZL OALs. However, hypermethylation status varies among different subtypes of non-EMZLs that includes follicular, diffuse-large-B-cell and Mantle cell lymphomas. Validation of this data is in progress using methylation enriched pyrosequencing. *Conclusions*. Many OALs demonstrated hypermethylation. Correlation of methylation analysis data with clinical presentation and follow-up may reveal epigenetic markers of prognostic value in these tumors.

0680

DETECTION OF PLASMACYTOID (PLASMACYTIC) DIFFERENTIATION IN MARGINAL ZONE B CELL LYMPHOMAS (MZL) BY CD138 EXPRESSION

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Background. MZL are usually characterized by a morphological heterogeneous lymphomatous population. The occurrence and frequency of plasmacytoid (plasmacytic) differentiation as well as its clinical impact have not been precisely defined yet. *Aims*. To evaluate the occurrence of plasmacytoid (plasmacytic) differentiation in MZL and to correlate these findings with clinical and laboratory characteristics as well as survival. *Methods*. We analyzed 71 MZL patients diagnosed and followed in our Departments. Among them, 37 were splenic MZL (SMZL), 20 non-gastric mucosa-associated lymphoid tissue (MALT) lymphomas, 7 nodal MZL (NMZL) and 7 primary bone marrow MZL (PBMMZL). The last category is not defined in the WHO classification and included cases with blood involvement and bone marrow infiltration without splenomegaly, lymphadenopathy or other extranodal localization of the disease. Anatomic localization of non-gastric MALT lymphomas were as follows: 5 salivary glands, 5 skin, 3 ocular adnexae, 3 lung, 3 Waldeyer's ring and 1 bladder. Plasmacytoid (plasmacytic) differentiation was defined on the basis of the expression of the plasma cell-related antigen CD138 by immunohistochemistry. *Results*. Tumors were all positive for CD20 and CD79a antigens. Nine out of 71 (13%) MZL cases demonstrated plasmacytoid (plasmacytic) differentiation as this was defined by a >20% CD138 positive lymphoma cells. Analytically plasmacytoid (plasmacytic) differentiation was identified in 2 (5%) of SMZL, in 4 (20%) of non-gastric MALT lymphoma, in 3 (43%) of NMZL, and in none of the PBMMZL patients. Among non-gastric MALT lymphomas only skin and salivary gland localization demonstrated plasmacellular differentiation. Correlation of plasmacellular differentiation with demographics, stage of disease, paraproteinemia, LDH, outcome and survival, on a univariate analysis, disclosed no statistically significant differences. *Summary and Conclusions*. Plasmacytoid (plasmacytic) differentiation in MZL as a whole is not a common finding. However there is significant variation between the different subgroups. NMZL and MALT lymphomas seem to display more commonly features of plasmacytoid (plasmacytic) differentiation. Furthermore in non-gastric MALT lymphomas this differentiation varies according to anatomic site of the disease.

0681

EPSTEIN BARR VIRUS PREDICTS OUTCOME IN PEDIATRIC B-CELL NON HODGKIN LYMPHOMA

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Background. In Western countries malignant non-Hodgkin lymphomas (NHLs) represent the fourth most common childhood cancer, accounting for about 6% of pediatric malignancies. The majority of pediatric NHLs derives from B cells (B-NHL) and represents primary high-grade lymphomas. The 3 most prevalent entities are Burkitt lymphoma (43%), B-lymphoblastic lymphoma (7%), and diffuse large B-cell lymphoma (13%). EBV expression as well as some apoptosis markers has been related to prognosis in adult B-NHL, but little is known in pediatric B-NHL. *Aims*. To quantitatively assess EBV, bax, bcl2, Ki67 and activated caspase-3 (casp-3a) expression in B-NHL, and to correlate each one with patients' event free survival. *Methods*. We analyze 40 pediatric B-NHL, 23 Burkitt lymphoma (BL) and 17 diffuse large B-cell lymphoma (DLBCL); age range 1 to 16 yrs, (median: 7 yrs); male: female ratio 5:3. Ki67, casp-3a, bax and bcl2 expression was assessed by immunohistochemistry, and EBV by EBERs *in situ* hybridization in formalin fixed-paraffin embedded lymph node biopsies. Results were expressed as (n° positive cells/field) / (n° total cells/field) - 100, in 10 high-power fields (x1000). *Results*. Ki67, casp-3a, bax and bcl2 were detected in 39/40 (97.5%), 34/40 (85%), 37/40 (92.5%) and 13/40 (32.5%) cases, respectively. Quantification of positive tumor cells for each cellular marker was: Ki67 1 to 97.6% (median 55), casp-3a 0 to 15% (median 1.75), bax 0 to 99.1% (median 9.65) and bcl2 0 to 70.6% (median 0). Significant positive correlation was found only between bax/casp3a (r=0.4504, p=0.0035, Spearman's correlation). The 5-year event-free survival of all patients was 65%. None of the cellular marker was associated with unfavorable outcome according to Kaplan Meier survival analysis, using each median as cut off points. Sixteen out of 40 cases (40%) showed EBERs positive hybridization (8/23 BL, 35%; 8/17 DLBCL, 47%). EBV expression was not statistically associated with Ki67, casp-3a, bax and bcl2 positive staining; however it was significantly associated with a worse event-free survival (EFS) according to Kaplan Meier Survival analysis (p=0.0155, log rank test). *Conclusions*. High expression of Ki 67 and the proapoptotic protein bax together with casp-3a, indicate that high proliferation index could trigger apoptosis, and both are essential on pediatric B-NHL development. EBV expression in pediatric DLBCL is higher than the observed in the adult counterpart. EBV could be a cofactor of both pediatric DLBCL and BL lymphomagenesis. EBV presence was statistically associated with worse EFS, and predicts highly unfavorable prognosis in pediatric B-NHL, thus it could be used as prognosis factor for this pediatric malignancy.

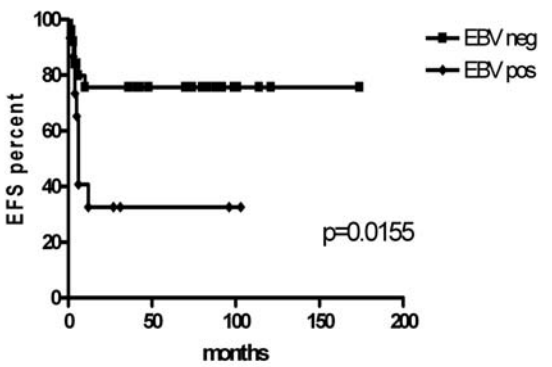

Figure 1. EFS correlated with EBV expression.

0682

EXPRESSION OF CD66ABCE ON TUMOR CELLS IN NON-HODGKIN LYMPHOMAS AND MULTIPLE MYELOMA

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Background. CD66 is expressed on myeloid cells at different stages of maturity in normal hematopoiesis, on epithelial cells, on lymphoid cell lines of T and B cell origin, in childhood acute lymphoblastic leukemias (ALL), chronic myeloid leukemias (CML) and in acute myeloid leukemias (AML). Little is known about the expression of CD66abce in non-Hodgkin lymphomas (NHL) and multiple myeloma (MM). 90 Y-labeled anti-CD66 antibody (clone BW 250/183) is a new radioimmunoconjugate developed for treatment of tumors with positive expression of CD66abce. Aim of this study was flow cytometric analysis of CD66abce expression on tumor cells in newly diagnosed untreated various types of NHL and MM to evaluate the possibility of treatment with 90 Y-labeled anti-CD66 antibody in these malignancies. *Methods*. 227 patients with newly diagnosed untreated hematologic malignancies were examined for expression of CD66abce on tumor cells in bone marrow between January 2007 and December 2008. All patients signed informed consent to participate in this study. The diagnosis of NHL or MM was confirmed histologically including bone marrow involvement in all specimens. Our group consisted of 74 B-chronic lymphocytic leukemias (B CLL), 22 mantle cell lymphomas (MCL), 18 follicular lymphomas (FCL), 15 marginal zone lymphomas (MZL), 13 lymphoplasmacytic lymphomas/M.Waldenstrom (LPL/MW), 13 diffuse large B cell lymphomas (DLBCL), 4 T NHL, 4 hairy cell leukemias (HCL), 3 B NHL not otherwise specified (B NHL NOS), 3 B ALL, and 58 MM. All 227 bone marrow samples were stained with monoclonal antibody CD66abce (clone Kat4C) fluoresceinisothiocyanate (FITC) - conjugated. 115 of 227 bone marrow samples were also stained with monoclonal antibody CD66abce (clone BW 250/183) FITC-conjugated. Expression of anti-CD66 was evaluated separately on unselected population of white blood cells and on malignant clone (positive expression ≥20% malignant cells was used as cut-off). Results are summarized in Table 1.

Table 1. Expression of CD66abce (clone BW 250/183 and clone Kat4C) in hematologic malignancies.

Diagnosis	Expression posit./negat. in bone marrow samples	
	CD66abce (clone BW250/183)	CD66abce (clone Kat 4C)
B CLL	6/30	60/14
MM	8/24	43/15
MCL	3/11	22/0
FCL	3/6	5/13
MZL	4/3	13/2
LPL/MW	2/2	13/0
DLBCL	2/4	11/2
T NHL	1/1	0/4
HCL	0/1	3/1
NHL NOS	0/2	3/0
B ALL	0/2	3/0
Total: posit./negat.	29/86	176/51

Conclusions. Positive expression of CD66abce was most frequently detected on tumor cells of B CLL, multiple myeloma, mantle cell lymphoma and lymphoplasmacytic lymphomas. In these diagnoses, treatment with radiolabelled anti-CD66 antibody would be possible. However, other clones of antiCD66abce than currently used BW 250/183 with better affinity to this antigen might be more beneficial.

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0683

SURVIVIN EXPRESSION IN AGGRESSIVE B-CELL LYMPHOMA

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Background. Survivin, a member of the Inhibitor of Apoptosis Proteins, has recently gained attention as a possible therapeutic target in neoplasia, due to its dual role both as an antiapoptotic protein and a cell cycle regulator. Survivin has been associated with an adverse outcome in several malignancies. However its prognostic significance in diffuse large B-cell lymphoma (DLBCL) is controversial. *Purpose*. To investigate Survivin expression and localization, its correlation with clinical and laboratory characteristics and its prognostic significance in DLBCL and follicular lymphoma grade III (FLGIII). Survivin was studied by immunohistochemistry in formalin-fixed paraffin-embedded tissues from patients treated and followed in our Unit. Positivity for Survivin expression was scored as follows: Nuclear Survivin was expressed as % of neoplastic cells with a positive nuclear staining. Cytoplasmic Survivin was considered positive for any % of positively stained cells in the cytoplasm and negative when all cells showed no cytoplasmic staining. *Results*. Among 66 patients [median age 63 years (17-90)], 62% were males, 39% had elevated LDH and 42% clinical stage III/IV. Histologic subtypes were: DLBCL 72%, primary mediastinal LBCL 6%, FLGIII 17% and transformed low grade lymphoma 4%. The vast majority of patients received R-CHOP. Nuclear expression of Survivin was detected in all patients at a median percentage of 11% of neoplastic cells (2-90%). Cytoplasmic Survivin expression was evident in 38% of the patients. Levels of nuclear Survivin expression were significantly lower in FLGIII compared to other subtypes (p=0.007). Cases with a high percentage of cells with nuclear Survivin expression tended to be cytoplasmic Survivin negative (p=0.11). There was no other significant correlation between nuclear or cytoplasmic Survivin expression and baseline patients' characteristics. 5-year failure free survival was 56% for all patients. Conventional prognostic factors were valid in this patient population. When FLGIII were excluded, higher nuclear survivin expression tended to correlate with inferior FFS at various cut-offs (25-50%), although differences were not statistically significant. Interestingly the presence of any cytoplasmic survivin was associated with superior FFS (5-year rates: 93 vs 50%, p=0.01). *Conclusions*. Our preliminary data show that Survivin is expressed in the nucleus of neoplastic cells in aggressive B-cell lymphomas. Moreover its prognostic significance may vary according to its nuclear or cytoplasmic localization.

0684

BCL-2, BCL-6 AND MYC REARRANGEMENTS AND CLINICAL OUTCOME OF DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of non-Hodgkin lymphoma (NHL) with clinical, morphological and biological features not well defined. Novel prognostic markers are needed to predict the outcome of patients with DLBCL; in this context, molecular and cytogenetic features may be helpful. The prognostic value of BCL-2, BCL-6 and MYC rearrangements has been investigated in several studies of DLBCL, but the results are still controversial. *Aims*. To study correlation between BCL-2, BCL-6 and MYC rearrangements and the clinical features in 64 patients with DLBCL. *Methods*. We performed a retrospective, single centre study on 64 patients diagnosed of DLBCL: 53 *de novo*, 6 transformed from follicular NHL and 5 transformed from not-specified low grade NHL. All patients were diagnosed and followed-up in our centre in a period 2003-2008. Clinical data were obtained from clinical records. The FISH study was performed in paraffin-embedded tissue and BCL-2, BCL-6 and MYC FISH DNA split-signal probes (Dako, Denmark) were used. *Results*. BCL-

2 rearrangement was found in 13/64 (20.3%), BCL-6 in 15/64 (23.4%) and C-MYC in 5/64 (7.8%) of analyzed patients. BCL-2 rearrangement was present in 6/6(100%) of patients with DLBCL transformed from follicular NHL and in 7/53 (13.2%) DLBCL *de novo*. BCL-2 rearrangement was absent in DLBCL transformed from other type of low grade NHL. BCL-6 rearrangement was found in 13/53 (24.5%) DLBCL *de novo* and in 2/5 (40%) in DLBCL transformed from other type of low grade NHL. There was no case of BCL-6 rearrangements in DLBCL transformed from follicular NHL. MYC was detected in 1/6 (16.7%) in DLBCL transformed from follicular NHL and 4/53 (7.5%) in DLBCL *de novo*. There were two patients with dual rearrangements: one with BCL-2 and MYC and one with BCL-6 and MYC. We observed a significant association (p=0.046) between BCL-2 rearrangement and a higher stage (III and IV) of NHL. No significant correlation was found between presence of BCL-2, MYC and BCL-6 and age, disease presentation, patient ECOG performance status, serum LDH level, response to treatment, overall survival and between BCL-6 or MYC rearrangements and stage of disease. *Conclusions*. According to our results, BCL-2, BCL-6 and MYC rearrangements have no impact on patients' prognosis. However, BCL-2 rearrangement appears to be associated with advanced-stage of disease. This finding and the rarity of BCL2 rearrangement in *de novo* DLBCL, raises the question about the possibility of these lymphomas representing a transformation from previously non-diagnosed follicular NHLs.

Transfusion medicine

0685

RHD GENE POLYMORPHISMS IN ALLOIMMUNIZED D-NEGATIVE INDIVIDUALS WITH HIGH RATE OF RACIAL ADMIXTURE

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Background. The D-negative phenotype is the result of the total RHD gene deletion in almost all Caucasians, but it accounts for only about 20% in Africans and 70% in Asians. In Africans the RHDæ that is an insertion of 37bp in exon 4 is one of the most important causes of the D-negative phenotype. *Aims*. Considering that few studies have investigated the RHD alleles in alloimunized D-negative individuals with high rate of racial admixture (Caucasians, Africans and Asians), in this study we investigated the RHD polymorphisms in D-negative phenotype mixed Brazilians who have developed anti-D alloantibody. *Methods*. Blood samples from 130 individuals previously typed as D-negative were phenotyped again using: (a) two tube reagents (Anti-D blend reagent, Cellular line TH-28, MS-26, DiaMed Latino America, Brazil; and Anti-D polyclonal, Fresenius Kabi, Brazil); (b) one gel test ID-Card for Rh subgroups including Cw and Kell antigens (DiaMed Latino America, Brazil); and (c) ABO/Rh (ID-Card DiaMed Latino America, Brazil) according to manufacturer instructions. The presence of anti-D alloantibody was confirmed by testing patients' sera against two commercial RBC panels (DiaMed Latino America, Brazil; and Fresenius Hemocare, Brazil). All sera were shown to contain anti-D alloantibodies with title ≥64. The method used for RHD screening detected the presence of RHD exon 10 and intron 4. After amplifying exon 10 and intron 4 two PCR reactions were performed to determine the presence of other RHD gene regions. The PCR reproducing exons 2, 3, 5 and 7 of the RHD gene, resulted in 180, 117, 125 and 95bp products, respectively. The exon 2 product was used as internal control of the reaction, as it is found in both RHD and RHCE genes. Sequence analysis was performed on PCR products amplified from genomic DNA for all 10 exons RHD gene. *Results*. We found that 118/130 (90.8%) of D-negative tested individuals had total RHD gene deletion, while 12/130 (9.2%) showed RHD gene polymorphisms. The RHDæ was found in 10 (7.7%) individuals who had the insertion of 37pb between the end of intron 3 and exon 4 and the mutations 609 G>A, 654 G >C, 667 T>G, 674C>T, 807 T>G. One sample (0.77%) hybrid RHD-CE-Ds /RHDψ, and another (0.77%) weak D type 4.2 were characterized due to mutations 186 G>T, 410 C>T, 455 A>C, 1025 T>C / 609 G>A, 654 G >C, 667 T>G, 674C>T, 807 T>G + the insertion of 37pb and 602 C>G, 667 T>G, 957 G>A, 1025 T>C, respectively. *Conclusions*. The presence of the RHD gene in D-negative subjects has been shown to occur in 27.7% of Japanese, in 26.0% of Koreans, and more than 80.0% of Africans. Our results showed that the RHD gene was present in 9.2% of racially mixed Brazilians who produced clinically significant anti-D alloantibodies. Therefore, the data showed that careful attention is necessary for clinicians in applying RhD genotyping to transfusion medicine in populations with high rate of racial admixture. The highly frequent discrepancies between phenotypes and RHD genotypes, specially RHDæ, urge for improvements in imunohematological tests applied for transfused mixed populations.

0686

ACTIVE HEMOVIGILANCE OF PEDIATRIC PATIENTS SUPPORTED WITH PLASMA COMPONENTS PREPARED WITH PHOTOCHEMICAL TREATMENT

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Background. Platelet components photochemically pathogen inactivated using the INTERCEPT Blood System™ have been transfused in routine clinical practice to a broad patient population in Europe for 5 years. Transfusion of INTERCEPT platelets to pediatric patients has been shown to be safe and effective (Vox Sang 2006;91(S3):177). The same INTERCEPT Blood System was CE Marked in 2006 for pathogen inactivation of plasma components and has been in clinical use for 2 years. An active hemovigilance program was implemented to gather safety

information on routine transfusion of INTERCEPT plasma components (IPL). *Aims*. This ongoing study evaluated the transfusion safety profile in pediatric patients receiving IPL. Results of 1,029 transfusions in pediatric patients including infants are reported. *Methods*. Centers participating in this study produced IPL (approximately 200 mL per component) in routine practice. Plasma transfusions were ordered by primary care physicians per standard of care. This study assessed the response to all study IPL transfusions within the first 24 hr. The primary endpoint was the incidence of acute transfusion reactions (ATR). An ATR was defined as an adverse event (AE) possibly related, probably related, or related to the plasma transfusion. For each transfusion, patient demographics and primary diagnosis/therapy were recorded regardless of whether an AE was observed. For AEs, the following data were collected: time of event following transfusion, clinical description, objective clinical parameters (vital signs), results from clinical and laboratory tests (radiographs, bacterial cultures), severity (grade 0-4), serious or non-serious nature, and causal relationship (unrelated, probably unrelated, possibly related, probably related, or related). *Results*. To date 1,029 transfusions, comprised of 1,641 IPL, have been administered to 348 pediatric patients (61.8% male, 38.2% female). The mean age was 4.2 years (160 children between 1-18 years, 188 infants <1 year). Of the 348 patients, 109 (31.3%) received IPL transfusion for a hematologic disorder (including 3 with congenital coagulation deficiency, 101 with acquired coagulopathy, and 2 with TTP), 83 (23.9%) for surgery, 156 (44.8%) with another diagnosis as an indication for plasma transfusion. Average number of transfusions (txn) per patient was 3.0 txn (range 1-55, median 2.0). Each patient received a mean of 4.7 IPL (range 1-99, median 2.0). Compared to frequency of transfusions in infants (<1 year), older pediatric patients (1-18 years) received higher numbers of transfusions and plasma components (3.4 txn/7.0 IPL vs. 2.6 txn/2.7 IPL). Patients 1-18 years old with hematology diseases received more transfusions and plasma products (mean 4.7 txn/7.5 IPL) than other diseases. One patient with congenital coagulation deficiency and two patients with TTP received the largest number of transfusions and IPL products (Table 1). In all patients, 153 (44%) patients had previous transfusions, of which 63 (18.1%) patients received INTERCEPT platelets. Among the 1,029 transfusions, no AE, ATR, SAE, deaths or episodes of TRALI due to an IPL transfusion were reported. *Conclusions*. To date no acute transfusion reactions with IPL transfusions were reported in pediatric patients including infants. These results provide additional indication that routine IPL transfusion is safe and well tolerated in this patient population.

Table 1. Transfusion frequency per patient by age group and by diagnosis (Mean txn/mean IPL).

	All (n=348)	Hematology (n=109)	Congenital coagulopathy (n=3)	Acquired coagulopathy (n=101)	TTP (n=2)
1-18 years old	3.4/7.0 (n=160)	4.7/7.5 (n=56)	36.0/37.0 (n=1)	3.3/6.1 (n=51)	28.0/34.0 (n=2)
<1 year old[a]	2.6/2.7 (n=188)	2.7/2.7 (n=53)	1.0/1.0 (n=2)	2.8/2.8 (N=50)	-
Total	3.0/4.7	3.7/5.2	12.7/13.0	3.1/4.5	28.0/34.0

[a] Volume of IPL transfused is based on the body weight of an infant. Any transfusion with a volume up to approximately 200 mL was counted as one IPL.

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TRANSFUSION OF PLATELET COMPONENTS PREPARED WITH PHOTOCHEMICAL PATHOGEN INACTIVATION FOR SUPPORT OF SEVERE GLANZMANN THROMBASTHENIA DURING SURGICAL PROCEDURES

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Background. Glanzmann thrombasthenia (GT) is a recessive autosomal severe bleeding disorder characterized by defective fibrinogen binding and the absence of platelet aggregation to all physiological agonists. GT is due to the absence (Type 1) or dysfunction (Type 2) of the Gp IIb-IIIa complex. A large consanguineous group of Gypsies, living in Strasbourg, affected by type 1 GT has been studied in our laboratory since 1971. Due to a splice site mutation at the 5' end of Intron 15 of platelet Gp IIb (Gypsy mutation) detectable Gp IIb-IIIa is not expressed on the platelet surface. In the past, female patients from this cohort with severe hemorrhage, such as menorrhagia, have required repeated platelet component (PC) transfusion resulting in alloimmunization against Gp IIb-IIIa. The requirement of GT patients for repeated PC transfusion increases the risk of transfusion-transmitted infection. *Aims*. We evaluated hemostasis of GT patients in response to transfusion with amotosalen and UVA pathogen inactivated (INTERCEPT Blood System™) PC. *Results*. Patient 1 was a 21 year old female, (1.73m / 81kg). During the last 13 years, she received many transfusions: 33 RBCC and 40 non pathogen inactivated apheresis platelet concentrates (APC). Recently, she was diagnosed with an ovarian cyst. Laparoscopy followed by laparotomy was required because of cyst volume. Before surgery, she received 4 buffy coat platelet concentrates (BCPC), 2 more BCPC were transfused during surgery, and 2 more 2 hours after surgery. Then, she received 2 BCPC every 12 hours for 10 days (until the day after staple removal). During surgery, bleeding occurred, and she received 3 red blood cell concentrates (RBCC) and 1 unit of fresh frozen plasma (FFP). No other complications were observed. The total platelet transfusion dose was 192.2×10^{11} platelets. Patient 2 was a 22 year old female, (1.62 m/82kg), with a normal term pregnancy. Presence of antibodies to platelet Gp IIb/IIIa complex was documented since 2004. No anti-HLA antibodies were detected. Before cesarean section she received 1APC, 2 BCPC during surgery, and 2 BCPC 2 hours after surgery. Then, she was transfused with 2 BCPC every 12 hours through day 11 (until the day after staple removal). The cesarean section delivery of a normal male (3 kg) was without bleeding complications; and no complications were observed through day 11. No RBCC or FFP were required during the hospital stay. The total platelet transfusion dose was 186.3×10^{11} platelets. Patient 3 was a 6 year old boy (16.2 kg) requiring avulsion of 2 molar teeth (previously with infected abscess). He received split APC before, after and once a day for 6 days. Because he experienced a moderate urticaria during the third APC transfusion, he received Claritin just before the following transfusions that were uneventful. The total platelet transfusion dose was 12×10^{11} platelets. *Conclusions*. Intensive transfusion with INTERCEPT PC was well tolerated and supported major surgery, without significant bleeding, in 3 Type 1 GT patients. The same total platelet dose was used previously with conventional PC in similar GT patients before implementation of INTERCEPT PC. This report demonstrates effective clinical hemostasis in GT, a severe hereditary platelet disorder, using PC prepared with INTERCEPT pathogen inactivation.

0688

LOSS OF THE RED CELL B ANTIGEN AND BLOOD GROUP CHANGE IS NOT ASSOCIATED WITH METHYLATION OF THE ABO GENE PROMOTER REGION IN A PATIENT WITH 5Q- MYELODYSPLASTIC SYNDROME

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Background. Blood group change with loss of red cell (RBC) antigen may occur in solid tumors and in hematological diseases, however the mechanisms involved in these changes are uncertain. Aberrant DNA hypermethylation is thought to be involved in myelodysplastic syndromes (MDS), and the methylation of cytosines residues in the dinucleotide CpG may account for the expression patterns of the ABO genes. *Aims*. Using phenotyping and genotyping studies we investigated the possible role of DNA methylation in the ABO promoter gene in a patient with MDS presenting loss of the B antigen. *Methods*. An 80-year-old man was admitted due to acute coronary syndrome with the following laboratory results: Hb=8.2g/dL, MVC=101fl/RBC, WBC=6.1×10^9/L (neutrophils=4.2×10^9/L, lymphocytes=0.6×10^9/L, no myeloblasts), platelets=833×10^9/L. Bone marrow examination showed an increased number of dysplastic megakaryocytes with monolobulated nuclei. Karyotype analysis revealed 46,XY,del(5)(q15q33)[4]/46,XY[16] leading to the diagnosis of 5q- syndrome. The patient became transfusion-dependent, and the immunohematological tests indicated that his RBCs agglutinated with anti-B, while his serum agglutinated type A RBCs. Four months latter no agglutination was seen when his RBCs were tested with anti-A or anti-B, resulting in type O. Since anti-A was still present in his serum the patient was transfused with type O RBCs until he finally died due to heart disease. The B and H antigens expression on the RBCs surface was accessed by flow cytometry, while ABH secreted antigens were investigated in saliva. A PCR-based ABO genotyping using restriction enzyme digestion (Alu and Kpn) followed by agarose gel eletrophoresis was also performed. Methylation of CpG islands was investigated using MSP technique with methylated and unmethylated primer sets for region from -200 to +26 sequence of the ABO gene. *Results*. Flow cytometric analysis with dual staining for H and B antigens showed that the patient's RBCs had a partial positivity with anti-

H/FITC, but negative reaction with anti-B/PE. Genotyping studies showed heterozygosity for the B allele indicating the BO genotype. A macroscopic positive reaction was seen in a tube containing A RBCs, patient's saliva and anti-A, but no agglutination was seen with B RBCs, patient's saliva and anti-B, indicating the presence of the soluble B antigen in patient´s saliva. The presence of a 280bp PCR product in the unmethylated lane associated with the absence of a PCR product in the methylated lane revealed that the evaluated ABO gene promoter region was not associated with DNA methylation. *Conclusion*s. Many patients in the early stages of MDS with symptomatic anemia can be managed supportively with RBC transfusions, therefore the blood group change may augment the risk of serious blood transfusion reactions in such individuals. Using serologic techniques, flow cytometry and ABO genotyping, we demonstrated a blood group change due to loss of the B antigen in a transfusion-dependent patient with 5q- syndrome. These investigations facilitated the transfusion management of the patient who did not experience transfusion reactions. Our data also indicated that the loss of the B antigen was not associated with DNA methylation of the accessed ABO gene promoter region in this particular patient with 5q- MDS.

0689

A 10-YEAR LOOK-BACK ON USE OF CRYOPRECIPITATE IN A TERTIARY CARE HOSPITAL

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Background. Much of the evidence on use of cryoprecipitate rests on clinical experience and peer-reviewed articles, which defies our current practice of adopting evidence-based medicine. Hence it comes to no surprise that literature to this date shows variable and often inappropriate use of this blood component. *Aims*. To retrospectively assess the pattern of cryoprecipitate use at Aberdeen Royal Infirmary and affiliated hospitals and whether it complies with current practice guidelines *Methods*. A retrospective analysis of use of cryoprecipitate from 01/01/1999 till 12/31/2008 was undertaken. At Aberdeen Royal Infirmary cryoprecipitate is issued under the guidance of a hematologist or transfusion medicine specialist. All cryoprecipitate components issued are documented in the *medical officer* logbook, which includes the entry of each unit of cryoprecipitate being issued to a patient against his/her name, date of birth, unit number, indication and pre-transfusion fibrinogen. The appropriateness of cryoprecipitate transfusions was based on the British Committee for Standards in Hematology, Blood Transfusion Task Force guidelines. These guidelines state that cryoprecipitate transfusion is recommended if patient is bleeding and has a fibrinogen level that is less than 1g/l or dysfibrinogenemia. Any transfusion out with this was deemed inappropriate. There is no evidence that prophylactic transfusions prevent disseminated intravascular coagulopathy or reduce transfusion requirements. Indication was considered as 'not determined' if a pre-transfusion fibrinogen level was not available. *Results*. A total of 6534 units of cryoprecipitate were transfused in 616 events to 531 patients during the study period. Indication for cryoprecipitate transfusion was documented in 82.2% of the events. Both cardiac and non-cardiac surgeries topped the indication list for cryoprecipitate use though cardiac surgical procedures were slightly more common (21% vs.19% respectively). 61% of cryoprecipitate transfusions were deemed appropriate, 27% were inappropriate (33% which represented the highest proportion was from cardiac surgery) and 12% could not be determined. There was no significant difference on analyzing each year separately. *Summary and Conclusions*. Our study revealed results similar to previously published data with variation in the use of cryoprecipitate. In the last decade, despite the availability of specialist guidance, the misuse of this blood component has neither been prevented nor reduced. It is likely that the lack of high-quality trials evaluating the efficacy of this blood component, particularly in the setting of cardiac surgery is a major contributing factor.

0690

THERAPEUTIC PLASMA EXCHANGE (TPE) FOR THROMBOTIC THROMBOCYTOPENIC PURPURA AND HEMOLYTIC UREMIC SYNDROME USING PLASMA PREPARED WITH PHOTOCHEMICAL PATHOGEN INACTIVATION: A ONE YEAR EXPERIENCE IN ROUTINE PRACTICE IN A REGIONAL BLOOD CENTER

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Background. INTERCEPT™ Plasma (I-FFP) prepared by photochemical treatment with amotosalen and UVA light to inactivate pathogens in single plasma units was registered by Afssaps in 2007. EFS-Alsace provides blood components for approximately 2 million inhabitants in the Alsace region. EFS-Alsace implemented universal routine production of I-FFP in 2007 in place of quarantine plasma (FFP). TPE is the mainstay therapy for thrombotic microangiopathies (TMA) including thrombotic thrombocytopenic purpura (TTP) and adult hemolytic uremic syndrome (HUS). A prior randomized, controlled clinical trial of TPE for TTP demonstrated I-FFP was comparable to conventional FFP (Transfusion 2006;46: 1693). *Aims*. To further assess the efficacy and safety of I-FFP for TPE of TMA we reviewed our experience during a 1 year period of routine I-FFP use and compared it to a year before where we used quarantine plasma (FFP) for TPE. *Methods*. Patients (pts) with a clinical diagnosis of TTP or HUS received TPE (1.0 to 1.5 blood volumes) with I-FFP per conventional standard of care. The proportion of patients achieving remission (platelet count $\geq 150\times10^9$/ L for 2 consecutive days without neurologic or renal progression) and the global safety profile were assessed. Secondary endpoints were: volume of plasma used and number of TPE. *Results*. 11 pts, mean age of 45 yr, 54% female, received TPE with I-FFP. 90.9% of patients achieved remission. The mean number of TPE was 16 (median 10, range 1 - 71). The mean plasma volume infused per patient was 33.2L (median 17.4, range 1.1 - 153.8). These results are not different from those observed with FFP (see Table 1). No patients had an adverse event associated with I-FFP. There were no deaths and no reports of TRALI. No relapses were observed in treated patients. *Conclusions*. This 1 year experience with routine use of I-FFP for TPE in TMA demonstrated efficacy and safety comparable to prior experience in the treatment of TMA using conventional quarantine FFP. I-FFP was well tolerated.

Table 1. Comparison od TPE using I-FFP vs FFP.

	FFP period (n=5)		I-FFP period (n=11)	
	Number of plasma exchanges/pt	Total plasma infusion Volume/pt (L)	Number of plasma exchanges/pt	Total plasma infusion Volume/pt (L)
Mean	12.2	22.1	16	33.2
SD	11.4	21.7	20.7	44.9
Median	8	14.9	10	17.4
Min	3	7.5	1	1.1
Max	32	60.3	71	153.8
p value			0.71	0.61

0691

UNDERSTANDING TRANSFUSION OUTCOMES THROUGH CLINICAL REGISTRIES: VALIDATION OF A LINKAGE TECHNIQUE

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Background. There is evidence of substantial variation in transfusion practice, but little understanding of how this variation affects patient outcomes. While information regarding blood products transfused is required to be retained, these data have not been correlated with clinical outcomes. However, several registries gather clinical outcomes data without transfusion information. An opportunity exists to use these two sources to explore the effect of transfusion on clinical outcomes. *Methods*. Collection of transfusion data by record review or expansion of registry datasets has practical drawbacks. These data are captured by transfusion laboratory information systems (LIS). However, the validity of LIS data has not been confirmed. Prospective validation of LIS data against individual patient records was undertaken at two major clinical centres in Victoria, Australia. Data regarding all transfusion episodes were compared over seven 24 hour periods at each centre. All clinical areas, all fresh blood product types, and each day of the week were included. *Results.*Data regarding 478 units were captured; 218 centre 1 (46%), 260 centre 2 (54%), comprising 315 red cells, 71 platelets, 67 plasma and 25

cryoprecipitate units. 176 (37%) units were issued to inpatient wards including 80 (46%) to haematology/oncology; 126 (26%) to intensive care units; 73 (15%) to outpatient units including 38 (52%) to haematology/oncology day wards; 46 (10%) to operating theatres; and 25 (5%) to emergency departments. The location of transfusion was unrecorded for 32 (7%) units. 360 transfusions (75%) occurred on weekdays, and 118 on weekends (25%). Time of issue was stratified according to nursing shift: 216 day (45%), 173 evening (36%), 89 night (19). All products recorded as issued by the LIS were transfused to the expected patient. Transfusion commenced within the recommended timeframe in 77% of cases for red cells, 68% platelets, 94% plasma and 88% cryoprecipitate. Type of blood product, time of day and time of the week all influenced the length of time between issue and initiation of transfusion. *Conclusions.* Across a range of blood product types and destinations, at two different institutions, comparison of LIS data with clinical records demonstrated concordance. The difference between LIS timing data and patient clinical records reflects the expected time to transport, check and prepare transfusion but does not affect the validity of linkage for most research purposes. Linkage of clinical registries with LIS data can therefore provide robust information regarding individual patient transfusion. This enables electronic analysis of joint data sets, either periodically or continuously, to determine the impact of transfusion on clinical outcomes in particular patient populations.

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PLASMA EXCHANGE IN PATIENTS WITH TTP/HUS IN A BLOOD TRANSFUSION CENTRE: RETROSPECTIVE STUDY OF 46 CASES

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Background and Method. Plasma exchange (PEX) is effective in TTP because it removes anti-ADAMTS13 autoantibodies and replenishes the missing vWF cleaving protease. Today high clinical suspicion and less stringent diagnostic criteria are used to avoid delay in the implementation of PEX. The aim of this study is to present our experience in the treatment of TTP/HUS with PEX from 1992 to 2008. Fourty six patients, 29 females and 17 males, are included, with the median age of 42 years (range17 to 79 years). Fluctuating neurological manifestations were common, while coma and convulsions were seen in 8 patients. A patient was inadvertently transfused with platelets and latter became comatose. Two of our patients had classical adult HUS, with hemorrhagic diarrhea and anuria. ADAMTS13 levels or anti-ADAMTS13 autoantibodies were not measured in any of our patients. Contraceptives, ticlopidine and hormonal therapy for IVF were confirmed. TTP/HUS associated with pregnancy, post-partum and caesarian section, SLE, APS, surgery and AMI were encountered. There was one intrauterine death and another patient had relapsed after IVF and caesarian section, 20 years after the first episode. PEX was administered within the first 24 hours in almost all patients. Two types of apheresis machines, mainly Cobe Spectra and Hemonetics V50 were used. Microaggregate filters were used to give the replacement plasma (FFP, cryosupernatant (CSP) and liquid plasma). Despite the severity of the disease, CSP was preferred whenever available. We tried to exchange 1.5 the plasma volume for the first 3-5 sessions. PEX was continued daily until complete remission (plt >150K/μL, Ht ~30%, LDH <300u/dL) and discontinued after 3-5 additional sessions. Adjuvant therapy included corticosteroids, antiplatelet drugs, vincristine, ivIg and lately anti-CD20 monoclonal antibody, Rituximab. *Results*. Eighty percent had complete remission (CR), while there were nine deaths. All patients in deep coma died of the disease. Two patients died of severe sepsis, including a 17 yrs male with refractory disease, a history of splenectomy at age 5 for ITP, and corticosteroid and retuximab therapy for the TTP. One patient with frequent relapses who refused PEX had CR with corticosteroids and FFP infusion. We are aware of six patients who relapsed after 1 1/2 to 10 years; three patients after surgery. The number of PEX sessions ranged from 5 to 32. Complications during apheresis were hypotension, allergic reactions (one severe), catheter-associated infection, hemopneumothorax, hematoma, and arrhythmias (atrial fibrillation, bradycardia) and hypocalcemic manifestations and machine dysfunction. Circulatory overload was the main problem whenever plasma infusion was used due to delay in PEX, patient instability or machine dysfunction. Low fibrinogen levels were common whenever CSP was used. *Discussion and Conclusions*. Plasma exchange remains the treatment of choice for TTP. Coma, delay in hospital admission and initiation of PEX, and platelet transfusion are most probably adverse prognostic factors. Surgery and pregnancy are predisposing factors for primary disease and relapse. Microaggregate filters may help in minimizing severe side effects to plasma. High dose plasma infusion is not practical for the treatment of TTP, due to circulatory overload. Mechanical irritation of the heart by the tip of the catheter and infusion of Calcium-poor plasma to the vicinity of the sinus node should be kept in mind.

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ALLOIMMUNIZATION TO ERYTHROCYTE ANTIGENS IN TRANSFUSION-DEPENDENT PATIENTS WITH HAEMOGLOBINOPATHIES: AN EXPERIENCE FROM A GREEK BLOOD TRANSFUSION SERVICE

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Background. Alloimmunization to red cells (RBCs) antigens is a frequent complication among immunocompetent transfusion-dependent patients. This retrospective study analyses the incidence and the clinical significance of the presence of RBCs alloantibody in hospitalized adult patients with inherited disorders in globin synthesis. *Methods*. Data on transfusion history, alloimmunization and transfusion reactions were retrieved from blood bank records, from 2000 to 2008. The routine laboratory procedure for this cohort of blood recipients consisted of: 1/serologic detection of RBCs antigens (ABO, Rh CcDEe, Kell, Kidd, Duffy, MNS, Lewis, Lutheran, P1), 2/antibody screening using indirect antiglobulin test (IAT), 3/antibody identification with a panel of eleven different group O cells, 4/direct Coombs test (DAT). None of the patients receive extended antigen matched RBCs. Leukocyte-reduced and/or washed RBCs were given according to preceding transfusion reactions. *Results*. Among 197 adult patients, RBCs alloantibodies were detected in 29 males (15%) and 38 females (19%), with a reduced incidence the last two years (26%). The median age was 36 (range 20-77) years and the distribution of ABO, Rh D blood group is similar to this of general population. Based on underlying disease the incidence of alloimmunization was: 39% for sickle cell-B-thalassaemia, 33% for sickle cell disease, 25% for thalassaemia major and 38% for thalassaemia intermedia. The most common alloantibodies were directed against antigens of Rh system [anti-D: 8%, anti-C: 9%, anti-c: 23%, anti-E: 24%, anti-e: 1%], Kell system 31%, Duffy system 13% and Kidd system 12%. Multiple blood group alloantibodies were formed in 30 and autoantibodies in four patients. The incidence of positive direct Coombs test did not differ among patients with or without alloimmunization (52% vs 51%). Delayed hemolytic reactions were occurred in nine patients, with clinical evidence of haemolysis in four and with isolated serologic evidence in five, beside the administration of RBCs compatible with their existing antibodies. *Conclusions*. In multiply transfused patients with haemoglobinopathies the estimated prevalence of alloimmunization was 34% and the lowest incidence was observed in thalassaemia major patients. More than three quarters of alloimmunized patients would have not developed RBCs alloantibody, if they had been transfused with limited phenotype matching RBCs (ABO, Rh CcDEe, Kell). Pretransfusion matching for Kidd and Duffy antigens could enhance the reduction of alloimmunization effectively. A limited number of delayed hemolytic reactions were not prevented by extended antigen matching, probably due to a multifactorial process.

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PLATELET TRANSFUSION THRESHOLD FOR CENTRAL VENOUS CATHETER INSERTION IN ACUTE LEUKEMIAS

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Background. Central venous catheters (CVC) are prerequisite for patients receiving high-dose chemotherapy for hematological malignancies. These patients often have hemorrhagic diathesis and many centers mandate a platelet transfusion limit of 50 G/L. However, the bleeding incidence after CVC insertion in this patient setting has never been formally analyzed and there is no appropriate threshold for platelet transfusions. *Aims*. To assess the safety of a more restrictive thrombocyte tranfusion policy regarding the incidence of CVC insertion related bleeding complications. *Patients and methods*. We performed a retrospective single center analysis of all CVC insertions from 2001-2007 in patients with acute leukemia, autologous or allogeneic hematopoietic stem cell transplantations (HSCT). 596 CVC were placed in 191 patients (median age 49 years, range 18-78) for the treatment of AML (82%), APL (6%) or ALL (12%). 507 (85%) received subclavian and 89 (15%) jugular

catheters. Patients were categorized in 4 groups according to their platelet counts [I <20 (n=92), II 20-49 (n=142), III 50-99 (n=93), and IV > 100 G/L (n=261)]. *Results*. The overall bleeding rate was 186/596 (31%). 184 events were mild; only 2 patients experienced a prolonged bleeding. The hemoglobin levels 24 and 48 hours after insertion and the number of transfused RBC did not differ among patients with and without bleeding after CVC insertion. Crude event rates were similar across the platelet groups, although there was a trend toward higher bleeding rate in the lowest platelet group. In multiple logistic regression, there was a significant positive linear association between platelet count and bleeding rate when controlling for additional bleeding risk factors (concomitant heparin and NSAR therapy, DIC, fibrinogen, gender and age, diagnosis, site of CVC insertion, C-reactive protein and fibrinogen levels, and platelet transfusions). As compared to platelet counts > 100 G/L (group IV), the odds ratio for bleeding events in the lowest platelet group was significantly higher (I: OR 3.8, 95%-CI 1.4-10.6, p=0.01), whereas there was no difference in the other groups (II: 1.8, 95%-CI 0.9-3.4, p=0.08, III: 1.8, 95%-CI 1.0-3.2, p=0.06). In total 144 platelet transfusions were administered before CVC insertion, 61 (42%) of which were given in patients with platelet counts > 20 G/L. *Conclusions*. Based on these retrospective data, we recommend a platelet transfusion threshold of <20 G/L before CVC insertion. Strict adherence to this transfusion trigger is not associated with higher bleeding risk and may save more than 40% of platelet transfusions.

Table 1: Bleeding rate according to platelet count.

Platelets (G/L)	Number	Bleeding rate – N (%)	Platelet transfusion rate – N (%)
I (<20)	92	37 (41)	83 (91)
II (20-49)	142	39 (29)	54 (38)
III (50-99)	93	36 (39)	7 (7)
IV (>100)	162	71 (28)	0

0695

RED CELL ALLOIMMUNIZATION IN MULTITRANSFUSED CANCER PATIENTS

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Background. early detection of red blood cell (RBC) alloimmunization, through pretransfusion serological testing, is of great importance, in order to avoid alloimmune hemolytic reactions. *Aims*. the purpose of this retrospective study is to estimate the frequency of allloimmunization in multitransfused adult patients with malignant diseases. *Methods*. 572 cancer patients (354 males, 218 females) who received multiple transfusions in our Hospital, during 2008, are included in the study. Only patients who received at least 5 RBC units were considered multitransfused, whereas patients who received up to four units because of active bleeding were excluded. Pretransfusion serum samples of all patients were tested against a set of three unpooled commercial antibody-screening reagent RBCs (Ortho Biovue, Surgiscreen) both by the Indirect Antiglobulin Technique (IAT) and by direct centrifugation at room temperature, using the glass beads centrifugation method (Ortho Biovue).All positive samples were further studied for antibody identification using an expanded panel of reagent RBCs (Ortho Biovue Resolve Panel C). Enzyme treated RBCs were used as indicated whereas Direct Antiglobulin Test (DAT) of patient's red cells was performed in all positive cases. All transfused units were ABO and Rh matched and screened for the corresponding antigen in alloimmunized patients. *Results*. The median number of RBC transfusions was 17 (5-111) in our study group. Alloantibodies were identified in 65/572 patients (11.3%). 12 patients had multiple (up to 3) alloantibodies. Anti-Kell alloantibody was the most frequent (26%), followed by anti-E (17%), anti-Lewis b (11%), anti-D (8%), anti-c (9%), anti-M (7.5%), anti-P1 (2.5%), anti-Lewis a (3%), anti-Jka (3.5%), anti-Jkb (2.5%) and five other alloantibodies of lower frequency. In four cases identification of alloantibody specificity was not possible through routine techniques as described. No case of alloimmune hemolytic reaction was reported. *Conclusions*. The incidence of RBC alloimmunization in multitransfused patients is 11.3% in this study. Universal pretransfusion antibody detecting tests in addition to standard crossmatching should guarantee prevention of most alloimmune hemolytic transfusion reactions.

0696

CORD BLOOD BANKING: THE CONTRIBUTE OF BLOOD GROUP MOLECULAR GENOTYPING IN UNITS' VALIDATION

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Large employ of umbilical cord blood (CB) for unrelated stem cell transplantation is constantly encouraged by CB banks (CBBs) spreading worldwide. Only fully characterized CBUs can be validated, included in the inventory and managed for stem cell donor search and procurement in the CBBs network. Complete CBUs characterization includes blood group typing. In fact, according to FACT-Netcord international standards, a sample must be taken from each CBU to perform ABO group and Rh type. Traditionally serologic blood group techniques are used on fresh CBUs samples but cannot be accurately performed after thawing due to haemolysis. In case serologic blood group phenotype is not carried out on a fresh sample, this testing remains unavailable; all the more so because the blood group of the neonate cannot be acceptable as a surrogate. As blood group testing represents a requirement for validation, CBUs missing this data are considered nonconforming and excluded from the inventory causing waste of money and human resources. Molecular testing methods were introduced to the transfusion medicine community more than a decade ago. Actually, the molecular basis for almost all blood group polymorphisms has been determined, making possible to predict blood group phenotypes with a reasonable degree of accuracy. Moreover, in several situations (multiply transfused recipients, prevention of anti-D alloimmunization caused by very weak RhD variants in blood donors, foetal typing from amniocytes or cell-free foetal DNA present in maternal plasma), molecular genotyping showed to be a superior, or the only, strategy. The availability of consistent amount of cord's DNA suggested our CBB to extend the use of molecular blood group genotyping techniques to CBUs lacking blood group typing with the aim of evaluating the feasibility and advantages of this approach. We reviewed our CBB data; 7 out of 3395 CBUs were identified to be potentially suitable for validation except for missing blood group testing. ABO and Rh CDE molecular blood group was determined for all 7 cases by PCR-SSP technique using commercial kit READY-GENE AB0 and READY-GENE CDE (INNO-TRAIN Diagnostik GmbH, Kronberg/Taunus, Germany). For this purpose, genomic DNA was obtained from various sources as shown in the Table 1.

Table 1. Results of molecular blood group genotyping:7 CBUs.

Sample #	ABO molecular genotype	Rh molecular genotype	Age of CB unit (years)	Source of DNA
1	O^1A	CcD-ee	12	attached segments
2	O^1O^1	CcD-ee	1	frozen DNA from the original CB collection
3	O^1B	CcD-Ee	3	chorionic villi
4	O^1O^1	CcD-ee	3	chorionic villi
5	O^1A^2	ccddee	8	frozen DNA from the original CB collection
6	O^1B	ccddee	12	chorionic villi
7	AB	CcDΩee	12	frozen DNA from the original CB collection

We succeeded in determining the molecular blood group for all the CBUs examined, the results of ABO and Rh genotypes being detailed in the table. Despite the time of storage, which in 3 cases reaches 12 years, and DNA coming from disparate origin, molecular blood group genotyp-

ing demonstrated to be feasible on CB. Furthermore, with a tiny increase of initial costs, this molecular tool offers economical and organizational advantages by making eligible for validation CBUs otherwise unsuitable and by recovering costs of their characterization and maintenance. In our opinion molecular blood group genotyping could be reasonably implemented in CB banking.

0697

HAEMOLYTIC DISEASE OF THE FETUS/NEWBORN SECONDARY TO ANTI-N/'N'

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A 32yr female West African patient in her 1st pregnancy had an alloantibody detected by IAT at booking at 12/40 weeks gestation. The antibody was strongly positive with all screening cells at 18 and 37 degrees by IAT. It was destroyed by enzyme. The antibody was identified as IgG Anti-N/'N'. All panel cells including M+N- cells which have no N on Glycophorin A but carry 'N' on Glycophorin B were strongly incompatible. M+N-U- and M-N+U- cells which lack GPB related 'N' but carry N on GPA were also strongly incompatible excluding the presence of anti-U. The only compatible cells were MkMk cells which lack all GPA and GPB antigens. (Only two compatible donors registered in UK). The mother was referred to the regional specialist centre (RHSC) for foetal monitoring. Foetal anaemia was detected by Doppler MCA ultrasound. An intrauterine transfusion at 27/40 weeks was proposed. One Frozen red cell unit was obtained from IBGRL Bristol, but unfortunately these proved to also be strongly incompatible with maternal serum. One autologous red cell unit was obtained from the mother by an emergency isovolaemic venesection, washed and split into two packs. One was frozen, the other resuspended and transfused. Baby's cells were DAGT-ve. However, an eluate contained weak anti-N by IAT. The baby typed as M+N-S-s+U+ (Probable MNS genotype MM/sSu). Therefore the one copy of 'N' on GPBs was responsible for the maternal-foetal incompatibility. There was no further evidence of foetal anaemia by serial MCA Doppler. Red cells compatible with maternal and baby's serum were obtained courtesy of the American Red Cross. The baby was delivered by elective C-section at 37/40 weeks. The baby was DAGT-ve and was not anaemic or jaundiced. Anti-N/'N' has only rarely been implicated in HDFN. To our knowledge this is the first reported case to require intrauterine transfusion.

0698

TREATMENT OF ORBISAC DERIVED BUFFY COAT PLATELETS WITH THE MIRASOL® PATHOGEN REDUCTION TECHNOLOGY AND STORAGE IN ADDITIVE SOLUTION

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Background. The Mirasol Pathogen Reduction Technology (PRT) system for Platelets and Plasma uses riboflavin and UV light to introduce irreparable lesions to nucleic acids thereby inhibiting pathogen and WBC replication and reducing the pathogen load in donated blood products. To further reduce adverse transfusion reactions storage of platelets in additive solution (PAS) has become an attractive alternative to storage in plasma. *Aims*. The aim of this study was to evaluate low plasma buffy coat units obtained from the Orbisac System (CaridianBCT) and to examine the effects of the Mirasol PRT treatment (CaridianBCT Biotechnologies) on platelet (PLT) storage lesion development during storage in SSP+. *Methods*. Low plasma buffy coat platelet concentrates (BCPC) were generated by pooling 5 buffy coat units using the OrbiSac System (CaridianBCT). Riboflavin was added during the final pooling step. The BCPC product was transferred into an illumination/storage bag, placed in the Mirasol Illuminator and exposed to UV light. The bag was removed after the target energy of 6.24 J/mL was delivered and 150 mL of SSP+ (MacoPharma) was added prior to storage. Platelet function was assessed by pH, swirl, CD62P expression, lactate production and glucose consumption rates over 7 days of storage. *Results*. BCPC generated contained on average $3.45x10^{11}$ platelets in 116±8 mL of plasma prior to illumination and 150 mL SSP+ addition. *In vitro* cell quality parameters of treated units are summarized in the Table 1. *Summary and conclusions*. Orbisac derived BCPC treated with the Mirasol PRT system can be stored in SSP+ for 7 days without compromising cell quality. Elevated CD62P expression, lactate production and glucose consumption rates after PRT treatment are due to cellular activation and increased metabolism of the cells. First clinical evaluation of PRT treated platelets in thrombocytopenic patients showed that patients receiving treated platelets had comparable hemostasis and support requirements.

Table 1.

Parameter	Day 5	Day 7
pH (22°C)	7.13 +/-0.005	7.13 +/- 0.005
Swirl	2.2 +/- 0.5	1.3 +/- 0.5
Glucose consumtion rate (mmol/10^{12} plts/hr)	0.041 +/- 0.007	0.035 +/- 0.006
Lactate production rate (mmol/10^{12} plts/hr)	0.098 +/- 0.019	0.083 +/- 0.024
CD62P (%)	77 +/- 4	84 +/- 3
LDH (IU/l)	103 +/- 45	111 +/- 23

0699

INTRAUTERINE TRANSFUSION RESULTS IN PRENATAL ANEMIA

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Background. The treatment of intrauterine prenatal anemia is safer and more profitable thanks to the developmnet of image techniques making the protocolisation of the proceedings possible. *Aims*. To describe the cases that have appearded in the Regional Centre for Blood Transfusion in Granada-Almeria, Spain. *Methods*. Assessment during the time period 2000-2008, of 174 pregnancies analisying the predictive value of the doppler ultrasonography of middle cerebral artery (MCA) and antibody titers in relation to the degree of anemia. *Results*. In the last 8 years 174 pregnancies have been studied, all of who have been subject to the Intrauterine Transfusion (IUT) 73:1 was treated for erythrocytic enzymopathy, 5 Hydrops nonimmun, 3 Twin-Twin Transfuion Syndrome, 2 neonatal autoimmune thrombocytopenia (anti HPA 1a) and, 62 alloimmunization erythrocytic: 55 for anti-D antibody, (37 anti-D; 18: anti-D+C, anti-D+E, anti-D+C+E, anti-D+Jka, anti-D+Lea and anti-D+Jka), 1 anti-c and, 6 anti-K. They only needed assessment with Peak Systolic Velocity of MCA doppler ultrasonography (PSV-MCA), without having the criteria for IUT, 101 pregnancies were alloimmunized (55 anti-D, 18 anti-D and others, 12 anti-K, 15 antibodies from different antigens and 1 thrombopenia autoimmune). More interventions were not needed during these pregnancies. There were only a total of 14 hydrops fetalis (8 anti-D, 1 anti-K, 5 nonimmunes), which were given a protocol of gradual elevation of the inicial hemoglobin concentration (HC) (around 1,6 -7,6gr/dL, who were previously given a intrauterine exchange transfusion to avoid volume overload in the fetus, arriving at the end of the pregnancy without aftereffects. Of the 110 anti-D alloimmunized pregnancies, 63 have presented anti-D antibody titers superior to 15UL/mL (titer >1:128). According to our own experiences, the IUT should be used, taking into account the aessement for PSV-MCAdoppler. In the 2 cases where the pregnant women have had high titers of anti-D at the expense exclusively of IgG3 antibodies (1:1024 and 1:2048), the fetuses presented very moderate anemia (HC: 11,4 and 10,2gr/dL, in preliminary cordocentesis, so that the presence of IgG3 antibodies, in our experience, does not condition a worst prognosis. Of the 18 anti-K (15 anti-K and 3 anti-K+E), 6 were subject to IUT in the 17-31 weeks of gestation (WG), with HC between 6.1-12,4gr/dL. 1 case suffered from hydrops fetalis in the 31 (WG), with a titers of 1:128 only of IgG1 antibodies and 7.6gr/dL of hemoglobin, which makes us suspect that the sensibilization/alloimmunization for anti-K antiboby and the precocious intervention can condition hydrops with levels of hemoglobin relatively higher that the ones presented in the alloimmunization for anti-D. *Conclusions*. The IUT programme has meant an important advance in the treatment of badly affected fetuses. 322 cordocenteses have been carried out, 259 were followed by IUT, all of them without complications. 72 pregnancies completed the IUT programme. There were 2 failures due to fetal death, both in the interval between the IUT. We have not had serious complications

associated to the procedure. The global fetal survival has been 97.3% and the hydropic fetuses treated according to our protocol have survived.

Table 1. Alloimmunization:prenatal anemia.

	Antibody	Nº Cases	IUT	MCA - Doppler	Hydrops
Immune	Rh Anti-D Anti-D + others[1]	110	55	55	8
	Rh (E,c)	19	1	18	
	Kell Anti-K[2]	18	6	12	1
	IgG S, Fya, Jka, IFC, M, Lea	15	0	15	
	HPA Thrombopenia	3	2	1	
TOTAL		**165**	**64**	**101**	**9**
Nonimmune	Hydrops, Others	6	6	1	5
	TTTS[3]	3	3	-	
TOTAL		**174**	**73**	**102**	**14**

1) 81 Anti-D; 29 Anti-D + : C, E, C+E, C+Jka, FyB, Lea, Jka
2) 15 Anti-K, 3 Anti-K+E
3) Twin Twin Transfusion Syndrome

0700

PREDEPOSIT AUTOLOGOUS DONATION IN RADICAL PROSTATECTOMY: APHERESIS VS WHOLE BLOOD

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Background. At our institution, 27.8% patients undergoing radical prostatectomy (RP) used to receive allogeneic blood transfusion (ABT). To reduce ABT rates and ABT related risks, a preoperative autologous donation (PAD) program for RP patients was implemented in 2003. We present data of the first 501 RP managed with PAD (2003-2008) and analyze a possible new protocol involving erythropheresis donation and IV iron administration. *Patients and Methods*. Two to three blood units were requested and donated on preoperative weeks 4, 3 and 2. Dereferral rate, autologous unit drawn and transfused, type of donation (standard or aphaeresis), ABT transfusion rate and volume, age, weight, complete blood cell counts, iron metabolism, B12 vitamin and folic acid, EPO and iron treatment were analyzed. *Results*. 482 (96%) RP patients (64±6 y old, 80±12 kg bw) were admitted to the PAD program and donated at least one autologous uint. Blood donation: A mean of 2.6±0.7 U/patient were collected (25.8% by apheresis, double unit); 23% received at least a 200 mg of IV iron; and 24 (4.9%) at least one EPO dose (11: beta 30.000 IU; and 13: α 40.000 IU). Blood Transfusion: 63.8% patients were transfused, but only 5.8% received at least one ABT unit. Patients that donated two units received less PAD units (0.92 vs. 1.39) and total units (1.02 U/pte vs 1.54U/pte) than those with three donations. Patients treated with IV iron showed lower ABT rate (3.7% vs. 6.5%) and index (0.06 U/pte vs 0.6 U/pte). In apheresis donations, those treated with 200 mg IV iron also showed lower ABT rate (4% vs. 15%; p: 0.036) and index (0.06 U/pte vs 0.46 U/pte; *p*:0.036). Adjuvant treatment with EPO did not further decrease ABT transfusion rates. However, 41 % of PAD units were discarded. *Conclusions*. At our institution, PAD seems to be an effective alternative to reduce allogeneic blood transfusion requirements in RP patients. Apheresis donation of only two units plus IV iron, and EPO if anaemia, seems to be a very interesting approach to further reduce ABT requirements and PAD units discarding rate.

0701

CONTRIBUTION OF HAEMATOTHERAPY TO THE TREATMENT OF MICROCIRCULATORY DISORDERS

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Background. Transfusion medicine and haematology contributed to the therapy of some serious microcirculatory disorders in the last years. Haemorheopheresis was specifically designed to treat microcirculatory disorders. The single rheopheresis treatment simultaneously eliminates an exactly defined spectrum of high-molecular weight rheologically relevant plasma proteins (i.e. α2-macroglobulin, fibrinogen, LDL-cholesterol, lipoprotein(a), von Willebrand factor (vWF), immunoglobulin M (IgM), fibronectin, and putatively multimeric vitronectin). This results in the immediate pulsed reduction of plasma viscosity as well whole blood viscosity, which with a series of treatments can lead to sustained microcirculatory recovery, and change significantly the natural course of a chronic disease. *Aims*. We describe the experience of our Hemapheresis Centre. *Methods and patients*. In the prospective trial presented here, 41 patients were treated - severe familiar hypercholesterolemia (FH): 5 pts; non-healing lesions caused by severe ischemic diabetic foot syndrome (IDFS): 6 pts; age related macular degeneration (AMD): 20 pts; acute sensorineural hearing loss (ASHL): 5 pts; thyreoid orbital endocrinopathy (TAO): 5 pts. Our own modification of rheopheresis was used: Plasma, free from cellular elements is obtained by blood cell separator (Cobe-Spectra, Denver, USA) in high-speed centrifugation. Then it is run through the *second stage* - a rheofilter (Evaflux 4A, Kuraray) with ethylene-vinyl-alcohol hollow fibres with holes of 0,03 micrometer. Plasma flow is continuous, anticoagulation done with heparin. Basic amount of processed plasma (calculated by Cobe computer) is 1,5 of body volume. The size of holes in the filter enables to retain the above mentioned high-molecular elements. Haematological, biochemical and haemorheological parameters were measured before and after procedures and after the finishing of therapeutic series (AMD 8 procedures, IDFS 10, ASHL 3, TAO 10). *Results*. Rheological procedures were very effective and resulted in significant decreases of pathologically effective substances: alfa2-macroglobulin 57,6%, fibrinogen 68,6%, IgM 61,5%, LDL-cholesterol 75,0%, apolipoprotein B 76,0%, lipoprotein(a) 63,2%. It resulted in blood and plasma viscosity decrease (14,13/12,5%). Diabetic foot ulcers were healed in 4 of 6 pts. No progression from dry to wet form of AMD has been observed during 2 years. 6,3% of side effects were observed; they were not severe, transient and easily controlled. *Conclusions*. Hemorheopheresis appears to be a method suitable adjunct therapy for diseases involving severe disturbance of microcirculation, especially when previous therapeutic options were not sufficiently effective or invasive procedures cannot be applied.

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0702

ROLE OF RITUXIMAB FOR TREATING PATIENTS WITH IDIOPATHIC TTP/HUS WITH OR WITHOUT ADAMTS-13 ANTIBODIES

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Background. Idiopathic TTP/HUS (Thrombotic Thrombocytopenic Purpura-Hemolytic Uremic Syndrome) namely defined as absence of congenital deficiency of ADAMTS-13 is a rare disease usually responsive to plasma exchange (PE). Because of the high rate of relapses, a useful strategy is that to select high-risk patients such as those with low level of ADAMTS-13 activity for alternative therapy. Conventional immunosuppressive therapy (cyclophosphamide, cyclosporine, azathioprine and intravenous immunoglobulins) have been shown to successfully treat patients with relapsed/refractory TTP/HUS; few evidences are currently available about therapy with Rituximab®. *Aims*. We tested the role of anti-CD 20 drug for treating patients with relapsed/refractory TTP/HUS. *Methods*. Rituximab® was administered to 4 patients with relapse/refractory idiopathic TTP/HUS, with or without deficient

ADAMTS-13 activity. Characteristics of patients are reported in the Table 1. Complete remission was defined if presence of PLT >100×10⁹/L and normal laboratory values (LDH, serum bilirubin, creatinine, hemoglobin). Three cases were due to ADAMTS13 deficiency antibody-mediated; among them, one relapsed 39 months after complete remission, obtained by PE, while the remaining 2 patients were refractory to PE. The fourth patient did not have ADAMTS13 deficiency and he was refractory to PE. All 4 patients received Rituximab® at the dose of 375mg/m2, one shot a week for one month; no other therapy were given except steroids low dose tapering. *Results.* Complete remission was achieved in all patients just after the first two infusions of Rituximab®. In patients with ADAMTS-13 antibodies, these were not longer detectable after all courses of Rituximab therapy. Remission was maintained for a median period of 6 months (range 2 to 10 months). *Conclusions.* The role of Rituximab® in patients with idiopathic refractory/relapsed TTP/HUS is still unclear in patients with or without ADAMTS-13 antibodies. In our experience, Rituximab® has been proven to be effective in producing a durable complete remission; this happened in 3 patients with deficient ADAMTS-13 activity and in the fourth patient with refractory TTP/HUS without deficient ADAMTS-13 activity. Properly designed studies are required to confirm safety and efficacy of such approach.

Table 1. Patients' characteristics.

Patient N	Age (years)	Sex	ADAMTS-13 Activity (IgG Anti-ADAMTS-13)	Diagnosis	Therapy before rituximab application	Therapy during rituximab application	Rituximab doses	Serious rituximab side effects	Follow-up (months)
1	28	M	< 20 %** (>100 U/ml)*	Relapsing idiopathic TTP	steroids	Steroids low dose tapering	375mg/m² X 4	none	3
2	53	M	100 % ** (12 U/ml) *	Refractory idiopathic TTP	PE, steroids	Steroids low dose tapering	375mg/m² X4	none	10
3	52	M	40 % ** (85 U/ml) *	Refractory idiopathic TTP	PE, steroids	Steroids low dose tapering	375mg/m² X 4	none	9
4	16	F	<5%** (>120 U/ml)*	Refractory idiopathic TTP	PE, steroids	Steroids low dose tapering	375mg/m² X 4	none	2

** N:V:50-150%
* N:V:<17 U/ml

0703

THE EFFICACY AND EFFICIENCY PLATELET TRANSFUSION IN BUFFY COAT PLATELET COMPONENTS TREATED OR NOT BY INTERCEPT BLOOD SYSTEM

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Since the epidemic of chikungunya in Reunion island, all platlet components (PC) are treated by the Intercept technology (Cerus corporation). The principle of this new technic is based on a photochemical treatment of the PC (PCT PC) by a chemical substance, the amotosalen, and the UVA so allowing the inactivation of a large number of viruses, bacteria, parasites as well as the residual leukocytes. We compared the efficiency platelet transfusion of 413 PC treated by IBS and 276 untreated PC transfusion for patients (Pts) followed in our department. The Pts presenting a febril syndrom, disseminated intravascular coagulation or a splenomegaly were excluded. The average quantity of transfused platelets was respectively 3.82 (± 0.90) and 4.04 (± 1.03) ×10⁹/L for the PCT PC and untreated PC. The corrected count increment (CCI) were respectively 450 (± 556) and 898 (± 680) for PCT PC and untreated PC; by taking the CCI, the transfusion was ineffective in respectively 30,3 and 5,7% for PCT PC and untreated PC. Clinical effectiveness, i.e. post transfusionnal rate of platelets higher than 20×10⁹/L, is respectively 74,3 and 45% for the PCT PC and untreated PC. This comparative retrospective study showed, that the efficiency of platelets transfusion as well as the CCI are clearly decreased after treatment of the CP, for viral inactivation, by Intercept technology.

0704

DATA REVIEW FROM THE PAVIA CORD BLOOD BANK: CORRELATION BETWEEN CORD BLOOD NUCLEATED AND HAEMATOPOIETIC STEM CELL COUNT AND BIRTHWEIGHT

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Umbilical cord blood (UCB) is a source of hematopoietic stem cells (HSCs) which offers substantial logistic and clinical advantages for allogeneic HSC transplantation to patients otherwise not eligible for this curative approach. A cord blood bank (CBB) consists of a repository of cryopreserved UCB units from healthy newborns. For each unit/baby enrolled for HSC donation, the CBB records HLA profile, cellular characteristics and physiological parameters into databases for subsequent transplantation requests. Thus, beyond immunogenetic data, a huge amount of information regarding cord blood volume, levels of total nucleated cells (TNCs) and CD34⁺ cells are available. Here we examined the relation between birthweight and UCB concentrations of TNCs and CD34⁺ stem cells in 1037 infants/UCB units. Healthy Caucasian mothers were recruited to participate voluntarily in the cord blood banking program of the Pavia Cord Blood Bank, all giving written informed consent. Preterm (<34 gestational weeks) and pathological pregnancies were excluded from UCB donation. Cord blood was collected from umbilical cord vessels by in utero technique immediately after birth. Only the units with net volumes ≥50 mL and containing ≥800 - 10(6) TNCs were banked. All the 1037 babies were classified into 6 centiles according to birthweight, sex and gestational age. A non-parametric statistical analysis was made using Spearman test, and considering $p<0.05$ as statistically significant. As expected by the selective criteria of the CBB, an inverse relation between birthweight and TNC concentration (graphic 1 top, $\rho = -0.07$; $p=0.02$) was found.

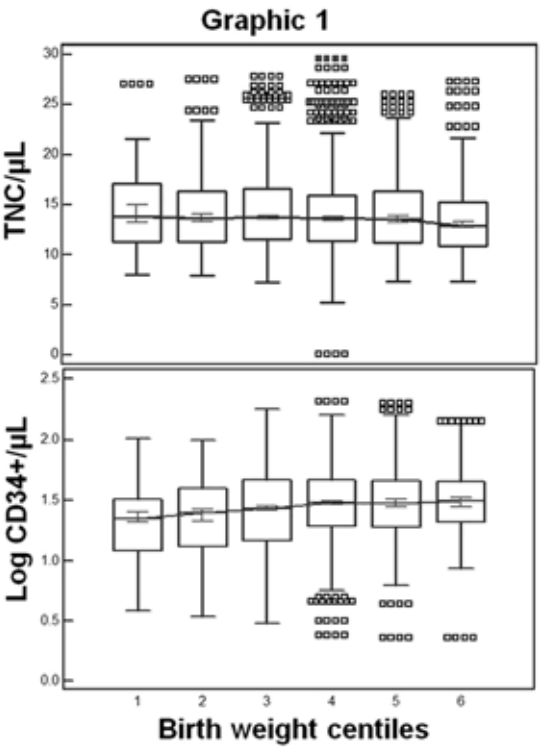

Figure 1. CD34⁺ and TNC count in the 6 birthweight centiles.

In fact, the low-weighted babies had a smaller volume than the big ones, but as they fit the ≥800 - 10(6) TNC total count cut-off, they must

have a higher TNC concentration. On the contrary, CD34+ cell count did not follow the TNC concentration trend. In fact, the heavy-weighted babies showed a higher concentration of early multipotent progenitor cells with respect to the small ones (graphic 1 bottom; ρ=0.11; *p*=0.0003). As expected by excluding preterm and pathological pregnancies, which are known to be characterized by high CD34+ cell levels due to environmental stress in utero, the low concentration of CD34+ cells in small UCB donors supports their healthiness during intrauterine life. Moreover, the amount of organ resident stem cell pools is reflected in the size of organs and consequently in birthweight. Cord blood CD34+ cell count is a good surrogate of the body overall stem cell content, including organ resident stem cells, therefore their highest concentration was found to correlate with the biggest birthweight centiles.

0705

HIGHER IRON SUCROSE DOSE IN A AMBULATORY ANAEMIA TREATMENT PROGRAM

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Background. Recently we have shown feasibility and safety of iron sucrose administration in anaemic out-patients awaiting for surgery.[1] At Spain iron sucrose maximum dose is 200 mg per session, but not in Europe. *Aim*s. We decided to treat with higher dose (300-500 mg, with 7 mg/kg/day dose limit) to those patients with higher needs, worse venous access or chronic bleeding. *Methods*. Since January 2004 we have recollected analytical and clinical data of 57 patients (40 females, 17 males). They were sent by ferropenic or mixed anaemia or symptomatic ferropenia resistant to oral iron. Most of them were scheduled for surgery, but 6 after surgery. Total iron dose was calculated with the classical formula [DTH: (14 - Hb) x 2.41 x weight + 500]. Since January 2007 we have included haematological, heart failure and chronic digestive bleeding (angiodisplasia) under anticoagulant or antiagregant treatment. And we begin to administer under 'compassionate use'/"off label" indication dose of 300 mg/day. *Results*. In the moment of this review at least 27 patients had received at least one 300 mg session and two at least 500 mg. Medium session was 4.2 (1-10) and 884 mg of iron sucrose (200-2900). Only 6 patients had been transfused, due their chronic blood loss. *Conclusions*. Higher dose of intravenous iron administration to anaemic ambulatory patients awaiting for major surgery or affected of chronic ferropenic anaemia seems to be safe, feasible and effective for correcting anaemia and reducing blood transfusion requirements. Further studies to confirm these promising results are warranted.

Table 1.

	Normal (n=28)		Higher (n=29)	
*p<0.05	Baseline	End	Baseline	End
Hb (g/L)	108.2	118.8*	98.7*	124.8
Ht0 (%)	34.1	35.9	30.7*	37.9
VCM (fL)	87.9	88.6	80.9*	87.5
Fe (mg/L)	49.3	51.4	19.8*	62.1
Ferritin	52.3	218	76.2*	248
TSI (%)	15.9	17	4.8*	17

Reference

1. Muñoz et al. Med Clin (Barc) 2009 (press)

Stem cell transplantation - disease related

0706

TARGETED INTRAVENOUS BUSULFAN IN CHILDREN PRIOR STEM CELL TRANSPLANTATION FOR THALASSEMIA: PHARMACOKINETICS, TOXICITY AND TRANSPLANT OUTCOMES

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Background. Busulfan (BU) is the mainstay of conditioning regimen for non-malignant diseases. Younger children have significantly high BU clearance than older children or adults which results in under-or over-exposure when standard doses are used. Intravenous BU (IVBU) is noted to have higher intrapatient pharmacokinetic (PK) consistency as compared to oral BU. No data are available on IVBU safety and PK in a large group of patients with thalassemia to date. *Aims*. Assessment of the IVBU PK in relation to patient and disease- related variables and the relationship of BU exposure to toxicities and transplant outcomes in children given SCT for thalassemia from HLA-matched related donors. *Methods*. 57 children and young adults with median age of 9 years (range, 1.6-24) were given IVBU (Busilvex, Pierre Fabre Medicament, France) as a part of conditioning regimen between 2006 and 2008. BU doses were based on actual body weight: <9-16= 1.2 mg/kg (n=10); 16-23= 1.1 mg/kg (n=13); 23-34= 0.95 mg/kg (n=23) and >34=0.8 mg/kg (n=11) and were given every 6 hours for 4 days. Anticonvulsant prophylaxis consisted of Valproic acid (Depakin). Most patients had liver disease and moderate to severe iron overload. Class 1 and class 2 patients (n=24) received IVBU in combination with CY200 ± thiotepa as conditioning regimen. Class 3 patients (n=33) before conditioning with IVBU/CY160 ± thiotepa were given hydroxyurea, azathioprine and fludarabine. Four blood samples were drawn after the 1st, 5th, 9th, and 13th doses for PK assessment by HPLC-MS. Dose adjustment (DA) was made at the 3rd dose as needed, to target an AUC range of 900- 1350 μmol/min. The influence of patient and disease-related variables on IVBU PK was investigated by a population PK-based approach using the NONMEM program. *Results*. Busulfan PK data are summarized in Table 1. In 58% of patients AUC was within, 37% below and 5% above the target range following the 1st dose of IVBU. Seventeen patients required dose elevations of 5-54% and 19 patents dose reductions of 5-34%. Following DA 79% of patients after the 5th and 9th doses and 91% after the 13th dose reached the target range. The inter-patient variability in IV BU clearance was moderate (CV=19%) and the intra-patient variability was low (CV=7%). We found that only weight or body surface area significantly explained the PK variability. Fifty three patients (93%) had sustained engraftment, 4 (7%) had graft failure. One patient had moderate hepatic VOD resolved with supportive care. Seventeen patients developed grade 2-4 acute GVHD. None of patients had seizure within 30 days post-transplant. Grade 1 or 2 ALT/AST increases or stomatitis/diarrhoea were observed in 19% and 44% or in 47% and 19% of patients respectively. Five patients died. No relationship was found between BU exposure and toxicities, engraftment time, chimerism, rejection, GVHD and survival. *Conclusions*. body weight based dosing of IVBU in children with thalassemia who have disease and treatment related important organ damage is well tolerated with no increase in organ system toxicity. Busulfan exposure did not predict rejection, GVHD, liver VOD or death.

Table 1.

	AUC μMol/min	Css ng/ml	Cmax ng/ml	Cmin ng/ml	Cl ml/min/kg	Vd L	T ½ h
Mean ± sd	982± 203	672± 139	1071± 207	239± 72	4.23± 0.95	16.8± 6.8	1.8± 0.2
Range	630-1621	431-1109	735-1614	101-450	2.31-6.43	7.3-43.6	1.2-2.3

0707

ADEQUATE MOBILIZATION OF CD34 IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS RECEIVING LENALIDOMIDE INDUCTION THERAPY

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Background. New drugs have been introduced as induction regimens before ASCT. Lenalidomide has raised concerns regarding its potential impact on the ability to collect stem cells. The use of Cyclophosphamide to mobilize stem cells may overcome these concerns. *Aims.* In the prospective randomized trial RV-MM-PI-209, Lenalidomide and Dexamethasone (RD) were used as induction and Cyclophosphamide (CY) was used to mobilize stem cells. The effectiveness of this approach was determined and compared with an historical control of patients treated at diagnosis with Vincristine-Doxorubicin-Dexamethasone (VAD) and mobilized with the same CY+G-CSF schema. *Methods*. Fifty newly diagnosed myeloma patients received Lenalidomide (25 mg/d for 21 days followed by a 7 days rest period) 28-days cycles in combination with Dexamethasone (40 mg days 1, 8, 15 and 22). Stem cells were mobilized with CY 4 g/m^2 /day, i.v. on day 1 and G-CSF 10 ug/kg/day from day 5 until the end of stem cell collection. Patients who failed to collect a minimum of 4×10^6/kg CD 34$^+$ cells received a second CY+G-CSF mobilization course. The historical control included 134 patients who received VAD induction and were mobilized with the same CY+G-CSF schema used in the RD protocol. The inclusion criteria for patients entering the RD or VAD trial were identical. *Results*. Baseline patients characteristics were similar in the 2 groups. Median time from CY to leukapheresis was 12 days (range 8-23) in the RD group and 11 days (range 7-15) in the DAV group (p=0.0003). The median days of leukapheresis was slighty superior in the RD patients, 3 (range 1-5) versus 2 days (range 1-3). A second mobilization was performed in 15% of patients in both RD and VAD groups. After the first mobilization course the number of patients who did not collect at least 2×10^6/kg CD34 was 4/50 (8%) in the RD and 22/134 (15%) in the VAD group. At the completion of the mobilization phase only 1 patient (2%) in the RD group and 10 patients (7%) in the VAD group did not collect a minimum of 4×10^6/kg CD34 and therefore could not receive the planned autologous stem cell transplant. The median yield of CD34$^+$ cells collected was lower in the RD group (RD: median 10×10^6/kg, range 0-25; VAD: median 14×10^6/kg, range 0-54, p=0.0005). *Conclusions*. RD as part of induction regimen allowed to collect an adequate number of stem cells to support autologous transplantation in 98% of patients, while VAD in 93%. The median number of CD34$^+$ cells harvested was 10×10^6/kg after RD and 14×10^6/kg after VAD. An update of the the first 100 patients enrolled in the RD trial will be presented at the meeting.

0708

STEM CELL TRANSPLANTATION IN DIAMOND-BLACKFAN ANEMIA: A RETROSPECTIVE ANALYSIS

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Objective. We retrospectively analyzed the characteristics and outcome of patients (pts) who received hematopoietic stem cell transplantation (HSCT) for Diamond-Blackfan Anemia (DBA) in Germany, Italy, Austria and Switzerland between 1987 and 2007. Engraftment, complications and survival were analyzed separately for patients transplanted from either a matched sibling donor (MSD) or an unrelated donor (UD). *Patients and Methods*. Thirty-seven pts (24 males and 13 females) were transplanted between 1987 and 2007 in Germany (23), Italy (12), Switzerland (1) and Austria (1). All pts received regular red blood cell (RBC) transfusions, and 19 pts were given iron chelation therapy for secondary hemosiderosis. Median age at HSCT was 5.8 years (range 1.2-15.1 years). Twenty-six pts were transplanted from a MSD, while 11 pts were grafted from a matched or 1 Ag mismatched UD. The majority of patients (24) received a conventional preparative regimen including busulfan (BU), cyclophosphamide (CY) ± thiotepa (TT). Ten pts were conditioned with BU, TT and fludarabine (FLU) and 2 pts received a reduced intensity regimen with TT and FLU. Stem cell source was bone marrow, peripheral blood and cord blood in 28, 4 and 3 pts, respectively. Two pts received grafts from combined stem cell sources. In 22 pts GvHD prophylaxis consisted of cyclosporine A, methotrexate ± antithymocyte globuline, while the other patients received various different prophylactic regimens. *Results*. Engraftment was achieved in all pts, the median time to neutrophil engraftment being 16 days (range 9-44). One pt grafted from an UD experienced secondary graft failure and subsequently died. One pt each developed acute GvHD grade > III and extensive chronic GvHD. Four pts, including two grafted from a MSD, experienced transplantation-related mortality. There was no veno-occlusive disease (VOD). With a median follow-up of 4.1 years (range 0.2-15.1) the probability of overall survival (OS) for patients transplanted from either a MSD or an UD is 0.92 (0.81-1.00) and 0.72 (0.44-1.00), respectively. *Conclusions*. HSCT offers an excellent survival for pts with DBA transplanted from a MSD. Results for patients receiving an allograft from an UD are encouraging and compare favourably with published experiences. Despite pre-existing hemosiderosis VOD does not seem to be a common complication.

0709

HLA-E UP-REGULATION ON IFN-G-ACTIVATED AML BLASTS IMPAIRED CD94/NKG2A-DEPENDENT NK CYTOLYSIS FOLLOWING HAPLO-IDENTICAL HAEMATOPOIETIC STEM-CELL TRANSPLANTATION (SCT)

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Background. We previously reported that NK-cell generated after haplo-SCT in high risk AML patients are characterized, during the first 6 months post-SCT, by specific phenotypic features and impaired functioning having potential impact for transplantation outcome (Nguyen *et al.*, Blood 2005). *Aims*. In the present study, we examined the impact of INF-γ on the impaired recognition of AML blasts by NK cells after haplo-SCT. *Methods*. We studied NK cells at 1 to 6 months (M1-M6; n=21patients) or at 24 months (M24; n=3 patients) post -SCT. Patients received CD34$^+$ haplo-transplant after a myeloablative conditionning regimen for high risk hematological diseases. *Results*. As previously observed,the subset of CD56brightCD94/NKG2A+ NK cells was increased and NK lysis of AML blasts was poor, suggesting an immature status of NK cells post transplant. However, at M24, CD56brightCD94/NKG2A+ NK subset and lytic functions restored and returned to donors levels. Blocking, *ex vivo*, the inhibitory CD94/NKG2A receptor on NK cells restored the lysis at M3 but not at M24. Higher level of intra-cellular IFN-g production was detected by FACS in CD94/NKG2A+NK at M1-M6 as compared to the donors and at M24, after incubation with IL-12+IL-18 (70% vs 14%). IFN-g treatment led to higher surface expression of HLA-E on AML blasts, resulting in decreased killing by NK cells from healthy donors. Actually, adjonction of IFN-g on AML blasts inhibited the lysis by healthy donor NK cells (12% specific lysis with INF-γ, vs. 37% without INF-γ). Blocking CD94/NKG2A restored the lysis previously inhibited by INF-γ. Neutralization of INF-γ during the interaction of NK cells post-SCT with mismatched AML blasts restored the lysis (16% lysis without INF-γ neutralization versus 44% after IFN-γ neutralization at M1). This effect was no more observed at M24 or in healthy donors. *Conclusions*. Increased secretion of INF-γ by immature NK cells generated after haplo-SCT upregulates the cell surface expression of HLA-E on AML blasts. Increased HLA-E expression protects AML targets from NK-mediated lysis through the inhibitory CD94/NKG2A receptor which is overexpressed on NK cells during the early period post-SCT. This suggests that HLA-E could be a component of an immune escape strategy against NK attack on AML blasts currently observed after haplo-SCT. By contrast, at 2 years post-SCT and in donors, mature NK cells were functionally, which is encouraging to develop clinical trials using adoptive transfer of healthy donor alloreactive NK cells.

0710

PLERIXAFOR CAN PREDICTABLY MOBILIZE HEMATOPOIETIC STEM CELLS IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA PREVIOUSLY TREATED WITH FLUDARABINE AND UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Purine analogues such as fludarabine are increasingly being utilized for patients with non-Hodgkin's lymphoma (NHL) and have been associated with unsuccessful mobilization of hematopoietic stem cells (HSC). This poses a challenge to pursue autologous stem cell transplantation (ASCT), a potentially curative procedure, in this patient population. *Aims*. The aim of this retrospective subgroup analysis is to examine the efficacy of mobilization with plerixafor + G-CSF among NHL patients previously treated with fludarabine. *Methods*. Patients with NHL who participated in the compassionate use program (CUP) who received plerixafor + G-CSF and had complete data on the number of cycles of fludarabine and total $CD34^+$ cells collected were analyzed. The methodology of the CUP has been described previously (Calandra BMT 2007). Briefly, patients in CUP received plerixafor + G-CSF as a HSC remobilization strategy after failing other mobilization regimens. Patients received G-CSF at a dose of 10µg/kg subcutaneously (SC) every morning for 4 days. At about 2200 hours, on day 4, plerixafor was administered at a dose of 0.24 mg/kg. The next morning, day 5, G-CSF was administered and apheresis was initiated. This was repeated daily (G-CSF, apheresis, plerixafor) until patients collected enough cells for transplantation, minimum of 2×10^6 $CD34^+$ cells/kg. *Results*. Of the 401 NHL patients in the CUP database, 48 (12%) had prior therapy with fludarabine. Complete data on number of cycles of fludarabine and total $CD34^+$ cells collected was available for 33 patients. The mean age was 56.5±7.9 years and 14/33 (42.4%) of the patients were men. The median number of fludarabine cycles prior to HSC mobilization was five (range 1-50). Table 1 shows the median number of $CD34^+$ cells collected and the percentage who collected $\geq2\times10^6$ $CD34^+$ cells/kg for those who had received prior fludarabine versus patients who were fludarabine naïve in the CUP. Among the patients that received prior fludarabine, administration of plerixafor + G-CSF resulted in a median collection of 2.8×10^6 $CD34^+$ cells/kg (range $0.3\text{-}6.6\times10^6$ $CD34^+$ cells/kg). A total of 21/33 (63.6%) of the patients collected $\geq2\times10^6$ $CD34^+$ cells/kg in a median of two apheresis sessions (range 1-6 apheresis days). Overall, 27/33 (81.8%) of the patients proceeded to ASCT. Median time to neutrophil and platelet engraftment was 12 days and 22 days, respectively. *Conclusions*. These preliminary results suggest that the majority of patients with NHL pretreated with fludarabine who have failed a previous mobilization regimen can successfully and predictably mobilize $CD34^+$ HSC with plerixafor + G-CSF and that they do not appear to differ from the larger group of NHL patients.

Table 1.

	Prior Fludarabine	No Prior Fludarabine
Median number of $CD34^+$ cells/kg (x 10^6)	2.8	2.5
Percent Patients collecting ≥ 2 x 10^6 $CD34^+$ cells/kg	63.6	64.4

0711

COMPARABLE OUTCOMES AFTER REDUCED-INTENSITY AND MYELOABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION IN ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA IN FIRST OR SECOND REMISSION: A MATCHED-PAIR ANALYSIS

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Background. It is reasonable to look to reduced-intensity conditioning (RIC) allogeneic stem cell transplantation (SCT) as a way to provide a graft-versus-leukemia effect with reduced toxicity for adults with high-risk acute lymphoblastic leukemia (ALL) with low leukemic cell burden who are considered poor candidates for myeloablative conditioning (MAC). Results of RIC-SCT have not been compared to those after MAC-SCT in adult ALL. *Aims*. With this aim, we compared the outcomes of 40 consecutive adults with high-risk ALL who underwent RIC-SCT in first or second remission to a historical group of 160 matched controls receiving MAC-SCT. *Methods*. The indications for RIC-SCT were: (1) aged more than 50 years 18 (45%) and (2) decreased organ function or active infections 22 (55%). Matching criteria included patients receiving SCT from a matched sibling or unrelated donor who (1) were aged 15-65 years; (2) were diagnosed as *de novo* ALL; (3) had received the same policy of pretransplantation chemotherapy; (4) were transplanted in first or second remission between 2000 and 2007; (5) had received RIC with fludarabine (150 mg/m^2) plus melphalan (140 mg/m^2); (6) had received MAC with total body irradiation (12 or 13.2 Gy)-based regimen. Both groups were comparable in terms of main patient-, disease-, and transplant-related factors. However, patients in the RIC group were older (median age, 46 years versus 29 years, $p<0.001$), had Philadelphia chromosome more frequently (48% versus 29%, $p=0.029$), and received peripheral blood stem cells more frequently (85% versus 13%; $p<0.001$) as compared to patients in the MAC group. All patients received the same graft-versus-host disease prophylaxis consisting of calcineurin inhibitor (cyclosporine for sibling transplants; tacrolimus for unrelated transplants) plus methotrexate. *Results*. During the observation period, the cumulative incidence of acute (grade II-IV) and chronic graft-versus-host disease was comparable in both groups. In the RIC group, 9 (23%) of the 40 patients relapsed. Fourteen patients (35%) had died; 6 of these 14 died of causes other than leukemic relapse, and the remaining 8 patients died of relapse and progression. After a median follow-up of 36 months (range, 12-103 months) for surviving transplants, the 3-year cumulative incidence of relapse and non-relapse mortality were 25% and 19%, respectively, and the 3-year disease-free survival and overall survival rates were 60% and 62%, respectively. In the MAC group, 28 (18%) of the 160 patients relapsed. Fifty-six patients (35%) had died; 30 of these 56 died of causes other than leukemic relapse, and the remaining 26 patients died of leukemia relapse and progression. After a median follow-up of 59 months (range, 12-103 months) for surviving transplants, the 3-year cumulative incidence of relapse and non-relapse mortality were 21% and 19%, respectively, and the 3-year disease-free survival and overall survival rates were 64% and 67%, respectively. Overall transplantation outcomes were not significantly different between the two groups. *Conclusions*. RIC-SCT may represent a valid therapeutic approach for adults with high-risk ALL in first or second remission who are not eligible for MAC-SCT. Large prospective studies are needed to elucidate the role of RIC in adults wit high-risk ALL.

0712

UNRELATED STEM CELL TRANSPLANTATION FOLLOWING TOTAL BODY IRRADIATION-BASED MYELOABLATIVE CONDITIONING IN ADULTS WITH PHILADELPHIA-NEGATIVE HIGH-RISK ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Stem cell transplantation (SCT) from a matched sibling or unrelated donor clearly benefits certain patients with high-risk acute lymphoblastic leukemia (ALL), such as those with Philadelphia chromosome (Ph)-positive ALL. In contrast, whether SCT from an unrelated

donor is a treatment option of equal value in Ph-negative ALL cases lacking a matched sibling donor remains unclear. Considering recent progresses including the improvement of human leukocyte antigen matching and the development of supportive care facilities, a detailed analysis is needed to clarify the role of unrelated SCT in adults with high-risk Ph-negative ALL. *Aims*. The aim of the present study was to identify graft-versus-leukemia effects and the factors that affect outcome in 53 consecutive adults with high-risk Ph-negative ALL who received unrelated SCT using total body irradiation (TBI)-based myeloablative conditioning (1997-2007). *Methods*. Median age was 21 years (range, 15-47 years). Patients in first complete remission (CR1) at SCT had at least one adverse prognostic factor, including (1) t(4;11), (2) high presenting leukocyte counts (>30×10^9/L for B-precursor ALL, >100×10^9/L for T-precursor ALL), or (3) CR requiring more than 28 days of induction therapy. Thirty-nine patients (74%) were transplanted in CR1; 3 (5%) in CR2; and 11 (21%) were resistant to chemotherapy before transplantation. All patients received unmodified stem cell grafts (43 bone marrow; 10 peripheral blood) following TBI-based myeloablative conditioning [TBI (13.2 Gy) + cyclophosphamide (120 mg/kg) for CR1; TBI (12 Gy) + cytarabine (12 g/m^2) + melphalan (140 mg/m^2) for >CR1]. Graft-versus-host disease (GVHD) prophylaxis was uniformly attempted by administering tacrolimus plus methotrexate. Antithymocyte globulin (2.5 mg/kg) was administered to 18 patients who received allele-mismatched grafts. *Results*. After a median follow-up of 59 months (range, 12-137 months) for surviving transplants, incidences of acute (grade II-IV) and chronic GVHD were 60% and 56%, respectively. The 5-year cumulative incidence of relapse and non-relapse mortality were 29% and 27%, respectively, and the 5-year disease-free survival (DFS) and overall survival rates were 51% and 51%, respectively. Disease status at transplantation (CR1 versus >CR1) was the most powerful predictive factor affecting relapse (14% versus 71%, p=0.001) and DFS (61% versus 21%, p=0.006) in multivariate analysis. The presence of chronic GVHD was also found to be significantly associated with lower relapse (10% versus 49%, p=0.005) and better DFS (79% versus 42%, p=0.006). *Conclusions*. TBI-based myeloablative unrelated SCT, especially in CR1, provides a curative option for high-risk Ph-negative ALL patients lacking a matched sibling donor. The presence of chronic GVHD results in better transplantation outcome, indicating a clinically important graft-versus-leukemia effect.

0713

ALLOGENEIC VERSUS AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH RELAPSING FOLLICULAR LYMPHOMA: USE OF THE PROPENSITY SCORE TO REDUCE RECRUITMENT BIAS IN A RETROSPECTIVE COMPARATIVE STUDY

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Autologous stem cell transplantation (autoSCT) is the standard treatment for relapsed follicular lymphoma (FL) in patients (pts) younger than 65. Allogeneic SCT (alloSCT) may offer higher curative potential but treatment related mortality (TRM) limits its use. Allografting with non-myeloablative conditioning (NMA-C) has therefore been considered to improve survival. Owing to non-randomized available data, there was a need to correct for potential recruitment bias. The propensity score (PS) methodology allows coping with the presence of such a bias. To assess the place of alloSCT for relapsing pts with FL, we compared pts who underwent consecutive allo or autoSCT at Saint Louis Hospital from 1989 to 2007 after propensity score (PS) matching (Stukel TA, JAMA 2007). PS was defined as the probability of receiving allo or autoSCT knowing age at relapse, number of pretransplant regimens and time from relapse treatment to SCT. To remove bias, matching 1/1 was done using PS and period of transplant (before/after 2000). Cox models were performed to compare allo vs autoSCT on OS and EFS with a fixed period effect. 27 pts were allografted (Figure). 5y-OS and 5y-EFS of alloSCT pts were 58% and 58% with a median follow up (FU) of 4 years (y). 13 pts (48%) died (TRM: 12/13) and 1/27 relapsed. 6 (22%) had grade 3-4 acute graft versus host disease (GvHD), and 9 experienced chronic extensive GvHD. 116 pts had autoSCT (Figure). 5y-OS and 5y-EFS were 73% and 46% with a median FU of 4.4 y. PS allowed 19 pairs to be matched, similarly distributed between allo and autoSCT pts. Based on these matched pairs, 5y-OS estimations were 67% and 77% (p=0.71), and 5y-EFS estimations were 66% and 52.4% (p=0.18) after allo and autoSCT (median FU 4.9 and 3.8), respectively. In conclusion, there was no significant difference in OS and EFS between allo and autoSCT matched-pts using PS between 1989 and 2007, despite a trend toward better EFS in alloSCT. For pts transplanted after 2000, this trend was confirmed, due to larger use of NMA-C alloSCT. As expected with the use of NMA-C we observed lower TRM and associated with very low relapse rate (none in our cohort) these data suggest that longer FU may result in significant better OS and EFS after alloSCT.

Table. Patients and disease characteristics of the entire cohort.

	AlloSCT	AutoSCT
Number of patients	27	116
Sex ratio male/female	14/13	68/48
Ann Arbor Stage IV at diagnosis (%)	27 (100)	82 (73)
Transformation before SCT (%)	4 (15)	18 (16)
Median number of previous regimens	4 [3-4]	3 [2-4]
Time from diagnosis to transplant (y)	3.2 [2.2-5.2]	3.7 [2.5-6]
Time from last relapse to transplant (y)	0.7 [0.6-0.9]	0.5 [0.4-0.7]
At transplant :		
Median age (y)	40 [36-46]	50 [46-55]
Chemosensitive disease (%)	26 (96)	102 (88)
Complete remission (%)	11 (41)	48 (41)
Partial remission (%)	15 (55)	54 (47)
Progressive disease (%)	1 (4)	1 (1)
Stable disease	0	3 (2)
Unavailable (%)	0	10 (9)
Period of transplant		
After 2000 (%)	16 (59)	48 (41)
Allogeneic transplant		-
MA-C (%)	14 (52)	-
AutoSCT before allo	5	-
NMA-C (%)	13 (48)	-
AutoSCT before allo	13	-
Matched sibling donor (%)	21 (78)	-
Unrelated donor (%)	6 (22)	-
Autologous transplant		
TBI in conditioning (%)	-	68 (59)

(MA-C : Myeloablative Conditioning regimen, NMA-C : Non MA-C)

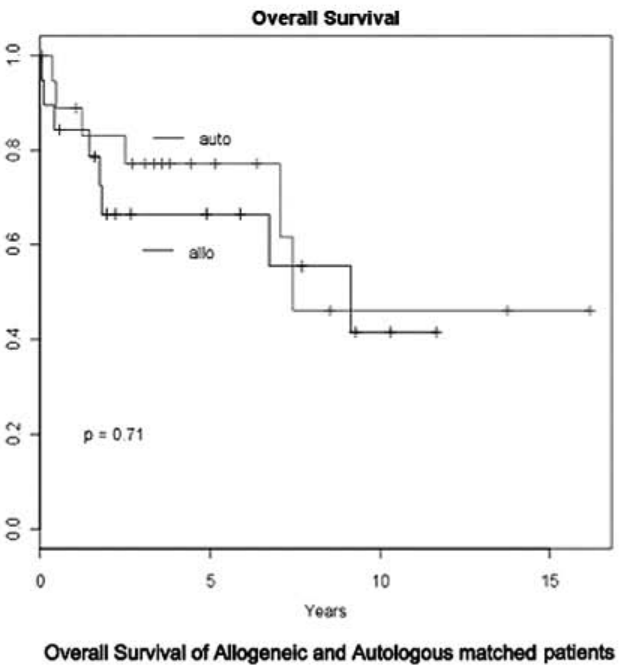

Figure 1. Characteristics of patients, and OS curve.

0714

TREATMENT OF MANTLE CELL LYMPHOMA BY ALLOGENEIC STEM CELL TRANSPLANTATION - A PRELIMINARY REPORT FROM THE TRIALS #060 AND #074 OF THE EAST GERMAN STUDY GROUP FOR HAEMATOLOGY AND ONCOLOGY (OSHO)

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Introduction. Mantle cell lymphoma (MCL) has a poor prognosis under conventional therapy. Allogeneic stem cell transplantation (SCT) after reduced-intensity conditioning is here a promising approach. Graft-versus-lymphoma effect has the power to eradicate minimal residual disease as shown by PCR-detection of chromosomal translocation t(11;14) or clone-specific CDR-III regions. *Aims and Methods*. To investigate the efficacy of chemotherapy-based conditioning followed by allogeneic SCT for treatment of MCL, two ongoing protocols have been established: Trial #074 is open for patients with *de novo* MCL and #060 for patients requiring salvage therapy. At least a partial remission to (re)-induction therapy is mandatory for proceeding to allogeneic SCT. Con (12 g/m^2) and fludarabin (150 mg/m^2). Busulphan (16 mg/kg) and cyclophosphamide (120 mg/kg) is optional for younger patients. ATG is given prior to mismatched or mud SCT. *Results*. 26 patients have been recruited into both ongoing trials (#060: n=10, #074: n=16) so far. Eighteen patients (69%) are male and 8 (31%) are female with a median age of 60 years (range: 32,6-69,1). Induction therapy prior to TX consisted mainly of R-CHOP or R-DHAP. Twenty patients proceeded to allo-TX from matched related or matched unrelated donors so far. One patient died from progressive disease prior to TX. Sixteen patients (80%) were conditioned with treosulfan/fludarabine and four (20%) with busulphan/cyclophosphamide. Sixteen patients are well (KI: 80-100%) and alive after SCT with a median follow-up of 12 months (0,3-78,9) in CCR. Three patients have died from early infections and one from infection related to an acute GvHD IV° due to DLI for relapse (blastic variant) ten months after SCT in complete remission of the lymphoma. Molecular analyses showed a 2-4log reduction of circulating lymphoma cells after chemotherapy alone. Blood became negative by qPCR after allogeneic SCT in all 5 patients analysed so far. An intermediate increase of circulating lymphoma cells in two patients was successfully treated by rituximab and withdrawal of Cy-A. *Conclusions*. Allogeneic SCT is a promising approach for treatment of MCL. Clinical long-term remissions can be reached and negativity of minimal-disease analyses by quantitative PCR strongly supports curative potential of allogeneic SCT for MCL.

0715

PRE-TRANSPLANT RISK ASSESSMENT FOR AML PATIENTS: COMBINED RISK FACTOR ANALYSIS MAY BE USED TO PREDICT OUTCOME AFTER ALLOGENEIC STEM CELL TRANSPLANTATION FOLLOWING REDUCED INTENSITY CONDITIONING

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Purpose. Allogeneic stem cell transplantation (AlloSCT) following reduced intensity conditioning (RIC) expands potentially curative therapy to patients with high-risk AML ineligible to transplantation following standard conditioning due to advanced age, a history of severe infectious complications, or major organ dysfunction. In addition to a number of disease-specific variables the overall outcome of these patients is determined by comorbidities, as indicated by an unfavorable hematopoietic cell transplantation-specific comorbidity index (HCT-CI), and transplantation-specific factors, i.e. the lack of an HLA-identical donor. Here, we investigated whether a combined risk-factor analysis may predict the overall outcome of AML patients after RIC-alloSCT. *Patients and Methods*. 90 patients with high-risk AML (median age 51 (17-68) years) who underwent alloSCT at our institution were retrospectively analyzed. 62/90 patients (71%) were in CR1 or CR2 and 26/90 (29%) had active disease. 57/90 patients (63%) had *de novo* AML and 33/90 patients (37%) had either secondary AML (sAML) or therapy-related AML (tAML). 4/90 patients (4%) had a favorable risk karyotype, 51/90 patients (57%) or 29/90 patients (32%) had an intermediate-risk or a poor-risk cytogenetics as defined by the SWOG/ECOG criteria. 18/90 (20%) patients had an intermediate-risk HCT-CI (1-2 points) and 72/90 patients (80%) had an unfavorable score (≥3 points). 74/90 patients (82%) were transplanted from a matched related or a matched unrelated donor. A mismatched unrelated donor was available in 16/90 patients (18%). As a preparative regimen all patients received RIC (fludarabine 180 mg/m^2 + oral busulfane 8 mg/kg + antithymocyte globulin 40 mg/kg). *Results*. Projected overall survival (OS) or disease-free survival (DFS) of all patients at 1, 3, and 5 years was 60%, 44%, and 39% or 56%, 46%, and 39%. 49/90 patients (54%) are in CCR. Causes of death were relapse [23/90 patients (26%)] or TRM [18/90 patients (20%)]. Depending on the presence or absence of at least one additional leukemia-specific risk factor (tAML, unfavorable-risk karyotype, disease status prior to alloSCT >CR2), or transplantation from a mismatched unrelated donor, patients were grouped into four categories: group I (HCT-CI <4, no additional risk factor), group II (HCT-CI >4, no additional risk factor), group III (HCT-CI <4, additional risk factor), and group IV (HCT-CI >4, additional risk factor). Projected OS in at 1, 2, and 4 years after alloSCT was 75%, 63%, and 63% (group I), 100%, 67%, and 67% (group II), 58%, 45%, and 36% (group III), or 31%, 15%, and 15% (group IV), which differed statistically significant between the 4 subgroups (p=0.016). In turn, there was no statistically significant difference in TRM between groups I - IV (p= 0.27). *Conclusions*. These results indicate that the combined risk-factor analysis may be useful in predicting the overall outcome of patients with AML after RIC-alloSCT.

0716

REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION DURING APLASIA IN PRIMARY HIGH-RISK AND RELAPSED ACUTE MYELOID LEUKEMIA

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Background. High risk acute myeloid leukemia (AML) as well as relapsed disease after conventional chemotherapy remains a challenging disease category. Immediate allogeneic hematopoietic stem cell transplantation (HSCT) during aplasia after cytoreductive chemotherapy harnessing reduced intensity conditioning regimens seems to be an option for remission induction and potential cure. *Methods*. This retrospective analysis reported here summarizes 82 consecutive patients with a median age of 50 years (range 17-71) who underwent allogeneic HSCT using reduced intensity conditioning on a chemotherapy- (n=67) or total body irradiation- (n=15) based regimen after a median of 34 days (range 11-91) after primary diagnosis or relapse of AML. Included were high risk AML patients defined by poor risk cytogenetics referring to SWOG criteria (n=38) or inadequate blast clearance after induction chemotherapy (n=20) and patients with relapsed AML (n=24). Allogeneic HSCT from related (n=29) or unrelated (n=53) donors occurred with engraftment in 81 patients. *Results*. With a median follow up of 39 months for patients alive (range 9 - 119 months) the probability of overall survival was 47% at two years after HSCT. The cumulative incidence of non-relapse mortality (NRM) and relapse were 25% and 35%, respectively. Reasons for death besides relapsing AML and acute or chronic Graft-versus-Host-Disease were bacterial infections (n=8), fungal infections (n=6), bleeding hemorrhage (n=3) and CMV infections (n=2). Analysis indicates that FLT-3 ITD mutations account for a lower OS and DFS in these patients as well as for a higher risk of relapse after HSCT. Age, karyotype and blast count after preceding chemotherapy were not predictive for overall outcome. Patients receiving transplants as part of primary therapy had a more favorable outcome (OS 53% vs. 27%, respectively, p=0.03). Patients with a Sorror score of 0 to 2 (n=63) had a better OS compared to patients with a Sorror score of 3 to 6 (n=19) (OS 54% vs. 30%, respectively, p=0.013). *Conclusions*. Immediate allogeneic HSCT performed during aplasia after induction or salvage chemotherapy is a feasible approach increasing the rate of remission and durable response in patients with high-risk AML. The identification of a suitable donor is an important part of this strategy. Given the increasing availability of unrelated volunteer donors, about 60-70% of patients with high-risk AML should be eligible for this treatment algorithm.

0717

IMPROVED SURVIVAL AFTER STEM CELL TRANSPLANTATION WITH KILLER-CELL-IMMUNOGLOBULIN-LIKE RECEPTOR (KIR)/HLA MISMATCHED ALLOGRAFTS IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA

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Background. HLA class I antigens are ligands for killer cell immunoglobulin-like receptors (KIRs). These receptors are expressed by NK and T-cells and thus modulate innate and adaptive immunity. KIR mismatches have been suggested to modify the outcome after stem cell transplantation. The clinical impact of killer cell receptors may vary between disease entities and pre- and post transplant therapeutic regiments. We have therefore performed a retrospective study with patients transplanted in our centre between 1998 and 2007. *Aims*. To analyze the impact of KIR / HLA mismatches on posttransplant relapse, transplant-related mortality and overall survival in allografted AML-patients. *Patients and Methods*. In a consecutive single center cohort of 147 AML patients, samples from 54 donor/patients, pairs were evaluable for retrospective KIR-ligand matching. The median patient age was 47 (25-66) years. Patients were transplanted with G-CSF-stimulated PBSC (n=51) or bone marrow (n=3) from HLA matched unrelated (n=31) donors or family related donors (n=23). KIR typing was performed as previously described. Patients were categorised according to their HLA inhibitory KIR ligand group C1, C2, Bw4, A3/A11 and presence or absence of KIR.

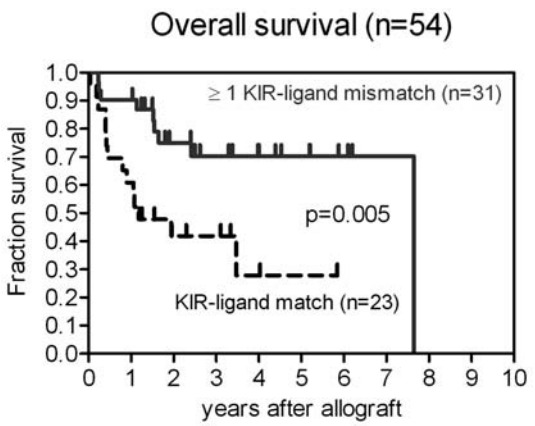

Figure 1. Survival in allografted AML patients.

Results. Overall survival (OAS) of all 54 evaluable patients at 5 years is 53%. The median follow up of surviving patients is 2,6 (range 1 - 6.2) years. A total of 23 patients died due to relapse (n=14) or non-relapse mortality (NRM, n=9). Patients with KIR-mm had a lower NRM (3/31 vs. 5/23, p=0.12) and a lower mortality due to relapse (6/31 vs. 8/23, p=0.15). OAS was superior in patients with KIR-ligand mismatches (KIR-mm) compared to the group of patients without mismatches (70% versus 28%, log rank p=0.005). Patients with 2 KIR mismatches (C1 and/or C2) had even better survival compared to patients with single KIR mismatches. *Conclusions*. Patients with AML benefit from KIR-ligand mismatched allografts. Allografted AML-patients with KIR mismatch have a significantly superior survival. The mortality due to both relapse and as well as NRM is reduced. KIR typing would be a useful tool to be included to define optimal histocompatibility of donor patient pairs.

0718

PLERIXAFOR CAN PREDICTABLY MOBILIZE HEMATOPOIETIC STEM CELLS IN PATIENTS WITH MULTIPLE MYELOMA PREVIOUSLY TREATED WITH LENALIDOMIDE

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Background. Emerging literature suggests that lenalidomide negatively affects the ability to collect CD34+ cells in patients with multiple myeloma (MM) undergoing hematopoietic stem cell transplantation. *Aims*. The aim of this retrospective subgroup analysis is to examine the efficacy of mobilization with plerixafor + G-CSF (P+G) among patients previously treated with lenalidomide. *Methods*. Patients with MM who participated in a phase III study (3102) assessing the safety and efficacy of plerixafor in hematopoietic stem cell mobilization, or in a compassionate use program (CUP) who received P+G and who had complete data on number of cycles of lenalidomide and total CD34+ cells collected were analyzed. Trial designs of the aforementioned studies have been described previously (DiPersio ASH 2007, Calandra BMT 2007). Briefly, in study 3102, patients received P+G as a first line mobilization regimen, whereas patients in the CUP received P+G as rescue therapy after failing other mobilization regimens.

Table 1.

	Study 3102 n=5	CUP n=22	All Patients n=27
Median Number of Lenalidomide Cycles (Range)	4 (1-5)	4.5 (2-20)	4 (1-20)
Median Collection of CD 34+ (x10^6 cells/kg) (Range)	14.8 (4.6-22.2)	3.29 (0.45-13.53)	4.3 (0.45-22.2)
Percent of Patients that Collected ≥ 5 x 10^6 CD34+ cells/kg	4/5 (80%)	7/22 (32%)	11/27 (41%)
Percent of Patients that Collected ≥ 2 x 10^6 CD34+ cells/kg	5/5 (100%)	15/22 (68%)	20/27 (74%)
Median Time (in days) to Neutrophil Engraftment (Range)	12 (11-13)	11 (10-16)	11.5 (10-16)
Median Time (in days) to Platelet Engraftment (Range)	18 (13-22)	18.5 (11-42)	18 (11-42)

Results. Of the 148 patients randomized to P+G in 3102, seven patients were previously treated with lenalidomide. In the CUP database, 60/708 patients were previously treated with lenalidomide. Complete data on number of cycles of lenalidomide and total CD34+ cells collected was available for 27 patients; five from 3102 and 22 from CUP. The mean age was 61.2 7.9 years and 14/27 (52%) of the patients were men. The median number of lenalidomide cycles prior to mobilization was four (range, 1-20). Administration of P+G resulted in a median collection of 4.3×10^6 CD34+ cells/kg. A total of 20/27 (74%) of the patients collected 2×10^6 CD34+ cells/kg in a median of two apheresis sessions. All the patients in 3102 collected 2×10^6 CD34+ cells/kg compared to 15/22 (68%) of the patients in CUP. Overall, 22 (81.5%) patients proceeded to transplant. The median time to neutrophil and platelet engraftment was 11.5 days and 18 days, respectively. *Conclusions*. These preliminary results suggest that the majority of patients with MM pretreated with lenalidomide can be successfully mobilized (collection of 2×10^6 CD34+ cells/kg) with plerixafor plus G-CSF

0719

INTRAVENOUS BUSULFAN IN AUTOLOGOUS STEM CELL TRANSPLANTATION CONDITIONING REGIMEN FOR ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA. MULTICENTRE RETROSPECTIVE ANALYSIS

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Background. Busulfan (Bu) is a common drug used in myeloablative hematopoietic stem cell transplantation (SCT) conditioning regimes for acute myelogenous leukaemia (AML) treatment. The incidence of sinusoidal obstructive syndrome (SOS) is 3 to 10% when oral Bu is used. Intravenous (Iv) Bu has a predictable pharmacocynetic profile and therefore, a reduced toxicity. *Aims and Methods*. In this retrospective analysis,

we show the results of autologous SCT with Iv Bu based conditioning regimen in 60 AML patients (27 males; 33 females) treated in 8 Spanish centres from 2003 to 2008. Median age at SCT was 56 years (18-76); 7 were more than 70. SCT was performed in first complete remission (RC1) in 55 (91.7%) patients, in second remission (RC2) in 4 (6.6%) and in partial response in one case. Cytogenetic risk at diagnosis was low in 6.9%, intermediate in 44.8% and high in 48.3%. The conditioning regimen was BuCy (Iv Bu 0.8 mg/kg/6 hours x 4 days + cyclophosphamide 60 mg / kg x 2 days), in 38 patients (63.3%); BEA (Iv Bu 0.8 mg / kg / 6 hours x 4 days + etoposide 40 mg/Kg + Ara-C 3 g / m^2 x 4) in 21 (35%); and other schema was used in one case. *Results*. Median time to obtain 0.5×10^9/L neutrophils was 11 days (7-52). Adequate platelet recovery was not reached in 3 cases (2 of them relapsed few months later); in the other 57 cases, median time to 20 $\times10^9$/L platelets was 15 days (7-122). One patient died before day +100 post SCT because an early relapse, thus, SCT related mortality was 0%. Only 1 patient died during complete response due to septic shock in the fourth month post SCT, with a global 2 year cumulative non relapsed mortality incidence of 1.6%. There were no cases of SOS. With a median follow up of 10.5 months for alive cases, the 2 year cumulative probability of overall survival (OS) was 53% (median OS: 38 months in RC1; 8 months RC2) and event free survival (EFS) was 44% (median EFS: 18 months in RC1; 6 months RC2). OS and EFS were better in patients < 60, although toxicity in older patients was no different. There was not any difference in OS or EFS in SCT conditioned with BuCy versus BEA. *Summary*. In this retrospective study of autologous SCT in AML patients with Iv Bu in the conditioning regimen, toxicity was low and results encouraging, with no worse EFS than the one obtained with other SCT modalities.

0720

HIGHER INFUSED CD34+ HEMATOPOIETIC STEM CELL DOSE CORRELATES WITH EARLIER LYMPHOCYTE RECOVERY AND BETTER CLINICAL OUTCOME AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN NON-HODGKIN'S LYMPHOMA

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Background. Lymphocyte recovery after autologous stem cell transplantation (ASCT) has been shown to be associated with positive clinical outcome in non-Hodgkin's lymphoma (NHL). Among the factors known to affect lymphocyte recovery following ASCT are infused lymphocyte count and NK cell count. However, the relationship between infused autograft CD34+ cell dose and lymphocyte recovery following ASCT has not been firmly established, although the number of CD34+ cells is commonly used to predict the potential engraftment of ASCT. *Aims*. This study sought to identify variables that affect lymphocyte recovery including infused lymphocyte count and CD34+ cell dose, and survival in patients with NHL receiving ASCT *Methods*. A retrospective analysis of outcomes in 97 consecutive patients who underwent ASCT for NHL in a single center from August 1999 to January 2008 was conducted. The relationships between infused lymphocyte count and infused CD34+ cell count with days to recovery of absolute lymphocyte count (ALC) were analyzed using the Spearman correlation coefficient. *Results*. We did not observe a significant relationship between infused lymphocyte count and days to recovery of ALC ≥500/mm^3 following ASCT (ALC500) (r=0.139, p=0.176), but there was a significant inverse correlation between infused CD34+ cell count and days to ALC500 (r=-0.333, p=0.001). Many prognostic factors were associated with OS and/or EFS by univariate analyses, including age-adjusted IPI (0-1 vs. 2-3), stage (I-II vs. III-IV), disease status at the time of ASCT (complete remission [CR] vs. not CR), immunophenotype (B- vs. T/NK-cell NHL), recovery of ALC500 (<20th vs. ≥20th day), collected CD34+ cell count (<10.4 vs. ≥10.4×10^6 cells/kg) and infused CD34+ cell dose (<8.2 vs. ≥8.2 $\times10^6$ CD34+ cells/kg). The median OS and EFS were not reached for patients with ALC500 < 20th day while they were 10.3 months and 4.8 months for patients with ALC500 ≥20th day, respectively (p=0.006 for OS and p=0.024 for EFS). The median OS and EFS were significantly longer in patients who received ≥8.2×10^6 CD34+ cells/kg than in those who received <8.2×10^6 CD34+ cells/kg (OS, not reached vs. 11.6 months, p=0.001; EFS, not reached vs. 4.8 months, p=0.003). No differences in OS and EFS by infused lymphocyte count (<0.98×10^8/kg vs. ≥0.98×10^8/kg lymphocytes) were observed (p=0.612 for OS and p=0.744 for EFS). Multivariate analysis showed that infused CD34+ cell dose was the most important independent predictor of OS (HR=0.314, p=0.017) and EFS (HR=0.175, p=0.002) for this sample set of patients who received ASCT for NHL. It did not provide evidence that other variables found to be significant by univariate analyses were independent predictors of survival once CD34+ dose is taken into account. *Summary and Conclusions*. In contrast to previous reports, we did not observe any association between infused lymphocyte count and kinetics of ALC recovery following ASCT. We found, instead, that higher infused CD34+ cell dose correlated with earlier lymphocyte recovery and better clinical outcome after ASCT in patients with NHL.

Table 1.

Prognostic factors	OS			EFS		
	P-value	HR	95% CI	P-value	HR	95% CI
Immunophenotype (B- vs T/NK-cell)	0.378	1.335	0.703-2.536	0.757	1.106	0.585-2.090
Stage <II vs ≥III	0.175	1.818	0.766-4.315	0.122	1.986	0.833-4.731
CR vs not CR	0.468	1.462	0.524-4.077	0.376	1.585	0.572-4.391
Age-adjusted IPI, 0-1 vs 2-3	0.174	2.023	0.733-5.577	0.281	1.758	0.631-4.896
Collected CD34+ cell count ($X10^6$/kg) <10.4 vs ≥10.4	0.589	0.724	0.224-2.339	0.111	0.354	0.099-1.268
Infused CD34+ cell count at ASCT ($X10^6$/kg) <8.2 vs ≥8.2	0.017	0.314	0.121-0.813	0.002	0.175	0.057-0.532
Recovery of ALC ≥ 500 cells/mm^3 <20 vs ≥20th day	0.247	1.620	0.716-3.667	0.366	1.441	0.653-3.179

0721

SEARCH OF AN UNRELATED DONOR FOR PATIENTS WITH HIGH RISK ACUTE LYMPHOBLASTIC LEUKEMIA: RESULTS OF A PROSPECTIVE, UNICENTRIC STUDY

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Background. Allogeneic hematopoietic stem cell transplantation (HSCT) plays a major role in the treatment of patients with high risk (HR) acute lymphoblastic leukemia (ALL) because of the features at diagnosis or in >II complete remission (CR); the search of an unrelated donor is therefore a common strategy for patients (pts) lacking a family compatible donor. *Aims*. To assess the effectiveness of the search on the outcome of pts with HR ALL. *Methods*. We analyzed prospectively 136 pts, 81 males and 55 females, median age 11 years (1-59), who were addressed, at our Center, to a search of an unrelated donor through the BMDWW Registry and cord blood (CB) banks between April 1995 and August 2008. *Results*. At the start of the search, 37 pts were in I CR, 70 in II CR, 3 in >II CR and 26 in relapse. The probability of finding a donor, CB or volunteer donor (VD), by cumulative incidence (CI) was 62% and 72% at 3 and 6 months, respectively; after this time, no increase in the CI of finding a donor was registered. The median time from the start of the search to finding a donor was 1.5 months for a CB and 3.5 months for a VD. Fifty % of the 110 patients who entered the search in CR showed a relapse at a median of 4 and 2 months from the start of the search for pts in I-II CR and >II CR, respectively. Of the 102/136 pts with a donor, 62 underwent a transplant: 25% of pts were in a more advanced phase compared to the start of the search; 35% of the pts who started the search in relapse obtained a CR at a median of 3 months and received an HSCT. Forty % of pts with a donor failed to undergo a transplant because of lost eligibility due to disease progression. The 10 year probability of OS was 23% for the whole population. In univariate analysis, relapse vs no relapse during the search (HR: 4.1; CI% 95: 2.75-6.12) and transplant vs no transplant (HR: 0.62; CI% 95: 0.4-0.98) significantly affected OS. For pts who underwent an HSCT, the phase of the disease at the time of transplant was the most important factor affecting OS. *Conclusions*. By decreasing the length of the search (waiting for more than 3 months increased the probability of finding a donor only by 10%, while half of the pts relapsed), the risk of relapse can be reduced, increasing the possibility of performing a transplant. The possibility of addressing simultaneously the search for a VD and a CB allows to

increase the probability of carrying out a transplant prior to the median time of relapse. During the search of a donor for pts with HR ALL, the major strategic factor should be "the time" of the transplant, rather than the type of the transplant; based on this strategy, the possibility of performing an haploidentical transplant must be considered.

0722

SINGLE CENTER COMPARATIVE STUDY OF UNRELATED UMBILICAL CORD BLOOD TRANSPLANTATION VERSUS HLA MATCHED FAMILY DONOR TRANSPLANTATION IN ADULTS WITH ACUTE LEUKEMIA AND HIGH RISK MYELODISPLASTIC SYNDROME

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Background. Allogenic cord blood transplantation (CBT) is usually considered as a secondary option for adult patients with acute leukemia, being the prioritary option a peripheral blood stem cell transplantation from HLA matched related donor (PBSCT). *Aims*. To show that cord blood can be a valid source of hematopoietic stem cells for adults with hematologic malignancies, with, at least, similar outcomes than PBSCT. *Methods*. We here present a retrospective study comparing outcomes of CBT done with our method of co-infusion of third party donor (TPD)-derived peripheral blood mobilized hematopoietic stem cells (MHSC), and outcomes of peripheral blood stem cell transplantation from HLA-matched related donors (PBSCT). 88 transplantations (in 79 patients) realized in our center between March 1999 and February 2008 have been included. All the patients undergoing CBT received a myeloablative conditionning regimen with reduced extra-hematologic toxicity. They were made with cord-blood units having 0-3 HLA missmatches and relatively low cell number: Total nucleated cell count after decongelation 1.14-4.57×10^7/Kg (median 2.39×10^7/kg) and $CD34^+$ cells precryopreservation 0.035-0.37×10^6/Kg (median 0.11×10^6/kg). All patients undergoing PBSCT received a sub-myeloablative conditionning regimen. *Results*. Medians and ranges of survival observed for both groups were similar, and Kaplan-Meier analysis determines a five-year overall survival slightly superior (0.53 vs. 0.41) for CBT (no statistical significance). In the cases of PBSCT, five-year OS is similar regardless of the diagnosis, with a slightly better OS for the patients with MDS diagnosis. In the cases of CBT there were no statistically significative differences in the OS depending on the diagnosis. Our study included comparative analysis of times from diagnosis to transplantation, disease status at transplantation (disadvantageous for CBT), as well as engraftment data, post-transplantation morbility (infectious complications, GVHD) and causes of death (data not shown). *Conclusions*. Our data are demonstrative of the safety and effectiveness of CBT as compared to PBSCT in adults with acute leukemia or high risk myelodisplasia. Thus, CBT must be considered as a primary unrelated stem cell source for these patients.

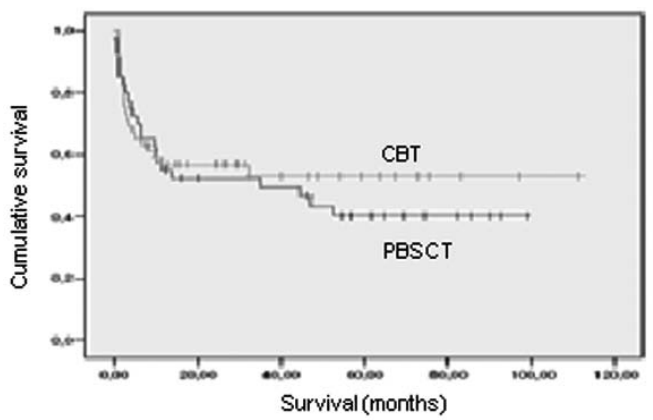

Figure 1.

0723

SECOND ALLOGENEIC STEM CELL TRANSPLANTATION AS SALVAGE TREATMENT IN RELAPSED HAEMATOLOGICAL MALIGNANCIES: FEASIBILITY AND OUTCOME IN 26 PATIENTS

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Background. Allogeneic hematopoietic stem cell transplantation (allo-SCT) provides for many patients (pts) with high risk acute leukaemia the best chance for long-term survival. Beside the classical matched related donors, a considerable amount of information about the use of unrelated volunteer donors (VUD), family mismatched donors (HAPLO) and unrelated umbilical cord blood (UCB) has recently become available. Instead of the deep effort in exploitation of allo-SCT, still remain a significant incidence of disease relapse. Often such pts still present with good general condition thanks to reduced toxicities conditioning regimens and better supportive therapy. In selected cases a viable options is represented by the possibility of a second allo-SCT, often an haploidentical one, in order to exploit the maximum graft-versus-leukaemia (GVL) effect through the haplotype mismatch. *Aims of study.* We report the retrospective analysis of outcome of 2nd allo-SCT at our Institution in the last 4 years. *Patients and Methods.* Between August-2004 and January-2009 26 pts -median age 45 years, range (r) 27-66, 9 male- received at our Institution a 2nd allo-SCT due to relapse after a previous one. Diagnoses were AML (n=21), ALL (n=3), CML (n=1) and multiple myeloma (n=1). First donor was an HLA identical sibling in 10 cases, an HAPLO in 11, a VUD in 4, a UCB in 1. Eleven pts were in complete remission (CR) at 1st transplant. Fifteen pts received a treosulfan based conditioning regimen. Median time from 1st allo-SCT to relapse was 153-days (r29-1174), median time from 1st to 2nd allo-SCT was 213-days (r53-1220), median time from relapse to 2nd allo-SCT was 47-days (r10-572). Thirteen pts received a 2nd allo-SCT from the same donor, 13 from an different one (all HAPLO); second donor was a VUD in 1 case, an HLA identical sibling in 5, an HAPLO in 20. Only 5/26 were in CR at 2nd allo-SCT. Eighteen pts received a treosulfan based conditioning regimen. The CMV host/donor serostatus was: +/+ 20, ±5, -/+1. *Results*. Engraftment was evaluable in 22pts, median time to neutrophils >500/ucl 17-days (r6-33) and to platelets >20.000/ucl 21-days (r10-39). Fourteen pts presented acute GVHD (grade I 1 pt, grade II 6, grade III 4, grade IV 3), 1 out of the 13 evaluable pts presented chronic GVHD. Overall there were 17 (65%) deaths, 8 caused by disease progression or relapse, 9 by infection (6), acute GVHD grade IV (2) and III (1). Of 22 evaluable pts 19 achieved CR, and currently 9 are alive and in CR (7/9 after a 2nd HAPLO). The median follow-up for pts alive is 234-days (range 28-687). At 1-year, the probability (95% confidence interval) of disease free survival is 25 (3-47)%, the non-relapse mortality is 44 (16-72)% and the overall survival is 34 (10-58)%. *Conclusions*. Second allo-SCT represents an effective therapeutic option for pts relapsed after a 1st allo-SCT. HAPLO will be a valid choice to better exploit GVL effect. A deeply evaluation of the state of the art and effectiveness of HAPLO as 2nd allo-SCT is now ongoing within EBMT Acute Leukaemia Working Party.

0724

OUTCOME OF ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN 30 ACUTE MYELOID LEUKEMIA PATIENTS AGED 60 YEARS OR OLDER

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Introduction. Older age is considered one of the most important adverse prognostic factors in Acute Myeloid Leukemia (AML). Allogeneic stem cell transplantation (allo-SCT) is one of the most recommended approach in poor prognosis AML but there are limited data about this procedure in elderly AML patients (pts). We report our experience about the feasibility and outcome of allo-SCT in AML pts aged 60 years or older. *Patients and results*. Between 2003 and 2008, 30 consecutive AML pts aged 60 years or older (median age 63; range 60-70 years) received an allo-SCT at our Center, and all of them were high risk at diagnosis. The median time from diagnosis to transplant was 7 months. At the time of transplant 12/30 cases (40%) had an advanced disease (relapsed or refractory), while 18/30 (60%) were in complete remission (CR). The hematopoietic cell transplantation comorbidity index (HCT-CI) was two or less in 12/30 cases (40%) and three or more in 18/30 cases (60%).

Donor were MUD in 12 (40%) and sibling in 18 (60%) of 30 allo-SCT. Twenty-six of 30 pts (87%) received a reduced intensity conditioning regimen (RIC). Conditioning regimen consisted of Thyotepa + Cyclophosphamide±ATG 14/30(46%), Fludarabine-based RIC 10/30(33%), Busulfan + Cyclophosphamide ± ATG 4/30(14%) and other regimens 2/30(7%). All patients engrafted. Acute GvHD was observed in 17/30 cases (57%) with 13 having grades I-II and 4 having grades III-IV. Data on chronic GvHD was available for 22/30 pts (73%); of those 3/22 (14%) developed extensive chronic GvHD. One year Nonrelapse mortality (NRM) rate was 20% (6/30 cases). At the time of analysis, after a median follow-up of 16 months (range 1-64), 17/30 pts (57%) were alive and in CR while 13/30 (43%) have died (leukemia refractory or relapse 7/13 and NRM 6/13). One year probability of Overall Survival (OS) of the whole population was 57%. The OS did not differ between unrelated and related donors. The pts transplanted in CR and those with a HCT-CI < 3 have a significantly better OS compared to those with refractory or relapsed AML at transplant or with HCT-CI > 3 (log rank=0,04 and 0,01, respectively). CONCLUSIONS. Our data indicate that allo-SCT is a feasible treatment option in selected poor prognosis AML pts older than 60 years. In this experience NRM rate is only 20% and OS rate (1 year 57%) is promising taking into account the poorer outcome of elderly AML pts. Favourable outcome was observed in cases with a low HCT-CI (2 or less) and in those transplanted while in CR. For older AML patients lacking a suitable family donor MUD can provide a suitable alternative option.

0725

TANDEM AUTO-SCT AND REDUCED-INTENSITY CONDITIONING (RIC) ALLO-SCT FOR RELAPSED OR TRANSFORMED AGGRESSIVE B CELL NON-HODGKIN'S LYMPHOMA (NHL)

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Background. Allogeneic stem cell transplantation (allo-SCT) can provide a potentially curative graft-versus-lymphoma effect. However, such benefit is usually offset by a significant incidence of non-relapse mortality (NRM), especially in advanced and/or elderly patients. With this background, RIC regimens for allo-SCT have arisen as an attractive modality in patients with NHL, but the role of RIC allo-SCT in aggressive B cell NHL remains a matter of debate because of a higher incidence of disease relapse/progression. *Aims*. This single centre study aimed to assess the potential benefit of a tandem auto-allo-SCT approach as salvage therapy in 23 consecutive patients with relapsed or transformed aggressive B cell NHL. *Methods*. Median age at time of allo-SCT was 55 (range, 44-65) y. NHL histology at diagnosis included 5 cases (22%) of DLBCL, 12 (52%) follicular NLH, and 6 (26%) other subtypes. Aggressive transformation was documented in 18 patients (78%; 26% of primary and 52% of secondary transformation). Patients received a median of 2 (range, 1-4) lines of previous chemotherapy regimens prior to allo-SCT, and all patients (n=21; 91%) but 2, could actually proceed to auto-SCT prior to allo-SCT. At time of allo-SCT, 9 patients (39%) were in first CR, 6 patients (26%) were beyond first CR and 8 (35%) in PR. Median times between diagnosis and allo-SCT and auto-SCT and allo-SCT were 25 (range, 7-131) and 4 (range, 2.4-71) m. respectively. The RIC regimen consisted of Fludarabine, Busulfan and ATG in 20 patients (87%) and Fludarabine and low dose TBI (2 Gy.) in 3 patients (13%). *Results*. After RIC allo-SCT, engraftment was achieved in all patients. With a median follow-up of 38 (range, 3-86) m., 13 patients had grade 2-4 acute GVHD (56%) and 10 patients had extensive chronic GVHD (48%). Overall, 9 patients died (3 from progression, 2 from acute GVHD, 2 multiorgan failures, and 2 other causes). The incidence of NRM was 26%, (95%CI, 8-44%). At last follow-up, 13 patients (57%) were in sustained CR. The Kaplan-Meier estimate of progression-free and overall survival rates were 51% and 64% respectively at 4 years. *Conclusions*. We conclude that a tandem auto-RIC allo-SCT approach is a potentially efficient salvage therapy for relapsed or transformed aggressive B cell NHL, with a relatively low toxicity, likely overcoming the poor prognosis usually associated with this phenotype. These results can also likely be improved with strategies aiming to enhance the GVL effect (e.g. inclusion of Rituximab as part of the RIC regimen, and/or as maintenance therapy).

0726

COMPLETE HEMATOLOGIC REMISSION BUT NOT MOLECULAR REMISSION IS ASSOCIATED WITH PROLONGED SURVIVAL IN PATIENTS WITH ACUTE LEUKEMIA BEFORE HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. In patients with acute myeloid (AML) or acute lymphoblastic leukemia (ALL), many trials proved that low or negative minimal residual disease (MRD) during consolidation treatment or after/before hematopoietic stem cell transplantation (SCT) is associated with prolonged survival. *Aims*. In this retrospective analysis we searched for significant risk factors including MRD level affecting progression-free (PFS) and overall survival (OS) in patients with AML and ALL before SCT. *Methods*. A total number of 34 hematopoietic stem cell transplantations performed in years 2003 to 2008 in 30 patients with a median age of 39.4 years (range: 18.3 to 60.8) were included. A molecular marker for MRD monitoring was available in all patients; fusion genes (NPM1, MLL, AML1/ETO, CBFβ/MYH, or CEBPA) in AML and bcr/abl or TCR/IgH gene rearrangements in ALL. The data were analyzed for complete hematologic remission (CR) and molecular remission rate, relapse rate, time to molecular and hematologic relapse, causes of death and risk factors affecting survival. *Results*. There were 29 allogeneic (9 sibling, 10 matched unrelated and 10 partially matched or mismatched unrelated donors) and 5 autologous SCT performed in 23 AML and 11 ALL patients. Myeloablative conditioning regimen was used in 20 (59%) cases, reduced intensity conditioning in 14 (41%) cases. Before SCT, 23 (68%) patients were in CR and 11 (32%) in molecular remission. After SCT, 28 (82%) patients achieved CR and 26 of 27 (96%) evaluable patients achieved molecular remission. Molecular relapse occurred in 15 (58%) cases within a median of 167 days (range: 56 to 490). Hematologic relapse occurred in 13 (46%) cases within a median of 212 days (range: 85 to 1128), while in all but one cases it was preceded by molecular relapse. Median PFS and OS of the whole set were 9.8 and 26.8 months, respectively. Out of all risk factors analyzed, only CR before SCT was associated with significantly longer PFS and OS (p=0.004 and p=0.005, respectively). Molecular remission before SCT had neither influence on PFS nor OS (p=0.30 and p=0.26, respectively). At the end of the follow-up period with a median of 9.9 months (range: 0.3 to 47.9) there were 16 (47%) patients alive in complete remission, 5 (15%) relapsed and 13 (38%) dead, with multiple organ failure, infection and disease progression being the most common causes of death. *Summary and Conclusions*. According to our analysis, in patients with acute leukemia before hematopoietic stem cell transplantation, complete hematologic remission but not molecular remission is associated with prolonged progression-free and overall survival.

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Stem cell transplantation - miscellaneous

0727

SMALL HUMAN ,-DEFENSIN-3 DERIVED PEPTIDE INDUCES RAPID AND PREFERENTIAL MOBILIZATION OF MOUSE HEMATOPOIETIC PROGENITOR CELLS VIA SDF-1 SECRETION

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Mobilization of stem cells from the bone marrow (BM) to the circulation by repeated G-CSF stimulations is widely used for clinical transplantation protocols. The chemokine stromal-derived factor-1a (SDF-1, CXCL12), expressed and secreted by BM stromal cells, and its major receptor CXCR4, expressed both by the stromal cells and hematopoietic stem/progenitor cells, are key players in G-CSF-induced mobilization (Petit *et al.*, Nat Immunol, 2002). The CXCR4 antagonist AMD3100 induces fast mobilization of hematopoietic stem/progenitor cells. Preliminary results from our studies revealed that rapid mobilization of immature murine progenitor cells (but not mature leukocytes) by AMD3100 is regulated by accelerated release of SDF-1 to the circulation from BM stromal cells in a CXCR4-dependent manner (Dar *et al.*, ASH abst., 2006). In addition, it has been found that human ß-defensin-3 (HBD3), a 45-amino acid peptide, is able to internalize CXCR4 and decrease SDF-1-induced migration of human T cells (Feng *et al.*, JI, 2006). ß-defensins are cysteine containing antimicrobial peptides that are major components of the innate immune system. We synthesized a short, 10-amino acid peptide comprising the C-terminal part of HBD3, named TL-1, which is not toxic. Unexpectedly, upon a single s.c. injection to C57BL/6 mice, TL-1 efficiently mobilized hematopoietic progenitor cells (but not mature leukocytes) within 1-2 hrs post injection in a dose dependent manner. Importantly, TL-1 synergized with G-CSF and with AMD3100 in their capability to mobilize both mature leukocytes and immature progenitor cells. In addition, injection of TL-1 was accompanied by a significant elevation in plasma SDF-1 levels, which is important for rapid progenitor cell mobilization. As expected, *in vitro* TL-1 treatment was accompanied by specific down-regulation of membrane CXCR4 expression, as well as by inhibition of SDF-1-induced (but not spontaneous) leukocyte migration in a dose and time dependent manner. The strongest effect (90% inhibition at 0.5-1.0 mM) was found upon testing immature $CD34^+$ enriched human cord blood cells. *In vitro* studies with isolated BM stromal cell cultures revealed that TL-1 directly targets the BM stroma, inducing them to release SDF-1 from intracellular storages to the culture medium, independently from *de novo* protein synthesis. In addition, TL-1 directly induces cultured BM stromal cells to release metalloproteinase-2. Altogether, these findings show that TL-1 is a potential, non-toxic small peptide, capable of preferential mobilization of hematopoietic progenitor cells. The reasons for preferential progenitor cell mobilization by TL-1 and mechanistic insights, in addition to SDF-1 release, as well as the physiological roles of ß-defensins with regards to hematopoietic progenitor cell motility are currently investigated.

0728

HEMATOPOIETIC CELLS RECEIVE A STRONG IMPRINTING WHEN PASSING THROUGH BONE MARROW: RESULTS OF A COMPARISON BETWEEN INTRABONE VERSUS INTRAVENOUS TRANSPLANTATION

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Background. Intravenous infusion (IV) is the method conventionally used to transplant hematopoietic stem cells (HSC) despite the relatively low seeding efficiency. To overcome this problem, intrabone transplant (IBT) of HSC has been introduced to improve HSC homing and engraftment and to reduce the incidence of graft versus host disease. However, the mechanisms underlying the possible beneficial effect of IB are largely unknown. *Aims*. To investigate whether the improved results of IBT derive from an increased local engraftment or from an improved kinetic behavior of HSC. *Materials and Methods*. Thirty-three male Sprague Dawley rats, were included in the study. CD90 positive cells were selected by FACS/Sorting. Labeling was performed by incubating 2×10^6 cultured cells with 37 MBq of TC-99m exametazime (HMPAO, Ceretec, GE Healthcare) according to a procedure validated in our lab that provides a labelling yield (defined as HSC counts / total dose counts) ranging 10-13%, by a remarkable stability (up to 98% of bound activity still present at 24 hours) and by the lack of effect on HSC survival. Labelled CD90+cells were infused via tail vein (IV, n=7) or directly into the tibia (n=7) of anesthetized rats positioned over the head of a large field gamma-camera. Regions of interest (ROIs) were positioned on lungs, heart, liver, spleen and forearms to plot time activity curves of ROIs counting rate (counts per second) after decay correction. At the end of the study, animals were sacrificed and lungs, spleen, liver and axillary/inguinal lymphonodes were harvested. Organ radioactivity was expressed as [%] of injected dose, to estimate [%] of injected cells present in each organ. *Results*. Fifteen seconds after IBT, >90-95% of cells escaped from the tibia. Despite this the short permanence in the bone marrow, these cells showed a remarkably different behaviour with respect to the same HSC injected intravenously: faster blood clearance (heart activity at 40 min 2±0.7% vs. 5±1.5% of the dose, $p<0.01$), lower lung sequestration (5.22±0.5% vs. 13±2.5%, $p<0.01$) and lower lymphonode uptake (0.01±.005% vs .04±.008% of the dose, $p<0.01$). Logan plot analysis - performed to define the ratio between cells delivered and cells released by the forelimb - documented an increased HSC uptake in the bone marrow after IBT with respect to IV (ratio between trapped and released cells 2.2±.8 vs. 1±0.2, respectively $p<0.01$). By contrast, no differences were observed in the time activity curves in the liver and in the spleen. The preconditioning of rats with total body irradiation (TBI) selectively decreased cell recruitment of injected cells in the forelimb after ITB (N=7) but not after IV (n=7). On the contrary, TBI aspecifically increased lung sequestration of donor HSC both after intravenous and intrabone administration. Kinetics of hepatoma line cells injected intrabone (n=5) displayed a virtually absent uptake in the forelimb and a very high sequestration in the lung (38.63±4.94%). *Conclusions*. Despite its extremely short duration, the contact with the bone marrow environment changes HC behaviour and this experience modifies the kinetic features of these cells by enhancing their migration in far bone marrow sites.

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HUMAN MESENCHYMAL STEM CELLS CAN AMELIORATE HEMATOPOIETIC FUNCTION AFTER CHEMO-RADIOTHERAPY INDUCED STROMAL DAMAGE

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Background. It has been shown that bone marrow (BM) stroma is damaged after allogeneic bone marrow (BM) stem cell transplantation and previous data from our group confirmed that although peripheral blood cell counts are recovered, BM hematopoietic function, assessed *in vitro*, is impaired. Mesenchymal stem cells (MSC), an important component of BM stroma, support and regulate hematopoiesis. *Aims*. We hypothesized in the context of allogeneic transplantation that if recipient MSC are altered, the addition of normal MSC may result in a better hematopoiesis in both, *in vitro* and *in vivo* models. *Methods*. For the *in vitro* experiments, stromal damage was induced by adding etoposide (50 µM) for 48 hours on human MSC (hMSC) confluent layers. Hematopoietic stem cell ($CD34^+$) with or without MSC were added to the layers. To be used as controls, both $CD34^+$ cells with or without MSC were plated on non etoposide-treated layers. Cultures were maintained for 5 weeks in all cases. Number of CFU-GM, adipocytes and cobblestone area were weekly calculated. For the *in vivo* assays human $CD34^+$ cells were administrated either by intravenous (IV) or intrafemoral (IF) injection in NOD/SCID mice after 300cGy total body irradiation. In order to see whether or not MSC favour engraftment both IV or IF $CD34^+$ cell-treated mice received or not IF MSC. Human BM cell engraftment was measured by flow cytometry using anti-human CD45, CD13 and CD19 antibodies at 3 and 6 weeks after transplantation. To test human chimerism at the stromal level in the mice transplanted with MSC, BM mononuclear cells from both femurs (the injected and the contralateral) were obtained and MSC were *in vitro* expanded following standard methods until 3rd passage, when FISH for human X,Y chromosomes was performed. *Results*. Our *in vitro* data showed that the number of cobblestone areas and adipocytes were significantly lower ($p<0.05$) in etoposide-treated layers as compared to non damaged cultures. After 5 weeks, the median number of CFU-GM was 720 (range: 50-907) in treated stromas versus 1727 (range: 645-5005) in normal ones ($p<0.05$). Nevertheless, when normal MSC were added to the damaged layers all the

above mentioned parameters returned to normal ranges. Regarding the *in vivo* engraftment assay results are shown in Table 1. At 3 weeks, the number of human CD45+ cells in BM was significantly higher in mice co-transplanted with human MSC. At 6 weeks, human chimerism was higher when IB infusion was performed independently of the addition of MSC. Regarding stromal chimerism, hMSC engraftment was detected in those femurs where human MSC were previously injected but not in the contralateral ones. *Conclusions*. Our data show that MSC can favour the hematopoietic function after chemo-radiotherapy stromal damage. However in the longer follow-up, IB injection enhanced hematopoietic chimerism.

Table 1. Human engraftment.

		CD34IV	CD34IV +MSC	p	CD34IF	CD34IF +CSM	p
3 weeks	hCD45	5.35	14.53	<0.05	6.58	22.39	<0.05
	hCD13	0.36	1.39	N.S.	3.49	17.13	<0.05
	hCD19	0.05	0.09	N.S.	0.10	0.17	N.S.
6 weeks	hCD45	2.67*	4.85**	N.S.	13.98*	14.02**	N.S.
	hCD13	0.39*	0.65**	N.S.	2.38*	4.20**	N.S.
	hCD19	0.30*	2.15**	N.S.	5.73*	6.60**	N.S.

Results expressed as % of human cells among mice BM mononuclear cells.
*p<0.05 between CD34IV and CD34IF; **p<0.05 between CD34IV+MSC and CD34IF+MSC; N.S.= Not significance; IV= intravenous; IF=intrafemoral; MSC=mesenchymal stem cells.

0730

CXCL12 RS1801157 POLIMORPHISM IS ASSOCIATED WITH DECREASED MRNA CXCL12 EXPRESSION AND HIGHER HEMOPOIETIC PROGENITOR CELL MOBILIZATION

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Background. Interaction between CXCL12 and its receptor CXCR4 is critical for hemopoietic progenitor cell (HPC) homing in bone marrow. Administration of G-CSF leads to degradation of CXCL12 and mobilization of HPC to peripheral blood. This is the basis of peripheral blood collection of HPC for hemopoietic transplantation. *Aims*. To evaluate a possible association between polymorphisms in CXCL12 and in other genes involved in homing and migration of HPC with the degree of CD34+ cell mobilization after G-CSF administration. *Methods*. Twenty-seven SNPs described in 15 genes (CXCL12, CXCR4, VCAM-1, VLA-4, G-CSF, CSF3R, CD34, ADRB3, CXCL2, CXCR2, CD44, Kit ligand, c-Kit, MMP-9, CTSG) were analyzed by allelic discrimination PCR in 112 healthy donors receiving G-CSF (filgrastim; 10 µg/kg; 5 days) in a single institution. Univariate and multivariate multiple 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.

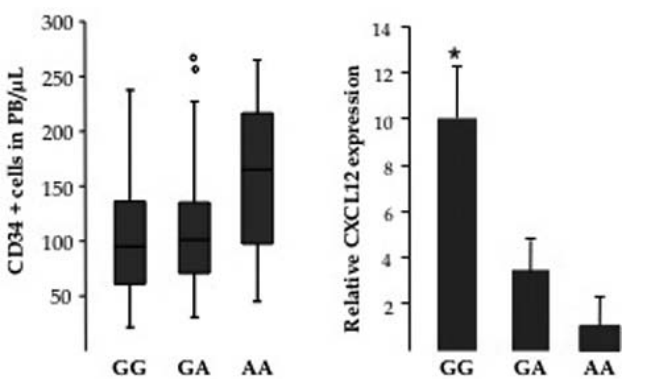

Figure 1.

Results. CD34+ cells/µL of peripheral blood after 5 days of G-CSF was of median 99 (21-267), and the total number of CD34+ cells collected with the first leukapheresis was of median 478x106 (83-2006). AA genotype corresponding to the homozygous less frequent variant at rs1801157 in CXCL12 was associated with a higher count of CD34+ cells in peripheral blood (median and range, AA 165, 44-265 vs. GA/GG 99, 21-267; p=0.018). Of note, AA genotype showed significantly lower mRNA CXCL12 level than GA and GG genotype, with a 3.5 fold and 10 fold lower expression, respectively (p<0.0001; Figure). CC genotype at rs3917924 in CSF3R corresponding to the homozygous most frequent variant and CC genotype at rs1041163 in VCAM1 corresponding to the homozygous less frequent variant were associated with a lower CD34+ cell count (median and range, CC 81, 21-176 vs CT/TT 106, 31-267; p=0.002 and CC 74, 53-141 vs TC/TT 102, 21-267; p=0.018, respectively). No differences were found in mRNA levels of CSF3R and VCAM1 classifying the groups by the pattern of SNPs. *Conclusions*. Genetic individual variability in CXCL12, VCAM1, and CSF3R seems to influence HPC mobilization. CXCL12 variant AA at rs18001157 was associated to lower mRNA levels of CXCL12, which may explain the higher CD34+ cell mobilization of this group of individuals. These findings might be useful in planning mobilizing strategies.

0731

REPORT OF 15-YEAR EXPERIENCE OF THE MILANO CORD BLOOD BANK: CLINICAL OUTCOMES OF SINGLE UNRELATED CORD BLOOD TRANSPLANTS

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Umbilical cord blood (UCB) is a useful alternative hematopoietic stem cell source for patients lacking a suitable donor. More than 400000 UCB units are stored in public cord blood banks and over 20000 procedures have been already performed worldwide. CB banks support this increasing clinical activity and aim to store good quality units and to maintain an inventory of CB units (CBU) with a large HLA diversity and adequate cell dose. Netcord works in collaboration with Eurocord to ensure good practice in CBU storage and to establish international standards and accreditation processes (eg Netcord-FACT). Eurocord provides annual analysis of biological and clinical outcomes of units provided by cord blood banks for accreditation requirements. We report the annual Eurocord analysis of the Milano cord blood bank (MICBB) activity as of December 2008. From 1994 to 2008, 379 CBU shipped by MICBB were reported to Eurocord. The units were used for 362 UCB transplants (UCBT) including 350 unrelated UCBT, 11 related transplants and one autologous transplant. Five CBU were not used (3 for disease relapse, 2 for unknown reasons). The 350 unrelated transplants included: 217 single unmanipulated UCBT (191 first transplant), 44 double UCBT, 2 single expanded UCBT, 2 single UCBT + haplo-identical PBSCT, 84 preliminary UCBT, 1 missing data. The CBU were issued to 24 countries and 122 centers (84 EBMT, 38 non EBMT). Forty-three percent of the units collected underwent volume reduction by semiautomated procedure. All units were cryopreserved in DMSO. At collection median volume was 117 mL (25-276); median TNC, CFU-GM and CD34+ counts were 16×10^8 (2.3-40.3), 6.44×10^5 (0.7-46) and 4×10^6 (1-19.8), respectively for units with available data. Among 191 first single unrelated transplants, analysis of outcomes was performed on 186 with available follow-up data. One-hundred-sixty hematological malignancies (acute leukemia=106, MDS=20, lymphoproliferative disorders=13, CML=11, histiocytosis=9, MM=1) and 26 non-malignant diseases (BMFS=9, metabolic disorders=9, immunodeficiency=8) were reported. Median age at transplant was 8.3y (0.2-65), (126 children, 60 adults) and median weight 27Kg (4-110). Nine percent of CBU were 6/6 matched to recipients, 53% of units had 1 HLA mismatch and 38% had 2 or 3 HLA disparities. Conditioning regimen was myeloablative in 86% of transplants. Graft-versus-host disease prophylaxis consisted of cyclosporine and corticosteroids in 60% of patients. Eighty percent of patients received ATG/ALG before day0. Median infused TNC dose was 4.02×10^7 TNC/Kg (1-25) and 1.70×10^5 CD34+cells/Kg (0.1-25). Median cell loss after thawing was 17%. Neither microbial contamination nor early post-infusion adverse reactions were reported. Median follow up was 35.2 m (2-122). Cumulative incidence (CI) of 60 day-neutrophil recovery (>500/mm^3), 180 day-platelet recovery (>20000/mm^3), 100 day-acute GVHD, 100 day-TRM were 81±3%, 67±4%, 34±3%, and 25±5%, respectively. Estimated overall survival at 36 months was 38±3%, with better survival for non-malignant diseases

(58±8% vs 34±4%) and for children (45±4% vs. 20±5%). Cord blood units provided by MICBB were of excellent quality according to Netcord-FACT standards guidelines and allow a high engraftment rate comparable with other reports on UCBT outcomes. Analyses such these are important in maintaining continuous high quality in UCBT.

0732

IRON OVERLOAD AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) EVALUATED BY SUPERCONDUCTING QUANTUM INTERFERENCE DEVICE (SQUID)

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Background. Iron overload (IO) is an adverse prognostic factor in patients who undergo allogeneic HSCT for thalassemia and appears to play a similar role in patients with other hematological disorders. *Aims*. to quantify IO by SQUID after HSCT and evaluate the impact on hepatic function and infections. Additionally, the feasibility of iron-depletion has been investigated. *Methods*. Between December 2005 and December 2007, 102 consecutive patients who received an allogeneic HSCT have been analyzed. Clinical characteristics were: median age 47 years (21-67), diagnosis of acute leukemia/MDS 61%, lymphomas 25% and others hematological malignancies in 14%. Assessment of IO after HSCT included serum ferritin; in those with hyperferritinemia (> 1000 ng/mL), liver iron concentration (LIC) was evaluated by SQUID magnetic susceptometry. Iron removal therapy was offered to patients with moderate (LIC 1000-2000 microg/gww) or severe (LIC >2000 microg/gww) IO. *Results*. Fifty-seven patients had a ferritin level <1000 ng/mL and the median time from HSCT to ferritin assessment was 1006 days, significantly different from the median time of 183 days of the 45 patients who had a ferritin level >1000 ng/mL. LIC evaluated by SQUID was available for 42/45 patients with elevated ferritin values. Overall, 29 patients had moderate to severe IO: median LIC values were 1493 microg/gww (range 1030-3253); 5 patients had normal LIC values (LIC<400 microg/gww) and 8 had LIC values between 400-1000 microg/gww. Potential confounding factors (hepatitis, cronicGVHD, disease status, timing of ferritin assessment) were tested in a multivariate analysis showing a significant correlation between ferritin levels >1000 ng/mL and the occurrence of liver dysfunction defined by the presence of at least one abnormal liver function test (LFT) on two or more occasions (OR 6.8; 95%CI 2.2-20.6). In addition, the rate of proven/probable invasive fungal disease was significantly higher among patients with hyperferritinemia as compared to patients with normal ferritin levels (13% vs 0%; p=0.006). Nineteen of the 24 patients considered eligible to iron depletion underwent regular phlebotomy: 13 completed the program after a median time of 10 months (3-13), reaching the target of ferritin <500 ng/mL; for 1 patient the program is still ongoing; 5 patients discontinued phlebotomy (relapse n=2; hypotension, n=1; anemia, n=1; fatal GVHD, n=1). Eight patients were revaluated by SQUID at the end of iron depletion program: LIC showed a significant reduction (median 1368 microg/gww to 606 microg/gww; p=0.005) consistent with the decrease of serum ferritin levels; one patient did not show a remarkable reduction of LIC despite serum ferritin normalization. Four of the 5 patients ineligible to phlebotomy were successfully treated with deferasirox and one with deferoxamine. *Conclusions*. The measurement of LIC obtained by SQUID documented the presence of moderate/severe IO in 69% of the patients with high ferritin levels. Our data showed that in HSCT recipients, high ferritin level is an independent risk factor for the occurrence of abnormal LFTs and IO may be considered a potential risk factor for fungal infections. A phlebotomy program resulted feasible in 58% of the patients who might benefit from a procedure of iron depletion.

0733

FREEZE-DRYING OF HUMAN UMBILICAL CORD BLOOD MONONUCLEAR CELLS

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Background. Today long term storage of human umbilical cord blood (HUCB) units is mainly done by freezing the mononuclear cell fraction with DMSO which results in 20-30% cell loss upon thawing. The units are usually stored in liquid nitrogen tanks. Long term storage in liquid nitrogen is expensive, requires means to avoid transient warming events and there is a risk (although small) of cross contamination between stored samples. Freeze-drying may be a superior alternative to avoid these problems. Freeze-drying is a process in which ice crystals sublimate (form vapor without going through the liquid phase) resulting in a dry, stable and low weight sample. We have developed a freezing apparatus, which is based on directional solidification technology, named the Multi Thermal Gradient (MTG) device. This device enables the precise control of ice crystals morphology during the freezing process, thus making the use of intracellular cryoprotectant agents redundant. *Methods*. The study was performed on mononuclear cells derived from HUCB. A freezing solution named IMT-2 was added prior to freezing. Freezing was done using the MTG-1314 freezing device. We tested the viability, number of $CD34^+$-presenting cells and ability of the rehydrated hematopoietic stem cells to differentiate into different blood cells in culture before freezing and after freeze thawing and freeze-drying. *Results*. The viability of the MNCs after freeze-drying and rehydration with pure water was 88%-91%. The total number of $CD34^+$-presenting cells and the number of colonies did not change significantly when evaluated before freezing, after freeze-thawing and after freeze-drying ($5.4\pm4.7\times10^4$, $3.49\pm6\times10^4$ and $6.31\pm12.27\times10^4$ cells, respectively, and 31±25.15, 47±45.8 and 23.44±13.3 colonies, respectively). *Summary*. In summary, we have developed a freeze-drying technique for maintaining the viability and functionality of cells. Using this technique hematopoietic stem cells have survived complete desiccation while maintaining their clonogenicity capabilities upon rehydration.

0734

IMPACT OF VITAMIN D RECEPTOR GENE POLYMORPHISMS ON CLINICAL OUTCOMES OF SIBLING HLA-MATCHED HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Vitamin D is a main regulatory hormone of calcium metabolism and bone mineralization. Its immunoregulatory action has been investigated recently, and polymorphisms of the vitamin D receptor (VDR) gene has been associated with some diseases, such as immune dysfunction, infection and malignancy. We hypothesized that polymorphisms of VDR may affect the clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT). *Aims*. This study was conducted to determine whether genotypes or haplotypes of VDR were associated with post-transplant clinical outcomes. *Methods*. Three VDR polymorphisms (BsmI G>A, ApaI G>T and TaqI T>C) were genotyped in 152 patients and their donors who underwent sibling HLA-matched allogeneic HSCT in single institution between 1998 and 2005. The median age of recipients were 40 years (range, 16-70) and eighty-six patients (57%) were male. The most common diagnosis was acute myeloid leukemia (AML 51, ALL 21, CML 21, MDS 12, NHL 10, Others 37). Reduced intensity conditioning was performed in 82 patients (54%) and conventional conditioning in 70 patients. Frequency of infection and graft-versus-host disease (GVHD), overall survival (OS) and disease-free survival (DFS) were compared according to genotypes and haplotypes.

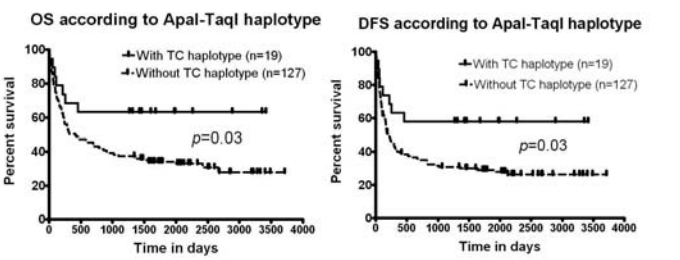

Figure 1.

Results. Patients with the ApaI TT genotype had less frequent infection and showed a tendency of less frequent acute GVHD than non-TT genotypes (p=0.01 and p=0.06, respectively). OS and DFS were longer for TT genotype (p=0.08 and p=0.045, respectively). For TaqI genotypes, there were no statistical difference in frequency of infection and acute GVHD (p=0.64 and p=0.30, respectively) but patients with the TC geno-

type showed longer OS and DFS than those with the TT genotype (p=0.03, both). These differences were not observed between BsmI GG/GA genotypes. In the ApaI-TaqI haplotype analysis, patients with the TC haplotype had significantly longer OS and DFS compared with those without the TC haplotype (p=0.03, both). In multivariable analysis to estimate the effects of VDR genotype or haplotype on survival, TaqI genotype and ApaI-TaqI haplotype of recipients were significant indicators of both OS and DFS. We found no associations between donor genotypes and clinical outcomes. *Conclusions*. Our results suggest that patient genotype and haplotype of VDR associate with clinical outcome of sibling HLA-matched HSCT. We need further studies to confirm these results in other populations and to reveal underlying mechanisms for these associations.

0735

IMMUNOBIOLOGICAL EFFECTS OF NKT CELLS FROM ACUTE MYELOID LEUKEMIA PATIENTS IN A PRE-CLINICAL ANTI-LEUKEMIC TRIAL

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Background. Recently, it was shown that natural killer T (NKT) cells play key roles in anti-tumor immune responses, and there is increasing evidence that NKT cells and NK-like T cells are involved in tumor resistance. However, their response to certain tumor cells and the molecules that are functionally critical are not known. *Aims*. In a new trial using an acute myeloid leukemia (AML)-specific animal model, we explored ways in which the anti-tumor activity of NKT and NK-like T cells could be used in cell therapy to suppress or even kill a patient's leukemia cells. *Methods*. First, we investigated the profiles of NK and NKT cells from both AML patients (n=46) and the HLA-matched normal donor population (n=20), using flow cytometry. We also used enzyme-linked immunosorbent assays (ELISA) to determine the expression of cytokines such as interferon-γ, interleukin (IL)-13, and IL-4 after stimulating peripheral blood mononuclear cell (PBMC) cultures with IL-2, IL-12, IL-18, and α-galactosylceramide, a CD1d-restricted NKT cell ligand. A cytotoxicity assay using the chromium release method was employed to examine the functional effects of defined NK and NKT cells against a patient's primary leukemic cells and leukemic cell lines. The NOD/SCID mice were transplanted with patient-derived leukemic cells at a dose of 2-106 bone marrow mononuclear cells (BMCs) and then infused with the same dose of either leukemic or normal PBMCs that had been cultured for 4 h with one of 4 cytokines at 1-week interval. The surviving mice were sacrificed and analyzed 8 to 24 weeks post-transplant; mice that died were analysed at the time of death. *Results*. The proportions of both $CD16^+CD3^-$ and $CD56^+CD3^-$ NK cells were lower in the AML group than in the control group, but the difference was not statistically significant. Based on a flow cytometric α-galactosylceramide-tetramer assay, the proportion of CD1d-restricted human NKT cells ranged from 0.2~0.4% of gated lympho-monocytic region in the AML group, compared with 0.2~0.7% in the normal controls. According to the cytotoxicity assay, the patient-derived and normal NK/NKT cells had similar cytotoxic activity at effector:target ratios of 1:20 to 1:40 against both primary leukemic cells and K562 cells. Of note, the CD56+CD1d-restricted cells in AML were more responsive to IL-12, IL-18, and α-galactosylceramide, whereas the CD16+CD1d-restricted cells were more responsive to IL-2. Of the 39 mice transplanted with cultured leukemic or normal PBMCs after exposure to each cytokine, only three mice were alive at 24 weeks. The other mice died beginning 10 days after transplant. Interestingly, after sacrifice, we found a much higher proportion (with 3.1~30.8% gated human $CD45^+$ bone marrow cells) of CD1d-restricted human NKT cells in the three mice serially transplanted with cultured leukemic and normal PBMCs in order, whereas splenic cells did not produce comparable results. *Conclusions*. These preliminary results suggest the high potency of autologous and allogeneic NKT cells specifically responsive to primary AML cells, as we had expected. We plan to develop a more practical AML-specific animal model for examining the clinical potential of certain immunobiological cells.

0736

THE EFFECT OF HUMAN UMBILICAL CORD BLOOD $CD34^+$ PROGENITOR CELLS TRANSPLANTATION ON DIABETIC MICE

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Background. Curative therapy for diabetes mellitus mainly implies replacement of functional insulin-producing pancreatic , cells, with pancreas or islet-cell transplants. However, shortage of donor organs spurs research into alternative means of generating, cells. Stem cells might represent a potential source of tissues for cell therapy protocols, and diabetes is a candidate disease that may benefit from cell replacement protocols. *Aims*. We examined the effect of transplanted human umbilical cord blood $CD34^+$ cells on some detailed parameters in streptozotocin- (STZ) induced diabetic mice. *Methods*. Thirty male albino mice 8-12 weeks were included and subdivided into 3 groups, first group served as normal control group, second group as diabetic control after induction of diabetes with STZ and third group treated diabetic mice by injection of positively selected CD34 progenitor cells from human umbilical cord blood(UCB) with a dose of one million cells/mouse. Blood glucose and serum insulin were measured at specific time interval and immunohistochemical analysis (IHC) and histopathology on pancreas were conduced. *Results*. Intravenous injection of $CD34^+$ cells caused significant improvement in blood glucose level (277.9±102.5 mg\dL in treated group vs 530.3±99 mg\dL in untreated group, p<0.01). Blood level of mouse insulin was higher in the treated group as compared with untreated diabetic mice (0.77±0.2 ng/mL intreated group versus 0.26±0.09 in untreated group, p<0.001). IHC analysis for detection of human insulin producing cells in pancreas of treated mice revealed that 33.3% positive cellular staining and 55.6% positive sinusoidal staining were detected. In conclusion, Transplantation of UCB - $CD34^+$cells appear to be a modality of stem cell therapy in diabetes mellitus of animal model.

0737

A COCKTAIL TO BYPASS FETAL BOVINE SERUM LIMITATIONS FOR THE PRODUCTION OF UMBILICAL CORD DERIVED MESENCHYMAL STROMAL STEM CELLS FOR CLINICAL USE

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Background. Wharton's jelly, which surrounds the two arteries and a vein of human umbilical cord (UC), derives from a mesenchymal precursor cell population. This primitive connective tissue contains a subpopulation that exhibits a functional mesenchymal phenotype and a fibroblast like appearance. In contrast to bone marrow (BM) and other adult derived mesenchymal stromal cells (MSCs), they have greater and faster expansion capability, higher frequency of colony forming unit fibroblast (CFU-F) than BM and UC blood (UCB) MSCs and an isolation efficiency of 100% when cultured in the presence of fetal bovine serum (FBS). They are karyotypically stable over many passages and easier to isolate than UC_MSCs, but they are slower to differentiate into adipocytes than BM. This aspect is partially in contrast with minimal criteria for defining multipotent MSCs established by the International Society for Cellular Therapies. *Aims*. Although the *in vivo* functions of UC_MSCs still need to be further investigated, we believe that these cells may represent a population able to potentially generate multiple doses of cells for cellular therapies. In addition, not all lots of FBS are equivalent in terms of their ability to maintain MSCs and there is a significant immunogenicity of FBS proteins. This has led many researchers to investigate alternative supplements. We investigated the effect of a growth factors (GFs) cocktail on UC_MSCs expansion, differentiation and phenotype, to define a FBS-free culture medium. *Methods*. MSCs were isolated from enzimatically digested UC (n=5). Cells were seeded at 1500 cells/cm^2 at the end of passage 0 (P0) and cultured in DMEM supplemented with FBS (10%); human platelet poor plasma (hPPP, 3%); hPPP (3%) with epidermal growth factor and platelet-derived growth factor-bb (EGF_PDGFbb, each at 10 ng/mL). Cells were harvested, counted and analysed by flow cytometer every 7 days for five passages (P5). The population doubling (PD) was determined at each passage to calculated the cumulative PD (cPD). Differentiation assays into adipogenic and osteogenic lineages were performed at the end of P2 and detected with Oil Red and von Kossa stain. *Results*. GFs cocktail supported UC_MSCs expansion better than hPPP (17.68±1.29 at the end of P5 and 12.00±2.06 respectively) and it showed a cPD similar to FBS (17.40±1.20. Figure 1A). In addition surface markers expression did not differ markedly in all culture conditions and

remained constant ($CD45^-/CD105^+/CD90^+$ about 96%, $CD45^-/CD105^+/CD44^+$ about 90%). Mineralization in the osteogenic differentiation was not appreciable. Cells cultured in the adipogenic medium appeared enlarged compared with negative control, but without lipid vacuoles in all medium supplements (Figure 1B). This result confirms that UC_MSCs are early-stage cells and that our cocktail does not induce maturation or differentiation. *Conclusions*. We defined a medium supplement, hPPP (3%) with EGF_PDGFbb (10 ng/mL), which permits us to bypass same limitations connected with FBS, including potential transmission of prion disease and the immunogenicity of the xenogeneic proteins. Furthermore, it allows us to fully exploit the potential of UC which represents a unique, easily accessible and not controversial source of early stem cells for cell therapy with low risk of viral contamination.

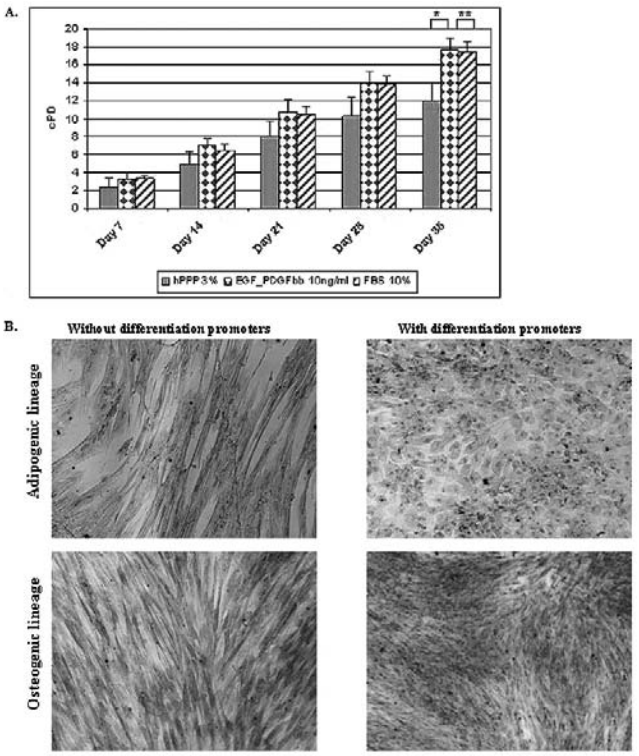

Figure 1. A. UC_ MSCs cPD (n=5) in three different medium supplements: FBS (10%); hPPP (3%) with and without EFG_PDGFbb each at 10 ng/mL. Our cocktail, defined in a previous study, can substitute FBS. *p<0.005; **not significant. B. Differentation of human UC_MSCs into adipogenic and osteogenic lineages after pre-treatment of 14 days with GFs cocktail. Differentation was detected with Oil Red O or von Kossa stain. Magnification 10X.

0738

POLYMORPHISMS OF MTHFR AND CLINICAL OUTCOMES IN ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Methotrexate (MTX) is an antifolate drug used to prevent graft versus host disease (GVHD) in allogeneic stem cell transplantation (alloSCT). Its function is due to inhibition of dihydrofolate reductase and limitation of function of other enzymes like MTHFR. It is well known that MTHFR 677 polymorphisms results in reduced activity of this enzyme and that the use of MTX in patients with 677 CT/TT variations may influence side effects like mucositis and bone marrow toxicity. *Aims.* In this study we try to correlate the impact of these polymorphisms on clinical outcome of patients submitted to alloSCT which received MTX in their GVHD prophylaxis. *Methods*. Samples of 47 pts submitted to alloSCT from 2002 and respective donors were analyzed for 677CT polymorphism. Pts' characteristics were: 30M/17F, median age 37years (range10-58). Underlying disease were 23 AML, 17 ALL, 2 IMF, 2 SAA, 2 CML and 1 CLL. In 39 case pts were submitted to standard conditioning regimen and in 8 cases pts were submitted to reduced intensity conditioning regimen. All pts were submitted to GVHD prophylaxis with CSA and short-course MTX. Stem cell source was BM in 5pts, PBSC in 38pts and CBU in 4pts. The donor were HLA identical siblings or parents in 32 cases and MUD in 11 pts. All CBU were unrelated. *Results*. Seven pts died early after tx (14.8%) at a median time of 45d (range 30-90) for progression of disease. Twenty-two out of 47 (46.8%) developed hepatic toxicity and the median time for PMN (>0.5×10^9/L) and PLT (>20×10^9/L) recovery was respectively 20 and 15 days (range 10-48 and 11-55days). Twenty-two pts developed aGVHD grade II-IV at a median time of 26d after tx (range 10-70). Twenty-five pts relapsed after tx at a median time of 4 months (range 1-34). At this time 24pts are alive with a median follow-up of 31 months (range 1-51). OS, RFS, NRM and GVHD curves were obtained by the Kaplan Meier method and statistically compared by log-rank test; while incidence of toxicities was evaluated with chi2test. Our data revealed that there was no correlation between 677CT polymorphisms and development of hepatic toxicity and/or delayed engraftment (χ^2 test=ns); aGVHD was significantly correlated with CC polymorphism in the donor (p=0.05) and there was a trend toward a lower incidence of aGVHD in pts with TT polymorphism. Regarding NRM at 100days after tx we found a strong correlation with CC polymorphism in pts (p=0.006), whilst we found a trend toward a higher incidence of relapse in pts with TT polymorphisms. *Conclusions*. We conclude that greater immunosuppressive effect of MTX due to low MTHFR enzyme activity in mutated pts (TT) decreases the risk of GVHD but increases the risk of relapse.

0739

PROGNOSTIC FACTORS FOR SUCCESSFUL MOBILIZATION AND COLLECTION OF PERIPHERAL BLOOD STEM CELLS (PBSC) IN PATIENTS WITH HEMATOLOGIC MALIGNANCIES. A STUDY OF 209 PATIENTS

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Background. High dose therapy and autologous stem cell transplantation is frequently applied in patients with multiple myeloma (MM), Hodgkin lymphoma (HL) and non-Hodgkin's lymphoma (NHL). This procedure requires successful mobilization and collection of PBSC. However there are patients, who are poor mobilizers and require multiple mobilization courses and/or apheresis procedures, or fail completely to collect PBSC. *Aims*. To study various clinical and laboratory parameters as possible predictive factors for successful mobilization and collection of PBSC in MM, HL and NHL patients. *Methods*. 209 patients who were mobilized in our Unit between 2004 and 2008 were studied. The following parameters were analyzed: age, gender, underlying disease, number of previous chemotherapies, mobilization regimen, disease status pre-mobilization and baseline laboratory parameters. Since more than one mobilization courses and/or apheresis procedures were performed in some patients, two methods of analysis were applied. Method A: All mobilization courses were analyzed with the following endpoints: maximum absolute number of circulating $CD34^+$ cells and number of $CD34^+$ cells collected for each mobilization procedure. Method B: Each patient was considered only once with the following endpoint: mean number of $CD34^+$ cells collected per apheresis. *Results*. Median age at mobilization was 42 years (13-70), 64% were men, 64% had received 0-1, 34% 2-3 and 2% 4 or more previous chemotherapy regimens, 43% had HL, 33% NHL, 21% MM and 3% acute leukemia. Eleven % received G-CSF as mobilizing regimen, 33% cyclophosphamide + G-CSF, 35%, 17% and 4% regimens containing platinum, ifosfamide and high dose cytarabine + G-CSF, respectively. Before mobilization, median Hb was 11.4g/dL (7.8-16), median WBC 5.96×10^9/L (0.79-27.3), median PLT 260×10^9/L (70-853) and median absolute lymphocyte counts 1.1×10^9/L (0.1-3.4). The maximum absolute number of circulating $CD34^+$ cells, as well as the number of $CD34^+$ cells collected per kg of body weight were statistically significantly higher for ifosfamide-, platinum-, high dose cytarabine-, cyclophosphamide- and G-CSF- containing mobilizing regimens in this order (p<0.001). The mean number of $CD34^+$ collected cells per kg of body weight was 8.89×10^6, 5.14×10^6 and 1.30×10^6 for patients who had received £1, 2-3 and 4 or more previous chemotherapies (p=0.001). Other significant factors were age (p<0.001), and base-

line PLT count (p<0.001). Underlying disease and disease status before mobilization were also statistically significant. Thus, HL patients achieved a higher number of CD34+ collected cells compared to NHL and MM patients (p=0.002). Interestingly, patients with refractory disease prior to mobilization had a higher number of CD34+ collected cells compared to patients in relapse and those in remission, (p=0.003). Similar results were obtained with the 2nd method of analysis. *Conclusions*. Factors that affect successful mobilization and collection of PBSC include the mobilizing regimen (superior outcome with ifosfamide-containing regimens), the number of previous chemotherapies (inferior collection with 4 or more), age, baseline PLT count, disease status pre-mobilization (superior collection in refractory patients) and underlying disease (best outcome n HL patients).

0740

AXP IN ROUTINE CORD BLOOD BANKING: CRITICAL ANALYSIS OF DIFFERENT VOLUME REDUCTION METHODOLOGIES

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Background. Volume reduction of cord blood (CB) units is a proceeding used in most cord blood banks in order to reduce cryogenic space. To date, many methods have been described for this purpose as red blood cell (RBC) sedimentation with hydroxyethyl starch (HES), semi-automated methods as top and bottom and automated systems as Sepax. The AXPTM system is an automatic method for CB volume reduction recently developed, that consists of a microprocessor-controlled device and disposable closed bag set. *Aims*. Our objective was to compare different volume reduction methodologies in routine CB banking. *Methods*. We compared three different methodologies for volume reduction in routine CB banking: 1. HES: HES was added to the anticoagulated CB in a proportion of 1:4 HES solution to blood and then centrifuged at 45 x g for 7 minutes at 10°C according to the method developed by Rubinstein and coworkers. The removed supernatant was centrifuged again at 600 x g for 15 minutes. 2. Top and Bottom: CB units collected in triple-bag system were centrifuged at 3000 x g for 12 minutes. A standard protocol programmed into Compomat G4 was used to process the CB units. 3. AXPTM: Cord blood was transferred to the bag set and centrifuged in the AXP device for 30 minutes. All CB units were volume-reduced to less than 30 ml. Volume and cell counts were determined before and after volume reduction process. *Results*. Final CB volume containing cryopreservation solution was 25.5±1.2 mL, 28.3±0.4 mL and 24.1±0.5 mL for HES, G4 and AXP systems respectively (p<0.001); while total nucleated cell content (TNC) recovery was 76.4±11.2% for HES, 71.9±6.8% for G4 and 76.7±8.0% for AXP (HES vs AXP p=ns). Red blood cell depletion was higher for HES and AXP (86.1±5.8% vs 86.8±4.7, p=ns) and lower for G4 (69.5±8.0, p<0.001). *Conclusions*. HES sedimentation and AXPTM CB volume reduction systems provide similar cell recoveries and RBC depletion. AXP system reduces CB volume automatically in a less-time consuming process, achieving acceptable cell recoveries and high red blood cell depletion in a closed system without HES.

0741

PLATELET ENGRAFTMENT IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES FOLLOWING UNMANIPULATED HAPLOIDENTICAL BLOOD AND MARROW TRANSPLANTATION: EFFECTS OF CD34+ CELL DOSE AND DISEASE STATUS

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Background. Unmanipulated haploidentical blood and marrow transplantation has been developed as an alternative transplant strategy for patients without a human leucocyte antigen (HLA)-matched related or unrelated donor [Annals of Medicine, 2008,40:444; Bone Marrow Transplant,2006,38:291]. In this transplant setting, factors associated with hematopoietic recovery have not been completely defined. *Aims*. The aim of this study was to investigate the effects of donor and recipient characteristics on neutrophil and platelet engraftment following unmanipulated haploidentical transplantation. *Methods*. Unmanipulated haploidentical blood and marrow transplantation was performed on 348 patients with hematologic malignancies at a single institution between 2002 and 2007. Factors correlating with neutrophil and platelet engraftment post-transplant were analyzed retrospectively. *Results*. All patients reached an absolute neutrophil count (ANC) of 500/μL in a median of 13 days (range, 9-49 days). Three hundreds and thirty-one of 348 patients (95.11%) reached an untransfused platelet count of more than 20 000/μL in a median of 16 days (range, 7-356 days). In the univariate analysis, six factors were found to be associated with platelet engraftment, age of recipients (p=0.022), HLA-match (p=0.022), disease status (p=0.011), infused nuclear cells/kg of recipient weight (p=0.045), infused CD8+ cells/kg of recipient weight (p=0.031), and infused CD34+ cells/kg of recipient weight (p<0.001). Among patients who reached a sustained platelet count of more than 20 000/μL, an increased CD34 cell dose was suggestively associated with a shorter time to platelet recovery, although the strength of the association was relatively weak (R=-0.237, p<0.001). A trend towards an association of sex (female vs. male) with neutrophil engraftment was observed (p=0.084). Finally, a trend for association between time to transplantation after diagnosis (≤210d vs. >210d) and disease stage (advanced stage vs. early stage) with neutrophil engraftment was also demonstrated (p=0.056, and 0.089, respectively). Multivariate analysis showed that infused CD34+ cells/kg of recipient weight (CD34+ cells>2.19-106/kg vs ≤2.19-106/kg, HR=1.695; 95% CI 1.361-2.112; p<0.0001), and disease status (advanced vs early, HR=0.724; 95% CI 0.577-0.907; p=0.005) were independently associated with an increased risk of platelet engraftment.(Figure 1A and 1B) *Conclusions*. Our results suggest that low number of CD34+ cells in allografts and advanced stage disease may be critical factors associated with delayed platelet engraftment after unmanipulated haploidentical transplantation.

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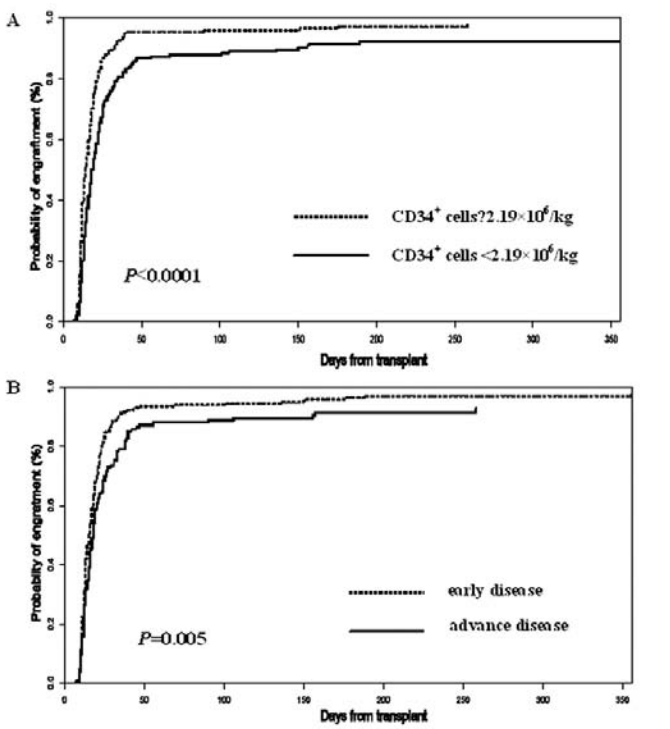

Figure 1.

Novel therapeutics, targeted therapies and gene therapy - Preclinical

0742

A VON WILLEBRAND FACTOR INHIBITORY ANTIBODY EFFECTIVELY INHIBITS ARTERIAL THROMBOSIS IN A HIGH SHEAR RATE MODEL IN BABOONS

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Background. Given the high incidence of cardiovascular events, there is a need for safer and more effective antithrombotic therapies. The fact that von Willebrand factor (vWF) plays a pivotal role in primary haemostasis, by initiating adhesion of circulating platelets to exposed subendothelium and aggregation of platelets at high shear stress conditions, makes the development of an antithrombotic agent with vWF as target a very attractive option. *Aims*. To determine the efficacy and associated bleeding risk of a vWF inhibitory (Anti-vWF A1-domain) antibody as antithrombotic agent in non-human primates, and to compare it with clopidogrel in the same model. *Methods*. The efficacy of the antibody was evaluated in a high shear rate arterial thrombosis model in baboons. Different concentrations of the vWF inhibitory antibody were tested in three baboons, and three baboons were tested with clopidogrel for comparison. The femoral artery of the baboon was shunted to the femoral vein. The artery was mechanically injured and external stenosis applied. This resulted in thrombus formation measured by decreased blood flow. Thrombi were mechanically dislodged resulting in a pattern of cyclic flow reductions (CFRs). Escalating doses of antibody were infused and the effect of this treatment on the CFRs measured. Bleeding was assessed with an incision bleeding model as well as a template bleeding time. A Full Blood Count (FBC), Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) were performed and Factor VIII (FVIII) and vWF levels determined. *Results*. The anti-vWF antibody completely inhibited arterial thrombus formation in a dose dependant manner. After complete inhibition, infusion of epinephrine did not reverse the inhibition, unlike observations with clopidogrel. This is significant in view of the fact that epinephrine levels drastically increase during stressful situations. Considerably less bleeding was observed than with clopidogrel. vWF levels were reduced in the course of the experiment, but FVIII levels were not affected by either surgical procedure or antibody. The FBC, PT and aPTT were not adversely affected. *Conclusions*. A vWF inhibitory antibody is an effective antithrombotic agent in a high shear rate arterial thrombosis model in baboons, and compares favourably with clopidogrel, but with less bleeding risk and better efficacy during stressful conditions.

0743

LEUKEMIA INDUCED BY ALTERED TRK-SIGNALING IS SENSITIVE TO TREATMENT WITH MTOR INHIBITORS IN A PRECLINICAL MODEL

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Neurotrophins (NTs) and their receptors play a key role in neurogenesis and survival. The TRK (tropomyosin-related kinase) receptor protein tyrosine kinases (TRKA, TRKB, TRKC) are high affinity NT-receptors that are expressed in a variety of human tissues. Their role in normal and malignant hematopoiesis is poorly understood. Recently, we and others have obtained evidence for potential involvement of this receptor system in leukemia. In a prospective study involving 94 adult patients, we demonstrated for the first time cell surface expression of the three TRKs and constitutive activation in blasts from patients with *de novo* or secondary acute leukemia. At least one TRK receptor was expressed in 55% of the analyzed cases (Li Z *et al.*, Blood 2009 in press). Altered TRK signaling efficiently transformed murine hematopoietic stem/progenitor cells (Meyer J *et al.*, Leukemia 2007; Li Z *et al.*, Blood 2009 in press). We observed constitutive activation of mammalian target of rapamycin (mTOR) both in murine and human leukemic cells. Murine leukemic cells induced by altered TRK signaling were very sensitive to rapamycin or RAD001 treatment *in vitro*. We next tested the therapeutic effect of rapamycin on altered TRK-induced leukemia in a mouse model (C57Bl/6J). Leukemic cells isolated from #483 mouse transplanted with primary hematopoietic stem/progenitor cells modified with deltaTrkA, an active mutant of TRKA isolated from a patient with acute myeloid leukemia, grew factor-independently. Treatment of #483 cells with rapamycin or RAD001 (10-50 nM) induced apoptosis and induced a dose-dependent growth inhibition (up to 100%) in colony forming assays. Consistently, mTOR was strongly dephosphorylated. In pilot studies, we found that i.v. injection of 10E6 #483 cells into recipients conditioned with sublethal irradiation (7.5Gy) induced leukemia in all animals after a latency of 8 weeks. Furthermore, daily i.p. injection of 2mg/kg rapamycin or 1mg/kg RAD001 in healthy animals mediated a high drug level in whole blood (around 50ng/mL and 175ng/mL, respectively). Thus, 20 sublethally irradiated animals were i.v. injected with 10E6 #483 cells and randomized in two groups. One group was treated daily with rapamycin 2mg/kg i.p., the other received only carrier (placebo). The treatment begun 3 weeks after cell injection and continued until the last animal succumbed to leukemia. Rapamycin treatment significantly prolonged the survival of animals compared with control group (mean survival 48.5 and 32 days, respectively, p=0.0087) (Figure 1). Concentration of rapamycin in whole blood at the time of end point analysis ranged 26- 151ng/mL. In a separate experiment RAD001 treatment (1 mg/kg) had a similar effect (Figure 2). Two RAD001-treated animals were even free of leukemia upon termination of the experiment (11 weeks). Concentration of RAD001 in whole blood at the time of end point analysis ranged 53-347ng/mL. Our findings suggest that mTOR plays an important role in leukemogenesis induced by altered TRK signaling, and might serve as a therapeutic target.

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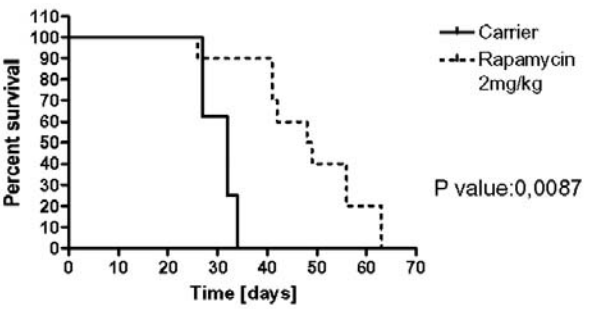

Figure 1. Kaplan-Meier estimate of survival of animals after treatment with rapamycin.

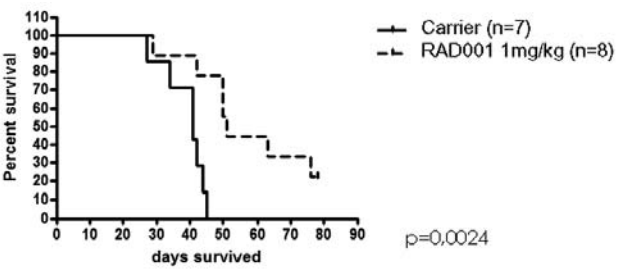

Figure 2. Kaplan-Meier estimate of survival of animals after treatment with RAD001.

0744

PRELIMINARY EVIDENCE OF CLINICAL ACTIVITY IN A PHASE 1 STUDY OF CAL-101, A POTENT SELECTIVE INHIBITOR OF THE P110DELTA ISOFORM OF PHOSPHATIDYLINOSITOL 3-KINASE, 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, including metabolism, proliferation and survival. The PI3K p110delta isoform is highly expressed in cells of hematopoietic origin and plays a key role in B cell maturation and function. CAL-101 is an oral, potent inhibitor of PI3K p110delta (IC50 of 2.5 nM against purified enzyme) with 40 to 300-fold selectivity compared to other PI3K isoforms. The lack of inhibitory activity against the PI3K p110α isoform should minimize the potential to alter insulin signaling and may provide a better therapeutic index relative to pan-PI3K inhibitors currently in development. *In vitro* studies of 0.1 to 10 uM CAL-101 showed inhibition of pAKT expression and/or apoptotic effects against primary chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) cells and against a range of leukemia and lymphoma cell lines. A Phase 1 study of CAL-101 in healthy volunteers showed that the drug was well tolerated and did not affect serum glucose or insulin levels at drug exposures that resulted in target inhibition. *Aims*. Evaluate the initial safety and clinical activity of CAL-101 in patients with select hematologic malignancies. *Methods*. In an ongoing phase 1 dose escalation study in sequential cohorts of 3 patients with relapsed/refractory CLL or select B-cell non-Hodgkin's lymphoma, CAL-101 is administered orally twice daily for 28 days per cycle. Clinical response is evaluated according to NCI criteria at the end of Cycles 1 and 2 and every 2 cycles thereafter. *Results*. To date, 9 patients have been treated in the first 3 cohorts at dose levels of 50 mg, 100 mg and 200 mg twice daily, with durations ranging from 1 to 7 months. Dose limiting toxicity has not been observed. There have been no significant adverse effects on hematological parameters or blood glucose. Mean peak and trough drug concentrations at the end of the first cycle were 1.5 uM and 0.3 uM in the 50 mg cohort, 2 uM and 1 uM in the 100 mg cohort and 6 uM and 1 uM in the 200 mg cohort, respectively. The trough concentrations are in the range required to inhibit PI3K p110delta in primary patient cells and cell lines *in vitro*. To date, three patients with the following diagnoses have attained partial responses: mantle cell lymphoma with 6 prior therapies (50 mg cohort); follicular lymphoma with 6 prior therapies and autologous stem cell transplant (100 mg cohort); CLL with 11 prior therapies and 17p deletion (200 mg cohort). Dose escalation is continuing and disease specific cohort expansion will occur at the maximally tolerated dose, at which time patients with AML will be added. Updated data will be presented at the meeting. *Conclusions*. Early results from a phase 1 study of the oral PI3K p110delta inhibitor CAL-101 show that it is well tolerated and has preliminary clinical activity in patients with B-cell malignancies.

0745

EPIGENETIC THERAPY WITH 5-AZACITIDINE, VALPROIC ACID, AND ATRA IN PATIENTS WITH HIGH-RISK AML OR MDS: RESULTS OF THE FRENCH VIVEDEP PHASE II STUDY

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Introduction. Promising results have been reported recently with a combination of 5-azacitidine (AZA), valproic acid (VPA), and all-trans retinoic acid (ATRA) in patients with AML/MDS (Soriano *et al.* Blood 2007). We report here on a similar study conducted in 9 centers between 7/2006 and 8/2007. *Methods*. Patients with high-risk AML (AML in patients aged 70y+ unsuitable for intensive chemotherapy or early relapsing/refractory AML) or MDS (int-2/high IPSS without possibility of allogeneic SCT) were eligible. Treatment consisted of 6 cycles with AZA 75 mg/m^2/d SC (d1-7), VPA 35 to 50 mg/kg/d PO (d1-7), and ATRA 45 mg/m^2/d PO (d8-28). Cycle 1 was initiated at the hospital but cycles 2-6 were planned monthly in out-patients if possible. Response was assessed after cycle 1, 3, and 6 (IWG AML criteria). VPA was started at 35 mg/kg/d, and then increased at 50 mg/kg/d if well tolerated. Sixty-five patients were enrolled and 62 are evaluable for response. *Results*. Patients characteristics were: M/F, 27/38; median age, 72y (50-87); median WBC, 2.3×10^9/L. Fifty-five patients had AML (42 untreated, 13 relapsed AML) and 10 had MDS. Cytogenetics was available in 58 patients with high-risk features in 30 of them (complex, -7, -5, 3q abnormalities). Thirty-one patients stopped the treatment after 1-5 cycles: 23 disease progression or early deaths, 5 toxic events (SAE), and 3 patient decision despite stable disease. VPA was associated with CNS toxicity at 50 mg/kg, but not at the 35 mg/kg dose level. ATRA-related symptoms (headaches, mucosal dryness) were noted. In the 34 patients who received the 6 cycles, 13 reached CR (8 after cycle 3), 2 reached PR (4 after cycle 3) and 14 had Stable disease. The overall CR/PR rate after 6 cycles was thus 24%, reaching 38% in the 34 patients who did not interrupt the treatment in the absence of progression or toxic event. Analysing best response obtained (14 CR + 4 PR), cumulative incidence of response or death before response after 6 cycles is 31% and 31%, respectively. Table 1 gives prognostic factors for CR/PR and deaths (for cumulative incidence of response or death before response). Advanced age, high-risk cytogenetics and poor PS did not influence the response rate, but were prognostic for early death before response. Median OS was 12.4 months for all patients and 19.6 months in responders. *Conclusions.* This study confirms that epigenetic therapy with AZA, VPA, and ATRA yields a promising 25 to 30% response rate in patients with high-risk AML/MDS. Although randomized studies are needed (AZA±HDAC inhibitors), this combined approach appears to be a good option to treat older patients, whatever their cytogenetics.Maintenance options should be investigated in responding patients.

Table 1.

	CR/PR (P value)	Death(P value)
Age > or = 70y	24% (0.50)	53% (0.025)
Female	25% (0.21)	47% (0.03)
OMS > or = 2	0% (0.10)	57% (0.008%)
WBC > or = 1,5.10^9/L	26% (0.47%)	31% (0.96)
Platelets < 50.10^9/L	19% (0.02%)	39% (0.10)
Marrow Blasts >30%	29% (0.44)	42% (0.05)
High-risk cytogenetics	24% (0.52)	48% (0.003)
Relapsed AML	31% (0.72)	62% (0.01)

0746

PRECLINICAL EXPERIENCES WITH THE NOVEL CALICHEAMICIN-CONJUGATED CD22 ANTIBODY INOTUZUMAB OZOGAMICIN (CMC-544) IN THE TREATMENT OF PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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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; Wyeth) is a newly designed drug consisting of a humanized CD22 antibody coupled to the anti-tumor antibiotic calicheamicin. CD22 antigens are specifically expressed on both immature and mature B cells, and it has already been shown that CMC-544 is effective in patients with B-cell lymphomas. *Aims*. To study the effectiveness of CMC-544 in precursor-B-ALL *in vitro* and to analyze which parameters determine its efficacy. *Methods*. Various ALL cell lines and primary precursor-B-ALL cells were tested for their sensitivity to CMC-544 and calicheamicin by quantitative flowcytometric measurement of viable ALL cells after 0 till 72 hours of incubation. CD22 saturation, defined as the percentage of CD22 antigens occupied by CMC-544, was determined by comparing the amount of bound CMC-544 with the maximal amount of CMC-544 that can bind to the cells. Binding of CMC-544 was analyzed by flow cytometry using anti-human IgG4-biotin and streptavidin-PE. The percentage of internalization of the CD22-CMC-544 complex was determined by comparing maximal CMC-544 binding at t=0 with maximal CMC-544 binding at the respec-

tive time point. *Results*. CMC-544 induced dose-dependent cell kill in ALL cell lines, with IC50 values at 72 hours of incubation varying from 1-10 ng/mL. High concentrations of CMC-544 (≥100 ng/mL) caused non-specific inhibition of proliferation and/or cell death in CD22 negative cells. Differential sensitivity of ALL cell lines to CMC-544 could not be attributed to differences in their sensitivity to calicheamicin, but seemed to be related to CD22 expression levels. However, if ALL cells expressing only moderate CD22 levels were incubated with CMC-544 for a longer time (>200 h), >99% of cells died, indicating that prolonged intracellular accumulation of CMC-544 contributes to killing these cells. Importantly, analysis of CD22 saturation and CMC-544-induced cell death showed that maximal saturation was not required to achieve efficient cell lysis. This is in contrast to earlier Mylotarg studies, where prolonged complete saturation was required to cause efficient cell death. CMC-544 was rapidly internalized both at low and at high CD22 saturation levels. Cell cycle analysis showed that, similar to calicheamicin, CMC-544 induced a G2/M arrest in its target cells. Primary ALL cells were also sensitive to CMC-544 treatment showing comparable IC50 values as ALL cell lines. CD22 expression levels were analyzed in a panel of 55 pediatric ALL patients. In all cases, ALL cells expressed CD22 and in >90% the CD22 expression exerted the expression level of CMC-544-sensitive cell lines. *Summary and Conclusions*. CMC-544 induced dose-dependent cell death in ALL cells, which seems to be related to the CD22 expression level. In contrast to previous data on Mylotarg-induced lysis of AML cells, our data indicate that prolonged and maximal saturation is not required for CMC-544-induced cell death of ALL cells. These data suggest that a fractionated scheme of administration of CMC-544 is less important than for Mylotarg, which has important implications for the design of future studies in children with relapsed/refractory ALL.

0747

INTERFERON-α-ENGINEERED MULTIPOTENT MESENCHYMAL STROMAL CELLS FOR THE TREATMENT OF MYELOMA

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Background. Multipotent mesenchymal stromal cells (MSCs) are non-hematopoietic progenitor cells with multilineage differentiation potential. Exogeneously administered MSCs preferentially survive and proliferate in the presence of malignant cells, become stromal cells and support tumor growth. Thus, MSCs are attractive candidates to deliver biologically active molecules in the tumor environment *in vivo* and to enhance specific immune responses. Interferon-α (IFNα) has been used for years for the maintenance treatment of multiple myeloma (MM), but its administration is limited by the temporary efficacy and the significant toxicity when used sistemically at high doses. *Aims*. We used bone marrow-derived MSCs (BM-MSCs) from Balb/c mice as cellular vehicles for IFNα, after their transduction with a lentivirus-derived vector carrying the murine IFNα gene (BM-MSCs/IFNα), to assess their effects *in vivo* in the Sp6 mouse model of myeloma. *Methods*. BM-MSCs were transduced with a lentiviral vector containing murine IFNα cDNA (efficiency = 70%). Two month-old Balb/c mice (Balb/cByJIco, Charles River Italia, Calco, LC, Italy) (H-2d), were injected subcutaneously (s.c.) with the tumorigenic dose of 0.5×10^6 Sp6 cells (H-2d). The same mice were then weekly injected with 0.5×10^6 BM-MSCs/IFNα (1, 4 or 8 doses), in the same anatomical quarter. As controls, some mice were injected s.c. either with Sp6 or with BM-MSCs/EGFP s.c. or intravenously (i.v.) to test *in vivo* homing. Tumor sections were processed for immunohistochemistry/immunofluorescence using the following antibodies: anti-CD31, anti-von Willebrand factor (vWF), anti-α-smooth muscle actin (α-SMA), anti-CD4, anti-CD8, anti-asialo GM1, anti-CD45, anti-CD90, anti-murine IFN-α and then analyzed using a Zeiss Axiovert Z1 microscope. *Results*. BM-MSCs were capable of homing into the subcutaneous Sp6 tumor and formed small clusters of cells inside the tumor. Treatment with BM-MSCs/IFNα resulted in a statistically significant delay in the onset of palpable tumors (event free survival, EFS, of 50% at day +17 for 1 dose, day +20 for 4 doses and day +64, for 8 doses; by contrast, with Sp6 alone or coinjected with BM-MSCs the tumor incidence was 100% 10-13 days after injection). The weekly administration of BM-MSCs/IFNα induced a statistically significant decrease of the tumor growth rate and improved the overall survival (OS) (median OS was 19 days in the control mice, 17 days for mice receiving unmanipulated BM-MSCs, 30-31 days for mice treated with 1 and 4 doses of BM-MSCs/IFNα, 77 days for mice treated with 8 weekly doses). The anti-tumor effect was associated with ischemic tumor necrosis, reduction in microvessel density, and NK cell infiltration. *Conclusions*. These findings show that transduced BM-MSCs are capable of delivering anti-cancer molecules in the microenvironment of myeloma and may be a promising tool for specific, low-toxic, and long-lasting anti-myeloma therapy.

0748

HEAT SHOCK PROTEIN 32-TARGETING DRUGS INDUCE GROWTH ARREST AND APOPTOSIS IN LEUKEMIC CELLS IN ACUTE LYMPHOBLASTIC LEUKEMIA

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We have recently shown that Hsp32 is expressed in leukemic cells in Ph^+ chronic myeloid leukemia and serves as potential therapeutic target. In the current study, we examined the expression and functional role of Hsp32 in acute lymphoblastic leukemia (ALL). Leukemic cells were obtained from patients with Ph^+ ALL (n=8) and Ph- ALL (n=12). In addition, Ph^+ ALL cell lines (Z-119, BV-173, TOM-1, NALM-1) and Ph- lymphoblastic cell lines (RAJI, RAMOS, REH, BL-41) were used. As assessed by immunostaining and qRT-PCR, ALL cells were found to display the Hsp32 protein as well as Hsp32 mRNA in all patients and cell lines examined. The Hsp32-inductor hemin was found to promote expression of Hsp32 in leukemic cells. To determine the functional role of Hsp32 in lymphoblasts, an siRNA against Hsp32 was applied. The siRNA-induced knock down of Hsp32 in RAJI and TOM-1 cells led to reduced growth and apoptosis compared to a control siRNA ($p<0.05$). Next, two pharmacologic inhibitors of Hsp32, pegylated zinc protoporphyrine (PEG-ZnPP) and styrene maleic acid-micelle-encapsulated ZnPP (SMA-ZnPP) were applied. As assessed by 3H-thymidine uptake, both agents were found to inhibit proliferation in $BCR\text{-}ABL^+$ cell lines and BCR-ABL-negative ALL cell lines. The effects of PEG-ZnPP and SMA-ZnPP on these cells were dose-dependent with IC_{50} values ranging between 1 and 10 μM, and were found to be associated with apoptosis as determined by microscopy as well as by flow cytometry and AnnexinV-staining. In NALM-1 cells, PEG-ZnPP and SMA-ZnPP also produced apoptosis and growth arrest, but the IC_{50} for SMA-ZnPP was slightly higher (20 μM). Hsp32-targeting drugs were also effective in producing growth inhibition in primary ALL cells (Ph^+ ALL and Ph^- ALL), with IC_{50} values ranging between 1 and 10 μM. No major differences were found when comparing results in imatinib-sensitive and imatinib-resistant patients. In drug combination experiments, Hsp32-targeting drugs were found to cooperate with imatinib and with nilotinib in producing growth-inhibition and apoptosis in all Ph^+ ALL cell lines. Moreover, cooperative antileukemic effects were obtained when Hsp32-targeting drugs were combined with bendamustine. Overall, these results suggest that Hsp32 may be a novel molecular target in ALL.

0749

PROTEOMIC ANALYSIS OF PKC-β INHIBITOR ENZASTAURIN-TREATED MANTLE CELL LYMPHOMA

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Background. Protein kinase C beta (PKCbeta), a pivotal enzyme in B-cell signaling and survival, is over-expressed in most cases of mantle cell lymphoma (MCL) and results in activation of PI3K/AKT pathway. Enzastaurin, an oral serine/threonine kinase inhibitor, suppresses signaling through PKCβ/PI3K/AKT pathways, induces apoptosis, reduces proliferation, and suppresses tumour-induced angiogenesis. Recently, a phase II study showed that enzastaurin is able to prolong the time free from progression in a subset of pre-treated refractory MCL. *Aims*. To optimize the treatment options with this promising inhibitor the goal of this study was to elucidate the molecular pathways influenced by Enzastaurin treatment in MCL. *Methods*. Four documented MCL cell lines (GRANTA 519, HBL-2, Jeko-1, Rec-1) were treated with Enzastaurin in a proliferation inhibiting dose of 10 μM, defined in initial pilot experi-

ments. After 2-8h of treatment, cells were harvested and analyzed by two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE)-based protein screening and mass spectrometric identification. The identified candidate proteins were mapped in a functional interaction network and pathway-selected candidate proteins were verified by Western blotting. *Results*. Enzastaurin (10 nM) exposure led to significant reduction of cell viability in all cell lines (15-20%). One hour after treatment distinct alterations of the protein pattern were recognized in the 2D-PAGE gels of all cell lines. Of a total of 977 concurrent protein spots 115 (12%) spots exhibited significantly (>3fold) altered protein levels after 4h of enzastaurin exposure, including increased (57 spots; 5,8%) and decreased (58 spots; 5,9%) protein levels. Sixty-two concurrent differentially expressed protein spots (39 increased at 4h; 23 decreased at 4h) were chosen for mass spectrometric identification. Mass spectrometry identified 108 different candidate proteins with significant identification confidence. The identified candidate proteins were classified in functional groups, including DNA-repair and -replication (RAD50, PCNA, RFC1, PSMC4), apoptosis (HIST1H1E, PSMC4, VIM, PLEC1, GDIR2), signal transduction (GRB2, ARHGAP25, EF1D, SNX25) and gene expression/mRNA processing (RPS20, EEF1D, HNRPF, SFRS7; SMC1A). The results were verified by Western blot in selected candidate proteins of the apoptosis (VIM, PLEC1), DNA-repair (RAD50, PCNA, RFC1) and gene expression (EEF1D, SMC1A) as well as several additional proteins mapped to the interaction networks (BMF, NOXA). *Summary and Conclusions*. Enzastaurin-treatment affects several crucial cellular pathways including apoptosis, DNA repair and gene expression. It thus affects three main cellular control mechanisms. Ongoing experiments incorporate this knowledge to select optimal combination partners of Enzastaurin.

0750

THE BH3 MIMETIC GX015-070 (OBATOCLAX) INDUCES APOPTOSIS AND GROWTH INHIBITION IN NEOPLASTIC MAST CELLS

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Background. Advanced systemic mastocytosis (SM) is an incurable neoplasm with short survival times. So far no effective therapy is available for these patients. We have recently shown that neoplastic mast cells (MC) in SM express various anti-apoptotic members of the Bcl-2 family including Mcl-1. *Aims and Methods*. In this study, we examined the effects of the Mcl-1-targeting BH3 mimetic Obatoclax® (GeminX, Montréal, Quebéc, Canada) on growth and viability of primary neoplastic MC obtained from patients with SM (n=3), the human MC leukemia cell line HMC-1, and the canine mastocytoma cell line C2. Two HMC-1 subclones, one lacking KIT D816V (HMC-1.1) and one expressing KIT D816V (HMC-1.2) were examined. *Results*. As assessed by RT-PCR and immunostaining, primary neoplastic MC and both HMC-1 subclones were found to express Mcl-1 mRNA and the Mcl-1 protein, but did not express proapoptotic Bim. Transfection of HMC-1 cells with Mcl-1-specific siRNA resulted in increased apoptosis compared to cells transfected with a control siRNA. Obatoclax was found to inhibit 3H-thymidine uptake and thus proliferation in HMC-1 cells in a dose-dependent manner, with higher IC_{50} values obtained in HMC-1.2 cells (0.5 µM) compared to HMC-1.1 cells (0.05 µM). Obatoclax also inhibited the growth and survival of the canine mastocytoma cell line C2 (IC_{50}: 0.5-1 µM). Moreover, Obatoclax was found to inhibit the proliferation of primary human neoplastic MC in all SM patients tested (IC_{50}: 0.05-0.1 µM). In all cell line models, Obatoclax induced apoptosis as determined by microscopy, Tunel assay, and caspase cleavage. We next combined obatoclax with a modulator of Mcl-1/Bim expression in MC, in order to enhance drug effects. Since Bim is degraded via the proteasome, we applied the proteasome inhibitor bortezomib. As assessed by real time PCR, bortezomib was found to promote Bim mRNA expression in these cells. In addition, bortezomib was found to suppress 3H-thymidine uptake in both HMC-1 subclones, and to cooperate with Obatoclax in producing apoptosis in neoplastic MC. Finally, Obatoclax was found to synergize with the KIT D816V-targeting drug PKC412 in producing apoptosis in HMC-1 cells and C2 cells. *Conclusions*. The Mcl-1-targeting drug Obatoclax exerts apoptosis-inducing effects on neoplastic MC. Whether Obatoclax will also act antineoplastic in patients with advanced MC disorders remains to be determined in clinical trials.

0751

QUANTITATIVE MEASUREMENT OF NUCLEAR TRANSLOCATION OF NF-KB AND STAT-3 AS PARAMETER OF RESPONSE TO TARGETED TREATMENTS IN IMMUNOPHENOTYPICALLY DEFINED CELLS IN ACUTE MYELOID LEUKEMIA

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Background. Acute Myeloid Leukemia (AML) has been associated with aberrant activities of various signaling cascades including FMS-like tyrosine kinase-3 (FLT3), signal transducer and activator of transcription-3 (STAT3), nuclear factor-κB (NF-κB) and vascular endothelial growth factor (VEGF) and the leukemia-specific nature of these anomalies make them attractive therapeutic targets in this disease. Activation of the VEGF and FLT3 cell surface receptors triggers the activation of key signaling pathways involved in cell proliferation and survival including the PI3K/AKT, Ras/MAP-K, STATs and protein kinase C pathways. STAT3 and NF-κB are down stream targets at the convergence of these and/or other signaling cascades. *Aims*. the aim of the study is to demonstrate the applicability of flow cytometry-based image analysis to quantitatively evaluate nuclear versus cytoplasmic localization of NF-κB and STAT-3 in immunophenotypically defined (target) cells as a parameter of response to treatments that target the activity of these pathways. *Methods*. Dose- and time-kinetics of NF-κB and STAT-3 nuclear localization following *in vitro* cytokine exposure was studied in AML cell lines and leucocytes from AML patients and healthy volunteers. Following the desired treatment, cells were stained with combinations of cell surface lineage specific antibodies (eg CD33), anti-p65 or anti-STAT3 antibodies and a nuclear DNA stain (DRAQ5). Multi-spectral images of at least 5,000 cells were then acquired with the ImageStream platform. Following hierarchical gating strategies to identify single, in-focus and $CD33^+$ cells, image analysis algorithms were applied to each cell to determine the 'similarity' of its nuclear image (defined by DRAQ5) and its p65 or STAT3 image (defined by FITC-labeled anti-p65 or anti-STAT3). Sensitivity and specificity was determined by titrating treated cells with nuclear translocation into untreated cell populations for which no or little nuclear translocation is expected. Image analysis results were compared with western blot and microscopy analysis. *Results*. the correlation between STAT3 or NF-κB images and DRAQ5 images was quantified for each individual cell by a 'similarity score', a log transformed Pearson's correlation between corresponding pixel values of each image. As such the similarity score is a measure of nuclear translocation that is measured as a continuous variable from -infinity to +infinity. The higher the score the higher the degree of nuclear translocation. In all model systems tested, dose- and time-kinetic changes of similarity scores corresponded well with expected nuclear translocation of NF-κB and STAT3 following exposure to TNF-alfa and IL-6, respectively. Cells with nuclear translocation events could be detected among cells without nuclear translocation with a sensitivity of at least 0.1%. The applied analysis was shown to be less bias prone than microscopy and more sensitive than western blot analysis. *Summary and Conclusions*. the data demonstrate that the ImageStream technology allows the quantitative study of nuclear translocation of NF-κB and STAT3 in immunophenotypically defined cells. These examples illustrate the applicability of this approach to study nuclear translocation events as a parameter of response in immunophenotypically defined target cells in patients undergoing therapy with agents targeted against these signaling pathways. Supported by NIH 1R21 CA126667.

0752

ACTIVITY OF MLN4924, A NOVEL FIRST IN CLASS SMALL MOLECULE INHIBITOR OF THE NEDD8 ACTIVATING ENZYME, IN PRECLINICAL MODELS OF ACUTE MYELOID LEUKEMIA

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Background. The ubiquitin-proteasome system (UPS) is responsible for the timed destruction of many proteins including key players in signaling cascades and critical regulators of cell cycle progression and transcription. The Nedd8 activating enzyme (NAE) has been identified as an essential regulator of the Nedd8 conjugation pathway, which controls the activity of a subset of ubiquitin E3 ligases, specifically, the cullin dependant E3 ligases. The cullins control the timely ubiquitination and

subsequent degradation of many proteins with important roles in cell cycle progression, DNA damage, stress response and signal transduction. Acute myeloid leukemia (AML) is a disease of the elderly and prognosis is extremely poor with a median overall survival of just 2 months for untreated patients. Novel therapeutic strategies are urgently needed for these patients. Considering that Nedd8-mediated control of protein homeostasis is vitally important for the survival of AML cells, we evaluated the pre-clinical anti-leukemia activity of MLN4924, a novel first in class small molecule inhibitor of the Nedd8 activating enzyme. *Aims*. (1) To determine the activity of MLN4924 in pre-clinical models of AML; (2) To elucidate the mechanisms of action of MLN4924 in AML cells. *Materials and Methods*. FLT3$^+$ and FLT3$^-$ AML cell lines, normal PBMCs and bone marrow stromal cells, and primary AML patient specimens were used to evaluate the preclinical activity of MLN4924. Cell viability and induction of apoptosis were assessed using PI/FACS and an ATP bioluminescence method. The prolonged *in vitro* effects of MLN4924 were assessed by MethoCult colony formation assays. Effects on protein neddylation and caspase activation were measured by western blot analysis. VEGF secretion was quantified by ELISA. *Results*. MLN4924 induced rapid and selective cell death at low nanomolar concentrations (mean IC_{50} of 175 nM) in AML cell lines independent of FLT3 expression, but not in peripheral blood mononuclear cells from healthy donors. Clonogenic assays confirmed impaired colony formation at 10 days in a dose dependant fashion. Importantly, MLN4924 also demonstrated significant activity in primary AML cells from patients. Kinetic analysis of drug-induced effects on cell cycle distribution revealed that AML cells treated with MLN4924 initially arrested at the G1 transition prior to their subsequent accumulation in the sub-G1 compartment. Activation of both caspases-8 and -9 following 2 hours of drug exposure confirmed potent pro-apoptotic effects. Notably, the activity of MLN4924 was preserved when cells were co-cultured with bone marrow stromal cells. MLN4924 induced a dose and time dependant increase in the expression of phospo-IKB, an important target for degradation through the Nedd8 conjugation pathway. The inhibitory effects of MLN4924 on NFKB were confirmed by demonstrating that the transcriptional activity of the NFKB p65 subunit and the secretion of VEGF, an NFKB transcriptional target were both reduced significantly. *Conclusions*. MLN4924, a novel first in class NAE inhibitor, overcomes the protective effect of the bone marrow microenvironment and rapidly and selectively induces apoptosis through caspase 8 and 9 activation and inhibition of the NFKB pathway. MLN4924 is a promising novel agent for the treatment of AML and warrants further evaluation in clinical trials.

0753

OVERCOMING DRUG RESISTANCE WITH SGI-1776: A NOVEL PIM KINASE INHIBITOR WITH POTENT PRECLINICAL ACTIVITY IN ACUTE MYELOID LEUKEMIA

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Backgroud. The PIM kinases (PIM1, PIM2, PIM3) are a small family of proto-oncogenes within the CAMK super-family. Overexpression of PIM kinases significantly augments cell survival and accelerates c-myc and N-myc-driven tumorigenesis in mouse models. Accordingly, overexpression of PIM kinases has been reported in a wide range of malignancies including clinical cases of acute myeloid leukemia (AML). In models of FLT3 positive AML, prolonged exposure to FLT3 inhibitors leads to resistance via activation of parallel signaling pathways, and resultant altered gene expression, including high levels of PIM1. Resistance to the mTOR inhibitor rapamycin, has also been reported as result of over-expression of PIM1 in AML cell lines. PIM1 is directly involved in the activation of efflux pumps which are strongly associated with clinical drug resistance in AML. Drug resistance is a major cause of treatment failure, particularly in the elderly, where the median survival is just 2 months for untreated patients. New treatments are desperately needed for these patients. Therefore, given the important roles of the PIM kinases in cell survival signalling and drug resistance in AML these kinases become very attractive targets for pharmacological inhibition. SGI-1776 is a novel orally available small molecule inhibitor of PIM kinase activity that has entered Phase I clinical trials. We hypothesized that SGI-1776 would have significant anticancer activity against AML cells due to their high intrinsic expression of PIM-1. *Aims*. 1)To determine the activity of SGI1776 in pre-clinical models of AML; 2) To elucidate the mechanism of action of SGI1776 in AML cells. *Materials and methods*. FLT3 positive and FLT3 negative AML cell lines, normal peripheral blood mononuclear and bone marrow stromal cells were used to evaluate the preclinical activity of SGI1776. Cell viability and induction of apoptosis were assessed using PI/FACS and MTT assays. The prolonged *in vitro* effects of SGI1776 were assessed by MethoCult colony formation assays. Effects on PIM kinase inhibition and caspase activation were measured by western blot analysis. FLT3 positive xenograft mouse models and primary patients specimens were used to establish the *in vivo* effects of SGI1776. *Results*. *In vitro* assays conducted in a panel of human AML cell lines demonstrated that low nanomolar concentrations of SGI-1776 potently diminished cell viability (IC_{50} 6NM). Clonogenic assays confirmed impaired colony formation at 10 days in a dose dependant fashion. SGI-1776 induced apoptosis in AML cells in a dose-dependant manner that correlated with a reduction in intracellular levels of phosphorylated BAD. FLT3 ITD positive AML cells were particularly sensitive to SGI-1776, likely due to the dual inhibitory effects of this agent against PIM and FLT3 kinase activity. Oral administration of SGI-1776 to immunodeficient mice bearing xenografts of human AML cell lines was very well tolerated. SGI-1776 inhibited tumor growth significantly and demonstrated a greater than additive effect when combined with the standard of care agent, cytarabine. *Conclusions*. These exciting data support the clinical study of SGI-1776 in patients with AML and further investigation of the role of PIM kinase in AML pathophysiology.

0754

INTERFERENCE WITH AML1/ETO LEUKEMOGENIC FUNCTION BY CELL-PENETRATING PEPTIDES TARGETING THE OLIGOMERIZATION DOMAIN

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Background. The leukemogenic fusion protein AML1/ETO is generated by the chromosomal translocation t(8;21) which appears in about 12% of all *de novo* acute myeloid leukemias (AMLs). It consists of the DNA-binding domain of the hematopoietic transcription factor AML1 and almost the entire ETO protein which functions mainly as a transcriptional repressor. Essential for the block in myeloid differentiation of AML1/ETO transformed cells is the oligomerization of the chimeric proteins and the formation of high molecular weight complexes (HMWCs). The α-helical nervy homology region 2 (NHR2) within ETO mediates oligomerization by tetramer formation as well as binding of AML1/ETO to members of the ETO protein family and corepressor molecules. *Aims*. Recent studies suggest the NHR2 domain as the only essential ETO domain crucial for AML1/ETO leukemogenic potential. Thus, selective interference with the oligomerization domain could inhibit the tumorigenic potential of AML1/ETO. *Methods*. By using retroviral transduction, the effect of different NHR2-containing peptides on the ability to prevent proliferation and induce differentiation of transformed cells was investigated. In order to directly deliver the inhibitory peptides to AML1/ETO expressing cells, the NHR2 domain was fused to the HIV-1 Tat protein transduction domain and the recombinant peptides were purified from bacteria and tested in cell culture. *Results*. We could show that expression of peptides containing the NHR2 domain inhibits AML1/ETO oligomerization. The binding affinity of NHR2 peptide mutants to ETO directly correlates with the degree of growth inhibition. A derivative of NC128 which retained all NHR2 amino acids (N89) maintained full binding capacity to ETO as well as antiproliferative effects, whereas a mutant of N89 lacking 7 C-terminal amino acids (N82) significantly lost binding capacity and its antiproliferative effect. A codon optimized expression construct was developed in order to increase the cellular expression levels of N89. Compared to N89, expression of this construct enhances growth arrest suggesting that the NHR2 peptides act in a dose dependent manner. Upon protein transduction into mammalian cells, recombinant NHR2 polypeptides were successfully detected in cellular lysates. Addition of the endosome inhibitor cloroquine further enhanced the intracellular stability of the cell-penetrating polypeptides. By co-immunoprecipitation experiments we could show that the transduced polypeptides are able to specifically interact with ETO protein in stably transfected cells. Protein transduction into the myeloid cell line Kasumi-1, which depends on AML1/ETO expression for growth, is possible in the absence of serum and can be further increased by pre-incubation of the cells with dextran. Treatment of Kasumi-1 cells for several days with the cell-penetrating NHR2 polypeptides resulted in a 22% reduction of c-kit surface marker expression. *Conclusions*. Our results propose that selective interference with NHR2-mediated oligomerization could provide a promising strategy for the inhibition of the oncogenic properties of AML1/ETO. The application of cell-penetrating polypeptides is possible but requires further improvement to achieve high bioavailability.

0755

TUMOR MEMBRANE-BOUND DIMERIC IL-2 AND A BISPECIFIC CD3X19 ANTIBODY INDUCE CYTOTOXIC T-CELL STIMULATORY CAPACITY OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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The graft versus-leukemia-effect in B-cell chronic lymphocytic leukemia (B-CLL) appears to be less efficient compared to myeloid leukemias. Since the aberrant coexpression of CD5 in association with CD19 is a hallmark of B-CLL, we asked if both molecules might serve as a combinatorial T-cell target. B-CLL cells as third party in mixed-lymphocyte reactions (MLR) confirmed their capacity to induce T-cell anergy. We used recombinant proteins comprising a single chain (sc) FvCD5 antibody (Ab) fused to human interleukin (IL)-2 via the hinge region of human immunoglobulin (Ig)G1 in conjunction with a bispecific CD3x19 Ab (bsAb; clone OKT3xHD37) to saturate CD19 and CD5 binding sites on B-CLL cells. Consistent with the absent immunogenic potential, B-CLL cells pretreated with scFvCD5 Ab, native IL-2 and CD3x19 bsAb were not recognized by allogeneic resting T cells. In contrast, B-CLL coated with the dimeric scFvCD5-IL-2 and CD3x19 bsAb induced profound stimulator cell dependent allogeneic and autologous T-cell proliferation. In addition, T cells from fresh peripheral blood mononuclear cell samples from B-CLL patients (n=4) pretreated with dimeric scFvCD5-IL-2 and cultured in the presence of CD3x19 bsAb expanded by 2-3 log within 7 days. Notably, addition of soluble exogenous IL-2 to both types of culture remained inferior compared to the CD5-targeted IL-2 delivery. T-cell proliferation and expansion also translated to cell-mediated cytotoxicity. In conclusion, a dual targeting approach directed against aberrantly expressed surface molecules for membrane delivery of dimeric IL-2 molecules and a surrogate second signal as shown for B-CLL represents a strategy to reverse tumor-induced T-cell anergy.

0756

BI-213 LABELED ANTI-CD20 MONOCLONAL ANTIBODIES INDUCE APOPTOSIS AND OVERCOME CHEMORESISTANCE IN NON-HODGKIN'S LYMPHOMA AND CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Background. Radioimmunotherapy (RIT) is an emerging treatment option for non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL). The development of RIT with α- emitters such as Bi-213 is promising for treatment of a variety of cancer and is attractive because of the high linear energy transfer (LET) and short path length, allowing higher tumor cell kill and lower toxicity to healthy tissues. *Aims*. In the present study we examined induction of apoptosis and the role of activation of apoptosis pathways using Bi-213 labeled anti-CD20 monoclonal antibodies ([Bi-213]anti-CD20) in sensitive and chemoresistant non-Hodgkin's lymphoma cells and CLL cells. *Methods*. Bi-213 was eluted from an Ac-225 generator and conjugated to anti-CD20 antibody (rituximab) with CHX-A'-DTPA as a chelator. The human non-Hodgkin's lymphoma cell line DoHH2 ($CD20^{+/+}$) and CLL cells, isolated from patients sensitive or resistant to fludarabine, were treated with 225, 75, 22.5, and 7.5 kBq/mL of [Bi-213]anti-CD20, using the specific activity of 3.6 MBq/μg anti-CD20. 24h and 48h after treatment with [Bi-213]anti-CD20, cell death, cell cycle and apoptosis were measured by flowcytometry and activation of apoptosis pathways was determined by Western Blot analyses. *Results*. 24h and 48h after treatment with 225, 75, 22.5, and 7.5 kBq/mL of [Bi-213]anti-CD20 we found a strong induction of apoptosis and activation of caspases and PARP cleavage in DoHH2 cells. [Bi-213]anti-CD20-induced apoptosis was completely inhibited by zVAD.fmk, a specific inhibitor of activation of caspases, indicating that caspases play an important role in [Bi-213]anti-CD20 induced cell death. Mitochondria were activated after [Bi-213]anti-CD20 treatment, resulting in caspase-9 activation. Bax, a death-promoting protein, was upregulated and Bcl-xL, a death-inhibiting protein, was downregulated after [Bi-213]anti-CD20 treatment. p21 were upregulated and cells showed a G2/M cell cycle arrest after treatment with [Bi-213]anti-CD20. In addition, [Bi-213]anti-CD20 induced cell death in CLL cells as well as in fludarabine-resistant CLL cells isolated from patients *ex vivo*, indicating that [Bi-213]anti-CD20 overcomes fludarabine-resistance. Furthermore, [Bi-213]-triggered apoptosis was not inhibited by the DNA-repair mechanism of non homologous endjoining, predominant for repairing double strand breaks in mammalian cells. *Summary and Conclusions*. Taken together, we found that [Bi-213]anti-CD20 antibodies induce apoptosis, arrest cell cycle in G2/M and activate apoptosis pathways through caspase activation in non-Hodgkin's lymphoma cells and in CLL cells *in vitro* and *ex vivo* and break fludarabine-resistance. [Bi-213]-mediated α-radioimmunotherapy seems to be a promising therapeutic approach for selectively killing tumor cells with limited side effects.

0757

LEUKEMIA CELL-SELECTIVE UPTAKE OF CYTARABINE AND DAUNORUBICIN IN THE BONE MARROW COMPARTMENT MEDIATED BY CPX-351 LIPOSOME INJECTION

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Background. Anticancer drug combinations such as cytarabine plus daunorubicin can act synergistically, additively or antagonistically depending on the ratio of the agents being combined. We have shown that delivering synergistic cytarabine:daunorubicin 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 without exacerbating myelosuppressive effects. This enhanced efficacy is associated with increased and prolonged delivery of the synergistic drug ratio to bone marrow. Promising signs of efficacy have been observed with CPX-351 in previously treated AML patients with poor prognosis. *Aims*. To investigate the pharmacodynamic basis for the potent therapeutic activity of CPX-351. *Methods*. A bone marrow-engrafting CCRF-CEM human leukemia xenograft model was developed. CPX-351 was injected i.v. and the bone marrows of femurs from tumor bearing mice were subsequently harvested. Cells were analyzed by flow cytometry and leukemia cells were separated from normal bone marrow cells using anti-CD45 coated magnetic nanoparticles. Cells were analyzed for intracellular cytarabine: daunorubicin and liposome content using 3H-Cyt, HPLC and 14C-lipid, respectively. *Results*. Twenty-eight days after i.v. tumor inoculation, femurs contained approximately equal numbers of CCRF-CEM and normal bone marrow cell populations which could be readily separated and quantitatively harvested by antibody coated magnetic nanoparticle-mediated isolation. Eighteen hours after CPX-351 i.v. injection, bone marrows of the tumor bearing mice contained 82.6 ng cytarabine, 63.0 ng daunorubicin and 1.2 μg liposomal lipid per total femur aspirate. Within the separated cell populations, leukemia cells contained 5.9 ng cytarabine, 8.2 ng daunorubicin and 0.16 μg liposomal lipid per 10^6 cells. These intracellular levels were 9.5-, 2.2- and 1.9-fold higher than those observed in the normal bone marrow cell population. Confocal microscopy on cells incubated with CPX-351 *in vitro* demonstrated that CPX-351 liposomes are taken up intact by human leukemia cells and subsequently release the drugs intracellularly. *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 molar ratio for extended times after injection. The potent anti-leukemic activity obtained in the absence of significant non-hematological toxicity with this formulation may be due, in part, to the selective accumulation of CPX-351 into leukemia cells.

0758

INDUCTION OF APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUCEMIA CELLS BY THE PARA-ISOMER OF NITRIC OXIDE-DONATING ACETYLSALICYLIC ACID (P-NO-ASA) AT LOW MICROMOLAR CONCENTRATIONS

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Background. Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of mature, non functional B cells. We and others have shown that WNT/β-catenin (CTNNB1)/TCF/Lef-1 signaling appears to be constitutively and aberrantly activated in these cells. Furthermore, it is already known that several compounds related to the non-steroidal antiinflammatory drugs (NSAID) can inhibit CTNNB1 stability and/or function in WNT active cancers *in vitro*. However, so far clinical studies with such substances generated disappointing results which is likely to the fact that therapeutic plasma concentrations could not be reached without producing significant toxicities; hence, these required high concentrations limit their clinical use. Recently, nitric oxide-donating acetyl-

salicylic acid (NO-ASA) has been shown to achieve high plasma levels in doses not leading to any side effects in humans. It consists of a traditional molecule of acetylsalicylic acid with a NO-moiety is covalently bound via a spacer part. In addition NO-ASA could disrupt complexation of CTNNB1 and TCF-4 *in vitro*, whereas the latter belongs to the transcription factors which posses a central function in mediating WNT signaling. *Aims*. Because the general structure features enable more variants of NO-ASA the aim of our study was to evaluate whether para-, and meta-NO-ASA have an effect on CLL cells and in affirmative case if the impact is dependant on the isoform. Further we were interested in the actual concentration range of the reagents and if the mechanism of action is based on β-catenin/Lef-1 disruption and subsequent downregulation of target genes. *Methods*. Primary CLL cells as well as healthy peripheral blood monocytes (PBMC) and healthy B-cells were treataed with varying concentrations of p- and m-NO-ASA. Cytotoxicity was assessed by microscopic cell viability testing and an ATP assay. Induction of apoptosis was investigated by Annexin V-FITC/Propidiumiodid (PI) staining and immunoblotting of PARP, caspases 3 and 9. Further, CTNNB1 protein amount was measured by immunoblotting and expression of WNT effector proteins like cyclin D1 (CCND1), C-MYC and LEF-1 was evaluated with immunoblot analysis as well. *Results*. The meta-isoform of NO-ASA did not have any effect on CLL cells whereas the para-isomer showed a selective cytotoxic effect. Mean lethal concentrations (LC50) values were 4.83 µM and 4.64 µM in CLL cells, respectively. LC50 values for healthy controls were more than 25-fold higher. Annexin V-FITC/PI staining revealed that the induced cell death is mediated by apoptosis and by Immunoblot analysis we show that p-NO-ASA cleaves PARP, caspase 3 and caspase 9, decreases CTNNB1 protein levels and downregulates WNT pathway target genes in a concentration dependent manner. *Summary and Conclusions*. Our findings show that the para- but not the meta-isomer of NO-ASA induces caspase-mediated apoptosis in CLL cells selectively. The mechanism of action might be mediated by inhibition of β-catenin/Lef-1 signaling since we observed downregulation of specific target gene expression. Therefore p-NO-ASA might be a valuable compound for the treatment of CLL. More investigations of the exact mechanism of action and the specific difference between the positional isomers are indicated.

0759

ZALYPSIS, A NOVEL AND SELECTIVE ANTILEUKEMIC AGENT TARGETING INMATURE BLAST CELL POPULATIONS

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Background. Acute Myeloid Leukemia (AML) is a heterogeneous group of diseases in which coexistence of leukemic cells at different stages of differentiation is frequently found. Zalypsis® is a novel alkaloid from marine origin with promising anticancer activity. *Aims*. To evaluate the activity, potential toxicity and mechanism of action of Zalypsis in AML *Methods*. The efficacy of Zalypsis® was analyzed in four AML cell lines, including drug resistant cell lines, and in cells from fresh bone marrow samples from eight newly diagnosed AML patients. We have used a multiparametric flow cytometry (MFC) technique that allowed us to study both mature and immature blast cell compartments. In addition, fresh bone marrow cells from 4 newly diagnosed Multiple Myeloma (MM) patients were used to evaluate the toxicity on normal hematopoietic progenitor cells (HPC). *Results*. Zalypsis showed a very potent antileukemic activity, with IC50s in the low nanomolar ranges. Zalypsis® potentiated several other antileukemic agents with a synergistic effect observed for the combinations with Doxorubicin, Cytarabine and Fludarabine. Regarding the mechanism of action, Zalypsis® provoked apoptosis with a prompt induction of Annexin V, and PARP, caspase-3, -8, and -9 cleavage in Western-Blot. Zalypsis® also induced loss of mitochondrial membrane potential measured by DioC6, decrease of Bcl-XL and BCL2 and cleavage of Mcl-1. Selective inhibition of caspases with Z-VAD-FMK, did not abrogate Zalypsis®-induced apoptosis, suggesting a role for caspase-independent apoptosis. Zalypsis® induced the phosphorylation of H2AX and increase of GADD45B, suggesting a DNA damage response which was concordant with the results of the GEP that demonstrated the deregulation of many genes involved in DNA damage response. Zalypsis® was also very effective in *ex vivo* experiments on freshly isolated patients' cells. Our MFC technique allowed us to study both mature and immature (defined as $CD34^+$, $CD38^-$ Lin^-) blast cells compartments in AML samples. Zalypsis® efficiently induced apoptosis in the inmature blast cell population, where leukemic stem cells are found. Interestingly, HPC from MM patients (and, therefore, theoretically, normal) were found not to be affected by Zalypsis while antimyeloma activity was preserved, suggesting that normal HPC are not affected by Zalypsis®. *Summary and Conclusions*. Zalypsis® induces a very potent and selective antileukemic effect *in vitro* and *ex vivo*, targeting inmature blast cell populations, which is, at least partially, mediated through a DNA damage response. Moreover, Zalypsis® is sinergistig with conventional anti-AML drugs. These results provide the rationale for the use of Zalypsis® in clinical trials for patients with AML.

0760

K-RAS SHRNA POTENTIATES FARNESYLTRANSFERASE INHIBITOR (FTI) EFFICACY IN CHRONIC MYELOID LEUKEMIA

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Approximately 95% of chronic myeloid leukemia (CML) cases are characterized by the Philadelphia translocation t(9;22)(q34;q11), which generates the constitutively active BCR-ABL fusion tyrosine kinase. Major down-stream effectors of BCR-ABL include signal transducers and activators of transcription (STATs), c-Jun NH2-terminal kinase, phosphoinositide 3-kinase and RAS. Deregulated RAS signaling plays an important role in the molecular pathogenesis of myeloid leukemias, and while strategies which target RAS signaling pathways are promising, their full therapeutic potential has yet to be realized. Blockade of RAS membrane localization by inhibiting post-translational modification (e.g. by farnesyltransferase inhibitors = FTIs) is one strategy to impede oncogenic RAS function *in vivo* and FTIs are currently being tested in clinical trials in myeloid leukemias. Importantly, alternative geranylgeranylation of K-RAS and N-RAS by geranylgeranyl transferase I (GGTase I) in the presence of FTIs allows membrane localization of RAS proteins and thus might represent a mechanism of FTI resistance. The aim of this study was to investigate the role of K-RAS in cellular resistance to FTI treatment. To analyze FTI effects on RAS post-translational prenylation, K562 cells were titrated with drugs, harvested, lysed, total cell proteins were separated by 15% SDS-PAGE and RAS-specific protein bands were detected by immunoblotting. Unprocessed RAS proteins were identified as band shifts, which migrate as slightly greater apparent mass than the farnesylated form. Induction of FTI-induced apoptosis was quantified by FACS analysis of Annexin-V stained cells. We generated shRNA constructs targeting two different regions of K-RAS, produced lentiviral supernatants and transduced K562 cells with greater than 96% efficiency as quantified by FACS analysis. Successful knock-down of K-RAS protein levels was detected by immunoblotting. We evaluated two clinically tested FTIs, namely BMS-214,662 (Bristol-Myers Squibb) and L-778,123 (Merck). Both FTIs blocked H- and N-RAS prenylation in K562 cells, but only FTI L-778,123, which possesses an additional activity against GGTase I, also inhibited K-RAS prenylation. FTI BMS-214,662 elicited apoptosis when used as a monotherapy, whereas no apoptotic effects were observed for L-778,123. The BMS-214,662-induced apoptosis was further enhanced by co-administration of FTI L-778,123. These results suggest that while inhibition of K-RAS alone is insufficient to elicit apoptosis, K-RAS inhibition does sensitize K562 cells to BMS-214,662-induced apoptosis. To further test this hypothesis, lentiviral sh-RNA constructs designed to target K-RAS were produced and used to transduce K562 cells. Interestingly, knock-down of K-RAS substantially potentiated BMS-214,662-induced apoptosis. Our results demonstrate that inhibition of K-RAS expression or blockade of its post-translational prenylation increase the ability of FTI BMS-214,662 to induce apoptosis in CML. As oncogenic K-RAS has been demonstrated to play an important role in generation of leukemic stem cells and as FTI BMS-214,662 is the first agent shown to specifically eradicate leukemic stem cells from CML patients, our observations may be useful in directing development of more effective therapeutic strategies.

0761

EXPLOITING THE UNIQUE MECHANISM OF ACTION OF SAPACITABINE (CYC682) TO OBTAIN SYNERGY WITH OTHER THERAPEUTIC AGENTS FOR CLINICAL USE IN ACUTE MYELOID LEUKEMIA

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Background. The nucleoside analogue cytarabine (AraC) forms the backbone of therapy for AML. As with other agents in its class, cytarabine is administered intravenously to patients and is thought to act by blocking DNA synthesis, causing cells to arrest in S phase. Sapacitabine (CYC682) is a novel nucleoside analogue that is orally available, is extremely well tolerated and has a unique mechanism of action involving the induction of DNA strand breaks, G2/M accumulation and apoptosis. As a result of promising clinical activity, sapacitabine is currently being evaluated in a Phase 2 trial comprising elderly patients with AML, as well as patients with myelodysplastic syndrome and is in a Phase 2 trial in cutaneous T-cell lymphoma (CTCL). *Aims*. Given that AraC, along with the majority of other anticancer agents are used in combination therapy in the clinic, we sought to perform a screen to evaluate the potential for combining sapacitabine with diverse classes of agents representing those in use or in testing in AML, such as daunorubicin, methyltransferase inhibitors and inhibitors of the histone deacetylases, together with compounds that exploit the mechanism of action of sapacitabine, including inhibitors of DNA repair, cell cycle checkpoints and cell survival. *Methods. In vitro* synergy was assessed in multiple cell lines including AML and CTCL using the Chou and Talalay median effect model, examining both sequential and concomitant treatments. The molecular basis for synergy was explored and the most promising schedules and drug combinations were tested in xenograft models. *Results*. Synergy was observed with demethylating agents, HDAC inhibitors, several topoisomerase inhibitors and inhibitors of the chk1 or aurora kinases. The HDAC combination was analysed further. Synergy was observed independently of the schedule of drug administration and resulted in synergistic increases in cell death. This was attributed to the activation of multiple BH3-only proteins, greater than additive depletion of several anti-apoptotic proteins, including MCL-1 and bclXL and activation of the pro-apoptotic proteins bax and bak. This combination has been evaluated in an MV4-11 model, where significant tumour growth delay and extension of survival was observed over the single agent treatments. Further mechanism of action and xenograft experiments are underway evaluating the most promising additional combinations. Results of the screen and more in-depth analysis of several of the most promising combinations will be presented. *Summary*. This screen has provided the basis for selecting several combinations of further interest and for consideration as part of the clinical development strategy for sapacitabine.

Red blood cells and Iron III

0762

THE EFFECT OF DEFERASIROX ON MYOCARDIAL IRON OVERLOAD AND CARDIAC FUNCTION: A PROSPECTIVE INDEPENDENT MONOCENTRIC STUDY USING CARDIOVASCULAR MAGNETIC RESONANCE T2*

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Background and Aims. Although deferoxamine in thalassemia major patients reduced organ damage and significantly prolonged life expectancy, its use has limitations that promote inconsistent adherence to treatment. Deferasirox, a new oral iron chelator, has been studied in adults and children. The aim of the study is to evaluate the impact of desferasirox treatment in thalassemia major patients in removing myocardial iron and in improving cardiac function by magnetic resonance T2* (MRI T2*). *Methods*. Forty-one patients (22 females, 19 males, mean age 32±6 years) affected by TM and treated with deferasirox performed 2 MRI evaluations. MRI was performed at baseline (T0) after a variable period of exposure to deferasirox (median: 12 months, range 4-48). The second one (T1) was after 13.6±2.5 months from T0. The MRI studies were performed using a 1.5 Tesla MR scanner (Avanto Siemens, Erlangen). Heart T2* normal values were defined above 20 ms, values under 10 ms were considered as severe iron overload. *Results*. In the overall population at T0 the mean cardiac T2* value was 27.41±10.48, the mean ferritin was 1300±985 and the mean iron intake in the last six months 0.34±0.07. The mean deferasirox dose was 26±7 mg/Kg/day. Despite a different length of deferasirox exposure and a different myocardial iron overload, an average improvement of myocardial T2* from 27.41 ms up to 29.83 ms was observed at T1 (p=0.004). Similarly, a trend of reduction in both end-systolic and end-diastolic left ventricular volumes was observed with an improvement of left ventricular ejection fraction (LVEF) from 63.59 up to 66.46% (p=0.013). The patients have been divided into two groups according to the myocardial iron overload at T0: group A with a T2* > 20 ms and group B with T2* between 10 and 20 ms. Only one male patient had a baseline cardiac T2* value under 10 ms (8.8 ms): at T1, after 13 months, with a daily dose of 40 mg/Kg/day of deferasirox he showed a significant improvement in cardiac T2* (8.8 vs 15.84 ms). In group A myocardial T2* changed from 33.10 to 34.71 ms (p=0.4) while in group B myocardial T2* changed from 15.67 to 19.61 ms (p<0.03). In group A LVEF increased from 65% at T0 to 68% at T1 (p= 0.01). End-diastolic volume (EDV) decreased from 142,7 to 134.8 mL (p=0.01), meanwhile end-systolic volume (ESV) passed from 48.89 to 43.43 mL (p=0.001). In group B LVEF increased from 59.66 up to 64.25% (p=0.04) at T1. EDV decreased from 139.83 to 130.41 mL (p<0.11) and ESV decreased from 56 to 48.25 ml (p=0.03). *Discussion and Conclusions*. Deferasirox reduces myocardial iron overload and improve left ventricular function in thalassemia major patients. Its efficacy is evident not only in those patients with cardiac iron overload but even in patients with heart T2* values above 20 ms. Myocardial T2* improves in concert with cardiac function in a relative short period of time. This confirm the prognostic significance of myocardial T2* and the relevance of the intensification of chelation therapy in patients with moderate/severe iron overload.

0763

DEFERASIROX REDUCES CARDIAC IRON BURDEN IN CHRONICALLY TRANSFUSED B-THALASSEMIA PATIENTS WITH MILD-TO-MODERATE CARDIAC SIDEROSIS AS DEMONSTRATED BY MRI T2*

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Background. Cardiomyopathy and congestive heart failure (CHF) resulting from myocardial iron deposition are the leading causes of death in regularly transfused patients with β-thalassemia major (TM). Preclinical and ongoing clinical studies suggest once-daily deferasirox effectively removes cardiac iron as well as controlling liver iron. *Aims*. To evaluate the effects of deferasirox on cardiac iron as examined by cardiac T2* magnetic resonance imaging (MRI) in patients with TM. *Methods*. Patients with MRI-evidence of cardiac iron (T2*<20 ms) and normal left ventric-

ular fraction (LVEF) [≥56%] were enrolled in a prospective, single-arm, multicenter trial. Deferasirox was administered at 30 mg/kg/day with the option to escalate to 40 mg/kg/day if there was <25% improvement in cardiac T2* compared with baseline, provided liver iron concentration (LIC) was ≥3 mg Fe/g dry weight (dw). Serum ferritin (SF) was assessed monthly and MRI assessments of LIC, cardiac T2* and LVEF performed every 6 months. Serum creatinine (SCr), biochemical and hematological status were also monitored. *Results*. Preliminary results from 26 evaluable patients (7 male:19 female; age 10-44 years) are reported. Mean baseline SF was 4307±613 ng/mL (312-12,655), geometric mean cardiac T2* 8.5±1.1 ms (7.7-9.4), mean LIC 19.3±3.2 mgFe/g dw (3.6-62.3) and mean LVEF 61.8±0.8% (55.6-73.3). At 12 months, 8/18 patients were receiving 40 mg/kg/day deferasirox. Cardiac T2* increased from baseline by 1.2 ms (n=18;+14.0%; p=0.02) and LIC decreased by 2.6 mgFe/g dw (n=18; -25%; p=0.02). SF fell by 606 ng/mL (n=17;-23%; p=0.13). LVEF remained stable. At 18 months, 6/15 patients were receiving 40 mg/kg/day deferasirox. Cardiac T2* increased from baseline by 3.2 ms (n=12;+33%; p=0.001) and LIC decreased by 4.7 mgFe/g dw (n=12;-50%; p=0.003; Figure). SF fell by 1057 ng/mL (n=15;-40%; p=0.03). Mean LVEF trended upward by 2.2% (n=12; p=0.15). Of 18 patients completing 12 months, 11 (61.1%) responded to treatment with >4% improvement in cardiac MRI T2*. Of 12 patients completing 18 months, 10 (83.3%) responded to treatment. Baseline cardiac T2* for all responders was ≥6 ms. The most common drug-related adverse events (AEs) were nausea (n=7), diarrhea and rash (n=5). One patient experienced a suspected serious AE (abdominal pain and vomiting requiring hospitalization) but completed the study. Six patients discontinued: two patient decisions, two AEs and two abnormal laboratory/test procedures. Both patients who withdrew due to AEs died; one enrolled with elevated baseline cardiac iron (T2*=1.8 ms) and died secondary to CHF 4 days after withdrawing. The other was due to sepsis and multi-organ failure 3 months after patient withdrawal. Three patients developed SCr >upper limit of normal (ULN) on two consecutive occasions. Two patients (8%) had abnormal transaminases (≥5xULN) on ≥2 occasions; both had abnormal values at baseline and were considered treatment-related. Both patients remained on study. *Conclusions*. Deferasirox (30-40 mg/kg/day) resulted in significant improvements to cardiac and hepatic iron after 12 and 18 months. Patients whose cardiac T2* improved over the course of treatment had a baseline T2*>6 ms, suggesting that for patients below this threshold, more intensive therapy is warranted. AEs were consistent with previous observations.

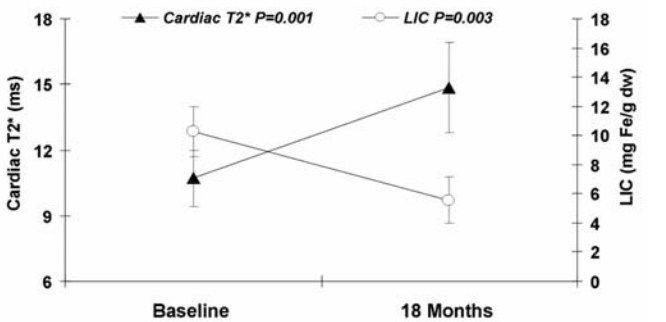

Figure. Mean change (±SEM) in cardiac T2* and LIC from baseline in patients completing 18 months of deferasirox therapy (n=12).

0764

FLUCTUATION OF CYSTATIN C CONCENTRATION DURING DEFERASIROX TREATMENT IN THALASSEMIC PATIENTS MAY REFLECT HEMODYNAMIC CHANGES

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Background. Deferasirox (Exjade®, ICL670) is an once-daily, oral iron chelator for the treatment of transfusional iron overload and its efficacy and tolerability have been established in adults and children with a range of transfusion-dependent anemias, such as thalassemia. We observed alterations in cystatin C plasma concentration during longterm treatment of thalassemia patients with deferasirox. Cystatin-C is a non-glycosylated protein that belongs to the cystatin superfamily of cysteine protease inhibitors. Plasma cystatin-C has been suggested to be an ideal endogenous marker of glomerular filtration rate (GFR). *Aims*. To investigate if these changes in cystatin C concentration were solely due to deferasirox treatment, or whether other factors contribute to these changes. *Methods*. 150 β-thalassemia patients treated with deferasirox at doses between 20-40 mg/Kg/day were followed longitudinally. Cystatin C concentrations were measured by an immunonephelometric technique (Dade Behring, Siemens Healthcare Diagnostics) at regular monthly intervals (5-12 monthly serial measurements per patient), while GFR was calculated according to the recently proposed cystatin C-based prediction equation using only its concentration in mg/L: GFR [mL/min/1.73m(exp)2] = 76.7 x Cystatin C(exp)-1.18. To further evaluate the effect of deferasirox on renal function we measured: a) plasma levels of Neutrophil-Gelatinase-Associated-Lipocalin (NGAL) (R&D Systems) a protein expressed on tubular cells and which production is markedly increased in response to harmful stimuli, such as ischemia or toxicity, b) N-terminal pro-B-type natriuretic peptide (NT-proBNP) (Roche Diagnostics), in order to correlate cystatin C fluctuation with left ventricular ejection fraction (LVEF) and c) ferritin level, as a marker of iron overload/mobilization. *Results*. The main results of the study showed that cystatin C concentration fluctuated during deferasirox treatment (ANOVA repeat measures p>0.850). Baseline mean values were 0.97±0.27 mg/L and reached a maximum of 1.01±0.29 mg/L at 4 months of treatment. According to the guidelines and as no significant increase of creatinine levels was observed, the drug dose was not reduced. Analysis of data showed that: a) there is no correlation between cystatin C fluctuation and NGAL levels, p>0.6745, evidence that the tubuloglomerular feedback is not activated b) cystatin C and NT-proBNP levels correlated positively with a binomial equation (p<0.004) and c) cystatin C and ferritin levels correlated positively with also a binomial equation (p<0.001). *Conclusions*. These findings suggest that cystatin C fluctuation during deferasirox treatment do not reflect renal injury. Hemodynamic signals, LVEF alterations and iron mobilization seem to play central role on changes of cystatin C.

0765

MALE AND FEMALE FERTILITY AND PREGNANCY IN THALASSAEMIA MAJOR, THE WHITTINGTON EXPERIENCE

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Background. Advances in the care with transfusion and chelation therapy has improved survival into adulthood and the health of adult thalassaemics giving us new challenges.Quality of life has improved as has expectation to have a family.The pituitary and hypothalamus are very sensitive to iron damage and hypogonadotrophic hypogonadism is a frequent complication.Ovulation induction or spermatogenesis techniques can be used to induce fertility. *Aims*. To examine both male and female fertility and pregnancy outcomes in patients with transfusion dependant thalassaemia who are under the care of the Whittington Hospital Thalassaemia Unit. *Methods*. An observational study spanning a twenty year period. *Results*. In total there were 41 successful pregnancies and 1 reported miscarriage in a total of 30 patients with transfusion dependant thalassaemia. Of 21 pregnancies in 16 female patients 11 were assisted conceptions, the majority being ovulation induction. 2 women had moderate cardiac iron overload at the time of conception one of which was given desferrioxamine from 25/40, and one woman had mild cardiac iron overload.2 women had severe liver iron overload at the time of conception one of which was started on desferrioxamine at 20/20. 4 had moderate liver iron overload and 2 had mild liver iron overload. Of the deliveries the majority were caesarean section. Of 21 conceptions in 14 male patients 6 were assisted either by spermatogenesis or invitrofertilisation (IVF)/intracytoplasmic sperm injection (ICSI). At the time of conception there was one patient with severe, 2 with moderate and 2 with mild cardiac iron overload.1 patient had severe liver iron overload with 5 having moderate and 5 having mild liver iron loading. *Conclusions*. Just over half of female thalassaemia major patients require assisted conception techniques. Pregnancy has been relatively uncomplicated for the majority even with some degree of cardiac iron overload. Desferrioxamine on a reduced dosing schedule in the second trimester onwards in patients with cardiac or liver iron overload has been used successfully. In male patients around a quarter require assisted conception techniques and there is often the need for intracytoplasmic sperm injection (ICSI). There have also been uncomplicated pregnancies with successful outcomes in patients who were on deferiprone at the time of conception.

0766

INFLAMMATORY MEDIATORS INDUCE AUGMENTED ADHESIVE PROPERTIES IN NEUTROPHILS FROM CONTROL AND SICKLE CELL DISEASE INDIVIDUALS, IN ASSOCIATION WITH ALTERED INTRACELLULAR CYCLIC AMP

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Background. Leukocytes play a role in the initiation and propagation of vaso-occlusion in sickle cell disease (SCD). *Aims*. To investigate whether the chronic inflammatory state, characteristically observed in SCD, may participate in the activation of SCD leukocytes, this study evaluated whether inflammatory mediators that are typically elevated in SCD plasma are capable of increasing the adhesive properties of neutrophils from healthy controls and from SCD patients and the signaling pathways that may be involved in this adhesion. *Methods*. Neutrophils (neu) were separated from the peripheral blood of healthy control individuals (CONneu) and SCD patients in steady state (SCDneu) over a ficoll-paque gradient. Following washing and lysis of contaminating red cells, neu were resuspended in RPMI medium and allowed to adhere to recombinant fibronectin (FN)-coated 96-well plates (5×10^6 cells/mL; 30 min, 37oC, 5% CO_2) in the presence of inflammatory mediators. Adhesion to FN was calculated as the percentage of the original cell suspension adhered. *Results*. As previously demonstrated, SCDneu adhesion to FN is significantly higher than that of CONneu (18.7±2.2%; 10.6±1.3%, respect. N≥10, $p<0.001$). The inflammatory mediators, prostaglandin E1 (PGE1, 10-50 μM) and Prostaglandin E2 (PGE2, 10-50 μM), did not significantly alter the adhesion of neither CONneu nor SCDneu to FN (results not shown, $p>0.05$). Co-incubation of neutrophils with the growth factor, GM-CSF (1-100 ng/mL), found in elevated levels in the plasma of our population of SCD patients, was capable of significantly increasing both CONneu and SCDneu adhesion to FN (11.7±2.4% basal adhesion, increased to 20.8±3.3% with 100ng/mL GM-CSF; n=7, $p<0.001$ for CONneu and 18.7±3.3% increased to 36.0±4.5% with 100ng/mL GM-CSF; n=4, p0.05). IL-8 (500ng/mL) and IL-6 (10pg/mL) also significantly increased both CON and SCD neu adhesion to FN. CONneu adhesion increased from 9.4±1.4% to 27.6±1.2% and 12.8±1.9% with IL-8 and IL-6, respect. (n≥3, $p<0.001$ and $p<0.05$, respectively). SCDneu adhesion increased from 16.7±3.0% to 30.3±4.5% and 22.4±3.5% with IL-8 and IL-6, respect., (n≥3, $p<0.05$). TNF-α significantly increased SCDneu adhesion (17.9±3.3% increased to 38.2±6.2% and 39.7±6.8% with 0.5 and 1.0 μg/mL TNF-α), but not CONneu adhesion (*data not shown*). Augmentation of CONneu adhesion by GM-CSF, IL-8, IL-6 and TNF-α is accompanied by significant increases in intracellular cAMP levels, a second messenger known to be encountered in elevated concentrations in SCDneu. Basal cAMP in CONneu was augmented by 135.8±20.3%; 127.7±22.1%; 60.1±24.2% and 64.7±20.2% following incubation (30 min, 37oC) with 10 ng/mL GM-CSF, 500 ng/mL IL-8, 10pg/mL IL-6 and 100 ng/mL TNF-α, respectively. N≥4; $p<0.05$. *Conclusions*. Results indicate that the chronic inflammatory state that is characteristic of SCD may contribute to the activation of leukocytes and their augmented adhesive properties, which in turn play an important role in the vaso-occlusive process. Alterations in adhesive properties are accompanied by alterations in intracellular cAMP, a second messenger known to alter leukocyte adhesive properties. Further studies may indicate whether the cAMP-protein kinase A pathway may represent a therapeutic target for the inhibition of leukocyte adhesion to the vascular wall.

0767

NEUTROPHILS ARE SUBJECT TO BOTH ANTI AND PRO APOPTOTIC STIMULI IN THE SERUM OF SICKLE CELL DISEASE INDIVIDUALS

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Background. Leukocytes play a central role in the sickle cell disease (SCD) vaso-occlusive process by adhering to the vascular endothelium and participating in oxidative stress and inflammation mechanisms. The relevance of neutrophil (neu) death to inflammatory disease pathogenesis is recognized, since alterations in leukocyte apoptotic processes may affect cellular function and inflammatory processes. *Aims*. This study investigated the effect of serum from control individuals (CON), SCD steady-state patients (SCD) and SCD patients on hydroxyurea therapy (SCDHU; 20-30 mg/kg/dayHU) on the apoptotic processes of neutrophils. *Methods*. Neu were separated from whole blood using ficoll-paque. Pools of serum were prepared by mixing serum from 18 individuals from each subject group. Neu (4×10^6cells/mL) were cultured in the presence of serum (10%v/v) for 16h (37°C, 5%CO_2) in RPMI. Apoptosis was evaluated by detection of annexin V binding; SOD levels were determined by ELISA. *Results*. CONneu and SCDneu were cultured in the presence of CONserum; SCDneu presented increased cell survival at 16h compared to CONneu cultured with CONserum (51.3±3.4; 40.6±3.0%; n≥12; p=0.02). In contrast, when CONneu were cultured in the presence of SCDserum, cells demonstrated a significantly lower percentage of non-apoptotic cells (40.9±15.1%; n=8), compared to CONneu cultured with CONserum (53.6±12.2%; n=8; p=0.0001 comp. SCD) or SCDHUserum (52.9±13.6%; n=8; $p<0.0001$ comp. SCD). CONneu were further co-incubated with serums and cytokine-neutralizing antibodies (IL-6; IL-8; TNF-α; GM-CSF). Anti-IL-8 (10μg/mL) reduced the number of non-apoptotic neutrophils following their culture in SCDserum (reduced from 42.1±2.3% to 37.3±2.5%; n=7; $p<0.05$); combinations of the anti-IL-8 with other cytokine-neutralizing antibodies did not further reduce CONneu survival when cultured in SCDserum ($p>0.05$). Despite the induction of apoptosis by SCDserum, activities of the caspase 3 and 8 enzymes were not significantly altered in CONneu cultured for 16h in SCDserum, compared to cells cultured in CON/SCDHUserum (data not shown), suggesting that SCDserum may exert a caspase-3-independent effect upon apoptosis. Intracellular reactive oxygen species (ROS) production has been reported to induce caspase-independent neutrophil apoptosis. Interestingly, significantly lower levels of superoxide dismutase (SOD), an enzyme that degrades superoxide, were found in SCDserum, compared to CONserum (12.4±2.8; 8.7±2.2; 14.2±4.7 U/mL; for CON, SCD, SCDHU resp, n≥8; $p<0.01$). When CONneu were co-incubated with serums and SOD (300U/mL), we observed a higher cell survival, compared to cells not incubated with SOD (survival increased by 24.8±8.2; 19.3±9.6; 11.9±5.4% for CON, SCD, SCDHU, resp.; n=7; $p<0.02$ comp to without SOD), indicating that superoxide production appears to contribute to neutrophil apoptosis. Conclusions: Whilst Neu from SCD may present an increased cell survival that could contribute to leukocytosis, these cells are subject to both anti- and pro-apoptotic stimuli in the serum of these individuals. Circulating cytokines, such as IL-8, may increase cell survival and production of ROS while decreased levels of serum antioxidants may augment cell death. Since alterations in neutrophil apoptotic processes may have significant effects on neutrophil function and number, in turn contributing to inflammatory processes and cellular damage at sites of inflammation, studies to understand the complex balance of these mechanisms in SCD are necessary.

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CREATING A EUROPEAN NETWORK OF EXPERT CENTRES ON RARE ANAEMIAS: A NEW CHALLENGE FOR ENERCA 3

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Background. ENERCA "European network for Rare and Congenital Anaemias" is co-funded by the European Commission (Public Health and Consumer Protection Directorate). Its website www.enerca.org is functional since 2001. The purpose of ENERCA is to improve the quality of life of patients with rare anaemia (RA) by increasing the efficacy of diagnosis, treatment and follow up. *Objective.* The main objective of ENERCA 3 is the establishment of a European Reference Network (ERN) of Expert Centres (EC) in RA. The ERN will serve as a platform for the provision of high quality information and services to health professionals, patients and other stakeholders such as health authorities and pharmaceutical industry. *Methods.* The partners involved in the Project will be 48 in total: 24 Associated and 24 collaborating covering the majority of MS. Most of the partners have been working together since 2002, and all are well known and recognized experts in their respective field. General methodology is designed on the basis of three transversal Work packages (WPs): WP1 "Networking of expert centres"; WP2 "Quality of patient care" and WP3 "Education and training" and three specific WP devoted to the public health issues and management of patients with RA: WP4 "Sickle Cell Disorders"; WP5: "Thalassaemia"; and WP6 "Very rare Anaemias". Three additional WP have been also designed in order to guarantee the Project management: WP7 "Evaluation", WP8 "Dissemination" and WP9 "Coordination". The methods to be undertaken are focused on creation of a consensus criteria proposal necessary to be fulfilled by an Expert Centre of the European Reference Network in Rare Anaemias, analysis of legal framework for referral of patients and samples among Member States in order to overcome the current legal barriers existing due to the different national rules and laws, the establishment of close links between experts in order to gather epidemiological data by means of the creation of a European Registry for patients with Rare Anaemias, elaboration of standardized guidelines for clinical procedures and the development of educational and training activities such as medical courses, continuous e-learning and material for the popularization of rare anaemias knowledge and assure quality services for patients. *Results*. Project outcomes will include the consolidation of the European Reference Network of Expert Centres in rare Anaemias, the promotion of the harmonization of diagnostic procedures by means of the elaboration of a comprehensive catalogue of External Quality Assessment Schemes (EQAS) in RA and the production of standardized guidelines, comparable epidemiological data and a better knowledge of the status of RA in the Member States due to the creation of the European Registry for patients with RA, and the increase of knowledge and awareness about RA by health professionals, patients and public in general by means of the production of training and educational material for professionals and patients. *Conclusions*. The achievement of the Project's objectives and outcomes will contribute to the improvement of health and quality of life of patients with RA by increasing the efficacy of patient's diagnosis, treatment and follow up.

0769

BILIRUBIN LEVELS AND TATA BOX POLYMORPHISM OF UGT1A1 GENE ASSOCIATED TO GILBERT'S SYNDROME IN THE DIFFERENT CLINICAL FORMS OF HEREDITARY SPHEROCYTOSIS

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Hereditary Spherocytosis (HS) is the most common non-immune hemolytic anemia in individuals of northern European ancestry, ranging from an asymptomatic condition to a severe life-threatening anemia. HS is usually classified as mild, typical or severe according to the severity of the symptoms and analytical presentation, namely, hemoglobin (Hb) concentration, reticulocyte count and serum bilirubin levels. Splenectomy, when performed, corrects the anemic state. In HS, because of hemolysis and consequent heme breakdown, plasma bilirubin increases. This toxic compound is metabolized in the liver by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1); however, when the activity of this enzyme is decreased, as occurs in Gilbert's syndrome (GS), a mild hyperbilirubinemia may arise. The co-inheritance of HS and GS can increase hyperbilirubinemia, and therefore, it may difficult the clinical classification of HS. The aim of our work was to evaluate bilirubin levels in HS patients according to UGT1A1 gene polymorphisms. We studied 132 Portuguese subjects: 106 were diagnosed with HS by standard screening tests (38 were splenectomized) and 26 were healthy individuals. The unsplenectomized patients were classified as having mild (n=43), moderate (n=18) and severe (n=7) HS, according to Guidelines for the diagnosis and management of Hereditary Spherocytosis (Bolton-Maggs *et al.*; Br. J. Haematol.; 2004). The TA insertion in the repetitive TATA box sequence (usually of 6 repeats) of the UGT1A1 gene promoter was screened by acrylamide gel (15%) analysis of polymerase chain reaction products. The studied subjects were grouped according the gene polymorphisms, namely, (TA)6/(TA)6 for homozygote individuals with normal UGT1A1 transcription, (TA)6/(TA)7 for heterozygote individuals and (TA)7/(TA)7 for individuals with decreased enzyme activity associated GS. We found that in splenectomized HS patients Hb concentration was similar to controls and that reticulocyte count and bilirubin levels were statistically and significantly increased. In unsplenectomized patients the same results were observed, with the exception of Hb concentration which was significantly lower. Also, Hb concentration decreased significantly and reticulocytes and bilirubin increased significantly with HS severity. Regarding the UGT1A1 polymorphism, we found that, in all of the studied groups (controls, splenectomized and total unsplenectomized patients), bilirubin was higher in (TA)7/(TA)7 individuals, followed by the (TA)6/(TA)7 and (TA)6/(TA)6 subjects, respectively. When we evaluated the unsplenectomized patients according to HS severity, we observed that in mild HS patients, (TA)7/(TA)7 individuals presented significantly higher values of bilirubin in relation to the other genotypes. Moreover, these individuals presented higher bilirubin levels even in relation to (TA)6/(TA)6 and (TA)6/(TA)7 moderate HS patients. Because we had only one patient with moderate HS and no severe HS patients with the (TA)7/(TA)7 genotype we could not ascertain about their bilirubin levels. Our data show that in mild HS patients with co-inheritance of GS, the observed hiperbilirubinemia was not in agreement with the typical mild HS presentation. Therefore, in HS clinical severity classification, it should always be considered the observed and the expected bilirubin values in accordance with the other clinical and analytical parameters used to classify HS.

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CARDIAC IRON OVERLOAD AFTER TREATMENT WITH ANTHRACYCLINES IS MODULATED BY HFE GENOTYPE

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The key role of iron in anthracycline-related cardiotoxicity, have raised the question that pre-existing genetic iron overload may amplify the iron-based toxicity of anthracyclines. Miranda *et al.* (Blood, 2003), in a murine model showed that mutations in the HFE gene are associated with higher anthracycline cardiotoxicity than expected in the presence of normal iron metabolism, however, there is not clinical data relating HFE mutations and anthracycline cardiotoxicity. *Aims*. The aim of this work was to evaluate the cardiac iron deposits induced by anthracyclines in necropsies obtained from cancer patients, and determine if iron deposits after treatment with anthracyclines is modulated by the HFE genotype of the patients. *Methods*. We retrospectively studied 97 clinical necropsies from patients with a solid or haematological tumour treated in the Hematology and Oncology Department between 1996 and 2005. Inclusion criteria were histologically confirmed diagnosis of cancer, availability of complete clinical record, existence of archived heart and liver histological tissue. Clinical, histopathology, molecular and iron deposition studies were performed. Determination of heart and liver iron concentration (iron mg/g of dry tissue) was determined in all cases in which formalin fixed tissue was available for atomic absorption spectroscopy. HFE gene mutation (C282Y, H63D), was determined by Light Cycler polymerase chain reaction. Statistical analysis was performed using SPSS program version 15.0 and SNPStats software. *Results*. DNA of enough concentration and integrity for genotyping C282Y and H63D polymorphisms was obtained in 93 cases. The distribution of HFE genotypes in the group of patients with data of heart iron load showed 13 cases heterozygous for H63D (G/C), and 4 for C282Y (C/T). Iron load was determined in 47 cardiac and 48 liver samples. 30 (63%) of them were included in the subgroup of patients treated with anthracyclines-based chemotherapy. C282Y heterozygous patients showed a trend to have higher iron deposits than patients not carrying mutations

(0.58 vs 0.31 mg/gr dw, p=0.08). Moreover, C282Y heterozygous patients treated with anthracyclines had a tendency to have higher iron load in heart (0.75 vs 0.08 mg/g dw, p=0.06) than patients not treated with anthracyclines, although these differences were not statistically significant. Haplotype analysis showed an association between higher iron deposits in heart tissue and the haplotype C282Y-T/H63D-G (OR: 0.3, 95% CI 0.02-0.57; p=0.03). Anthracycline treatment increased the OR to 0.55 (0.22-0.87, p=0.09) in patients with this haplotype. A cumulative anthracycline dose greater than 200 mg/m^2 was associated with a higher iron concentration in heart tissue (0.49 vs 0.24 mg/gr; T-Student, p=0.01). Although the number of red-cell transfusions was correlated with liver iron load (R: 0.75; p<0.001), an association of transfusion history with heart iron deposit was not found in our series (p=0.32). Ferritin plasma levels (p=0.31), liver iron load (p=0.24), or diagnosis of hemosiderosis (p=0.16) were also not related to heart iron load. *Conclusions*. Our study strongly sustains that anthracycline treatment induces a specific heart iron overload independent of systemic iron load. Furthermore, anthracycline induced heart iron overload can be influenced by the HFE genotype of the patient, which is in accordance with previously published experimental data.

0771

MRI LIVER IRON MEASUREMENT IN PATIENTS WITH TRANSFUSIONAL IRON OVERLOAD: DIFFERENT TECHNIQUES, DIFFERENT RESULTS, DIFFERENT THERAPEUTIC CONSEQUENCES?

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Background. Liver iron measurement plays a central role in the management of patients with transfusional iron overload. The development of non-invasive methods to assess liver iron requires calibration by parallel liver iron analysis in biopsy samples. For institutions with a limited number of patients this is not feasible. Therefore, the use of elsewhere established algorithms to calculate liver iron using locally generated MRI data represents an attractive alternative diagnostic possibility. *Aims*. To compare the results of liver iron measurement assessed by two MRI methods based on published validated protocols for liver iron calculation from standardized MRI sequences. *Methods*. Twenty-four patients with transfusional iron overload (median age 18.3yrs., range 2.9-38) underwent MRI for liver iron measurement. Examination with spin echo (SE) was performed according to the protocol developed by St. Pierre *et al.*[1] Gradient echo (GRE) sequences were acquired as published by Gandon *et al.*[2] In four patients with high liver iron additional GRE sequences were evaluated as proposed by Rose *et al.*[3]

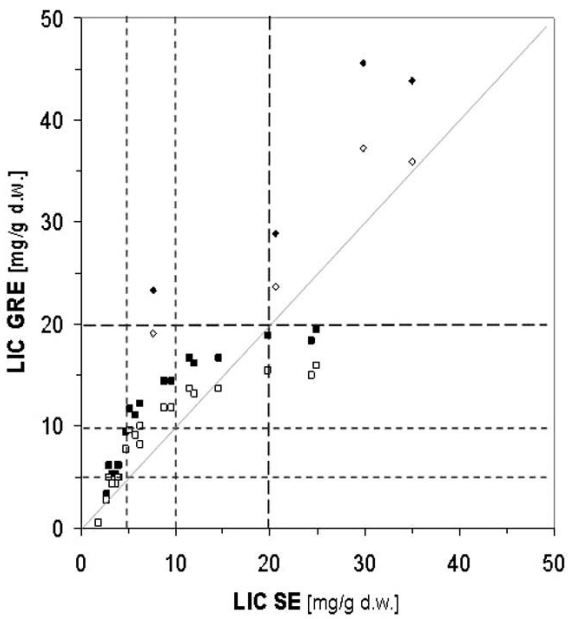

Figure 1. Liver iron assessed by GRE and SE MRI. Filled symbols represent GRE values before, empty symbols those after conversion (F=0.818). GRE values > 20mg/g d.w. were calculated according to Rose et al.[3], lower values according to Gandon et al.[2]

Results. The two MRI methods revealed different results for the liver iron concentration although a significant correlation between them was found (r=0.89, p=0.001). Values generated by GRE sequences (median 13.3mg/g dry weight (d.w.), range 0.6-45.6) were generally higher than those obtained by SE examinations ((median 6.9mg/g d.w., range 0.8-35). Since the calibration of both methods based on direct liver iron analysis in differently processed biopsy specimen (paraffin-embedded samples vs. fresh tissue) published conversion factors were applied to harmonise the results. However, even thereafter, data obtained by GRE remained higher than those from SE analyses. Regarding suggested values defining the optimal range for chelation therapy and the increased risk of cardiac complications, the discrepancies between both MRI methods may be of clinical relevance: 15/24 SE and 12/24 GRE liver iron values (after conversion) were lower than 9.6 mg/g d.w., 4/24 SE and 9/24 GRE values were greater than 9.6 but lower than 20 mg/g d.w., and 5/24 SE and 3/24 GRE values were above the critical threshold of 20 mg/g d.w.. Both, SE and GRE liver iron values correlated to serum ferritin levels (r=0.85 and r=0.81, p=0.00025). *Conclusions*. According to the original publications both MRI methods compared here were accurately calibrated and validated. Nevertheless, in our study they revealed different results which in individual patients could lead to different decisions concerning the management of chelation therapy. Further studies are necessary to confirm this single centre experience. If absolute values of a specific method are used to adjust treatment in an individual patient it is essential to consider the original source of the calibration data of the MRI method with regard to the processing of liver biopsy specimen.

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0772

EFFICACY AND SAFETY OF DEFERASIROX IN CHELATION-NAÏVE PATIENTS WITH B-THALASSAEMIA MAJOR: RESULTS FROM THE LARGE-SCALE EPIC STUDY

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Background. Patients with β-thalassaemia major are diagnosed during infancy, requiring lifelong transfusions and early chelation therapy to prevent complications related to iron overload. Previous deferasirox studies in β-thalassaemia major patients predominantly included those who had received prior chelation therapy; a further focus of clinical interest is to assess the safety and efficacy of deferasirox in patients who are chelation-naïve. The large, 1-year, prospective, multicenter EPIC trial, evaluating the efficacy and safety of the once-daily oral chelator deferasirox in patients with transfusion-dependent anaemias, included 937 patients with β-thalassaemia major, of whom 66 were chelation-naïve. *Aims*. To assess the efficacy and safety of deferasirox in chelation-naïve patients with β-thalassaemia major participating in the EPIC study. *Methods*. Patients enrolled in the EPIC study were aged ≥2 years with transfusional iron overload as shown by serum ferritin (SF) levels of b1000 ng/mL, or <1000 ng/mL, but with a history of multiple transfusions (>20 transfusions or 100 mL/kg of blood), and liver iron concentration (LIC) of >2 mg Fe/g dry weight confirmed by R2 magnetic resonance imaging. Deferasirox was dosed at 10-30 mg/kg/day dependent on transfusion requirements; dose adjustments of 5-10 mg/kg/day (range 0-40 mg/kg/day) were performed every 3 months based on SF trends and safety markers. The primary efficacy endpoint was change in SF from baseline to 52 weeks. *Results*. 66 patients with β-thalassaemia major (37 male:29 female; mean age 6.5±5.97 years) aged b2-<6 (n=40), 6-<12 (n=20), 12-<16 (n=1) and 16-<50 years (n=5), who were chelation-naïve at study entry were included in this analysis. Median SF at baseline was 3080, 6045, 8526, and 3745 ng/mL in the b2-<6, 6-<12, 12-<16, and 16-<50 years age groups, respectively. The Table 1 below summarizes the SF results by age group. Of 66 patients, two (3%) discontinued due to

gastrointestinal adverse events (AEs) and abnormal laboratory values (both n=1). The most common investigator-assessed drug-related AE was rash (n=3, 4.6%). There were no investigator-assessed serious drug-related AEs. 23 patients (34.8%) had serum creatinine >33% above baseline on two consecutive visits, and one patient (1.5%) had serum creatinine >33% above baseline and the upper limit of normal (ULN) on two consecutive visits; there were no progressive increases.

Table 1. Median change from baseline in SF (ng/mL) by age group.

			Baseline		End of study	
Age groups	Mean actual dose ± SD, mg/kg/day	Mean iron intake ± SD, mg/kg/day	n	Median SF	n	Median change from baseline in SF
≥2–<6 years	23.8 ± 3.0	0.72 ± 0.28	40	3081	39	440
6–<12 years	23.7 ± 2.8	0.61 ± 0.21	20	6045	20	–620
12–<16 years	25.1	0.48	1	8526	1	–2174
16–<50 years	23.0 ± 4.9	0.43 ± 0.39	5	3745	4	21
All patients	23.7 ± 3.0	0.66 ± 0.28	66	3439	64	98

Summary and conclusions. In this cohort of chelation-naïve patients with β-thalassaemia major, the effect of deferasirox on SF was reflective of baseline SF, transfusional iron intake and average actual dose received during the study. This effect is similar to that seen in the overall population of patients with thalassemia major enrolled in the study, which included 93% of patients who had received prior chelation therapy.[1] Patients aged 6-<12 years had a substantial reduction in SF with an average actual dose of 23.7 mg/kg/day. In patients aged ≥2-<6 years, despite receiving a similar average actual dose, this dose was suboptimal because of the high transfusional iron intake and known pharmacokinetic profile (lower steady state exposure compared to adults) in paediatric patients.[2] These results further highlight the need of having individualized dose titration guided by iron burden, iron intake and SF trends. In this chelation-naïve young patient population, deferasirox was safe and generally well tolerated with a very low discontinuation rate.

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0773

GENE EXPRESSION OF THE IL-6/STAT-3/HAMP SIGNALING PATHWAY IN MONONUCLEAR CELLS IN SICKLE CELL DISEASE

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Hepcidin is a key regulatory hormone of iron metabolism and its upregulation in monocytes causes ferroportin internalization and iron retention in patients with anemia of inflammation mediated by stimulation of the IL-6/STAT-3 signaling pathway. Sickle cell disease has been recently associated to a chronic inflammatory state. We hypothesized that sickle cell disease could cause elevation of the expression of hepcidin, leading to iron retention in mononuclear cells by activating the same pathway. We determined gene expressions of the IL-6/STAT-3 pathway in samples of healthy controls (n≥10), steady-state sickle cell anemia (SCA) patients, SC disease (SC) and three groups of HbS-β thalassemia (HbS/β0-CD39 C->T; HbS/IVS1-nt5 G->C and HbS/IVS1-nt6 T->C) (n≥5 for each group), transfusion-independent patients and not on hydroxyurea treatment. Mononuclear cells were separated from whole blood before extracting mRNA, and synthesizing cDNA. Quantification of gene expression was performed by Real-Time PCR for the following genes: HAMP (hepcidin), SLC40A1 (ferroportin) and IL-6 relative to β-actin and GAPDH genes. HAMP gene expression was significantly increased in sickle cell anemia compared to controls (0.97±0.27 vs. 0.20±0.03, p=0.017), but not in other sickle cell disease groups (SC 0.62±0.35, HbS/β0-CD39 0.54±0.43, HbS/IVS1-nt5 0.24±0.06, HbS/IVS1-nt6 0.40±0.08, p>0.05). SLC40A1 (SS 0.51±0.13, SC 0.57±0.12, HbS/β0-CD39 0.71±0.11, HbS/IVS1-nt5 0.65±0.13, HbS/IVS1-nt6 0.38±0.06, control 0.57±0.08, p>0.05) and STAT-3 (SS 1.16±0.23, SC 1.02±0.23, HbS/β0-CD39 0.82±0.13, HbS/IVS1-nt5 1.23±0.32, HbS/IVS1-nt6 0.82±0.08, control 0.89±0.21, p>0.05) expression did not differ among groups. IL-6 was studied only in sickle cell anemia samples and confirmed no difference compared to controls (SS 0.18±0.02 vs. control 0.17±0.06, p>0.05). Our data suggest that although hepcidin is upregulated in mononuclear cells of sickle cell anemia patients, it is probably not influenced by the IL-6/STAT-3 pathway since there is no accompanying upregulation of IL-6 or STAT-3. Absence of downregulation of SLC40A1 probably correlates with the fact that hepcidin is able to internalize ferroportin, rather than downregulate SLC40A1 gene transcription. Iron retention in mononuclear cells in sickle cell disease has different physiopathological mechanisms from those found in mononuclear cells in anemia of inflammation and further studies should elucidate the underlying pathway and its function in sickle cell disease monocytes.

0774

OXIDATIVE STRESS AND GENE EXPRESSION OF INFLAMMATORY MEDIATORS IN MONONUCLEAR CELLS IN SICKLE CELL DISEASE

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Inflammation, cell adhesion to vascular endothelium, and endothelial injury appear to contribute to sickle cell anemia (SS) vaso-occlusion. Furthermore, blood levels of inflammatory and anti-inflammatory cytokines (TNF-α, IL-6, IL-10), as well as reactive oxygen species (ROS) are reported to be elevated, both in steady state and during crisis, but reports have been conflicting and a conclusive role for these molecules in the disease remains to be established. The aim of this study was to evaluate gene expression of inflammatory mediators and ROS production in mononuclear cells in healthy controls (n≥6) and SS, SC disease and three groups of HbS-β thalassemia (HbS/β0-CD39 C->T; HbS/β+-IVS1-nt5 G->C and HbS/β+-IVS1-nt6 T->C, n≥5 for each group), transfusion-independent patients, not on hydroxyurea treatment. qRT-PCR analysis was use to examine expression of the following genes: TNF-α, IL-8, IFN-γ, IL-10 and heme oxygenase-1 (HMOX-1), which breaks down heme released during hemolysis and protects tissue against oxidative stress. For ROS measurement, the isolated cells were incubated with 2'-7'-dichlorofluorescin diacetate (DCF) and analyzed by flow cytometry. TNF-α expression was significantly higher in SS individuals, when compared to control individuals (0.34±0.06 vs. 0.13±0.03, p=0.0089), but not in the remaining groups (SC 0.07±0.04, HbS/β0-CD39 0.06±0.02, HbS/β+-IVS1-nt5 0.02±0.004, HbS/β+-IVS1-nt6 0.08±0.03, p>0.05). IL-8 expression was significantly higher in SS, HbS/β0-CD39 and HbS/β+-IVS1-nt5 individuals, when compared to control individuals (0.07±0.02, 0.15±0.05, 0.12±0.02 vs. 0.02±0.006, respectively, p<0.05), but not in the remaining groups (SC 0.06±0.04, HbS/β+-IVS1-nt6 0.08±0.02, p>0.05). No differences were found in IFN-gamma expression among groups or compared to controls (SS 0.20±0.06, SC 0.16±0.14, HbS/β0-CD39 0.16±0.10, HbS/β+-IVS1-nt5 0.15±0.07, HbS/β+-IVS1-nt6 0.13±0.05, control 0.29±0.06, p>0.05). IL-10 expression was significantly higher in SC, HbS/β0-CD39 and HbS/β+-IVS1-nt5 individuals, when compared to control individuals (1.20±0.43, 0.89±0.02, 0.38±0.06 vs. 0.17±0.04, respectively, p<0.05), but not in the remaining groups (SS 0.28±0.05 and HbS/β+-IVS1-nt6 0.34±0.03, p>0.05). HMOX-1 expression was significantly higher compared to healthy controls in all groups (SS 1.14±0.45, HbS/β0-CD39 2.20±0.43, HbS/β+-IVS1-nt5 2.15±0.64, HbS/β+-IVS1-nt6 1.30±0.30, SC 1.70±0.34, control 0.24±0.11, p<0.01). ROS production was significantly increased in mononuclear cells compared to the same cells in control individuals (SS 1339±56, HbS/β0-CD39 1160±121, HbS/β+-IVS1-nt5 1166±100, HbS/β+-IVS1-nt6 633±94, SC 909±148, control 369±50, p<0.05). An investigation of the production of inflammatory cytokines, anti-inflammatory cytokines and oxidative stress in SCD patients may further elucidate the pathogenesis of the disease and its complications and help in assessing disease severity and prognosis according to genotype.

0775

ACUTE HUMAN PARVOVIRUS B19 INFECTION AND NEPHROTIC SYNDROME IN PATIENTS WITH SICKLE CELL DISEASE

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Background and Aims. Human Parvovirus B19 (HPV B19) infection has previously been thought to be a trigger of nephrotic syndrome. The virus is associated with significant morbidity in patients with sickle cell disease (SCD) and a major cause of transient red cell aplasia (TRCA). We investigated possible links between acute HPV B19 infection and the development and nephrotic syndrome in our cohort of SCD patients. *Methods*. We also examined the HPV B19 seroconversion status and indices of proteinuria and renal function in a cohort of SCD patients who attended our specialist clinic between July and December 2008, as well as 37 cases of acute HPV B19 infection in SCD patients admitted to King's College Hospital between November 2002 and July 2008. *Results*. Of the 37 cases (average age 11 years) of acute HPV B19 infection, 29 cases were in children below the age of 16 years, 8 in patients over the age of 16 years. Three patients developed NS within 4 months of acute HPV B19 infection and TRCA. Two of these patients were aged 17 and 26 years at diagnosis (patient 1 and 2 respectively), whereas the third was a child aged 11 (patient 3). Renal histology demonstrated the collapsing variant of focal segmental glomerulosclerosis (FSGS) in the acute phase of NS in patient 1, a characteristic finding associated with HPV B19-associated nephrotic syndrome. A subsequent biopsy in the same patient two years later demonstrated non-collapsing FSGS and marked interstitial fibrosis. Patient 2 had a renal biopsy performed 4 months after the onset of NS. This demonstrated non-collapsing FSGS, acute sickle nephropathy and significant chronic tubular atrophy. Patient 3 also had a renal biopsy 1 year after her acute HPV B19 infection which demonstrated the cellular variant of FSGS. All 3 patients have persistent significant proteinuria, with impaired renal function in the 2 older patients. *Conclusions*. Consistent with previous reports, we observe that NS is a relatively rare complication of HPV B19 infection in young children with SCD (one case in 29 in our cohort). However, 2 out of 8 patients older than 16 years developed this complication. Proteinuria in patients with SCD is common and gradual in onset, and eventually may progress to NS. NS of acute onset in adults with SCD is rare, only two cases were observed during a 6-year period among our cohort of over 400 adult patients with SCD. In both cases, they were associated with recent HPV B19 infection. It has previously been noted that acute HPV B19 infection in older patients with HbSC SCD has a more severe clinical course than younger children (Smith-Whitley *et al.*, 2004). Our data, though limited by small numbers in the cohort, suggest that older SCD patients with acute HPV B19 infection may be more susceptible to chronic complications including the development of NS and progressive renal fibrosis.

0776

WITHDRAWN

0777

C-TERMINAL DELETION IN THE ALAS2 GENE AND X-LINKED DOMINANT PROTOPORPHYRIA

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Background. Erythropoietic protoporphyria is an inherited disorder caused by partial mitochondrial deficiency of ferrochelatase (FECH), the terminal enzyme of heme biosynthesis. Most patients have autosomal-dominant EPP (dEPP), in which clinical expression normally requires coinheritance of a FECH mutation that abolishes or markedly reduces FECH activity trans to a hypomorphic FECH IVS3-48C allele. About 4% of families have autosomal-recessive EPP. In Italy, mutational analysis fails to detect FECH mutations in about 20% of EPP families, of which about 50% are homozygous for the wild-type FECH IVS3-48T allele, suggesting possible involvement of another locus. Recently, Whatley *et al.* described a previously unreported form of X-linked dominant protoporphyria (XLPP) associated to c.1706-1709 delAGTG and c.1699-1700delAT deletions in ALAS2 exon 11. In their paper the authors suggested that a modification of the C-terminal region of ALAS2 may be responsible for a protophorphyria phenotype by gain of function mechanism. The marked increased ALAS2 activity in these cases that could explain the over-production of protoporphyrin IX, despite of normal FECH activity, and the reduction of iron stores that are often observed in these patients. *Aims*. In view of this report we re-examined 7 Italian unrelated FECH-negative EPP families for a total of 19 subjects. *Methods*. The ALAS 2 exon 11 has been amplified by PCR and submitted to direct automated sequencing. *Results*. In 4 families, 6 males and 3 females carried the deletion c.1706-1709 delAGTG. This supports Whatley observation and suggest that defects in ALAS2 could be an alternative genetic background for protoporphyria. In contrast to the authors, we found a remarkable heterogeneity of phenotypes between females that could result from X-chromosome inactivation. Out of three unrelated females with the ALAS2 deletion, one, mother of a proband, was asymptomatic while the other two showed increased level of protoporphyrin causing severe photosensitivity, despite normal FECH activity and homozygosis for the wild-type FECH IVS3-48T allele. Otherwise we found no evidences that x-inactivation could lead to a milder disease in symptomatic females. In fact, these latter showed a similar erythrocytes protoporphyrin concentration and liver involvement as symptomatic males. *Conclusions*. The molecular defect in 3 FECH-negative EPP families remain still unknown, indicating that new gene targets can potentially offer new opportunities for diagnosis and treatment of EPP.

Platelets and thrombocytopenia II

0778

ACCURACY OF PLATELET COUNTING BY HAEMATOLOGY ANALYSERS AND ITS POTENTIAL IMPACT ON THE TRANSFUSION DECISION-MAKING PROCESS

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Background. Although haematology analysers provide reliable full blood counts, they are known to be inaccurate at enumerating platelets in severe thrombocytopenia. If the thresholds for platelet transfusion, currently set at 10×10^9/L, are to be further reduced, it is vital that the limitations of current analysers are fully understood. Almost all automated haematology cell analyzers use methods based on either the impedance (PLTi) or the optical (PLTo) properties of the cells to count platelets. Cell-Dyn automated blood analyzers have incorporated in the last years methods based on the use of anti-GPIII (CD61) monoclonal antibodies (ImmunoPLT method) to improve the accuracy of platelet counting in peripheral blood (PB). *Aims.* To evaluate the correlation among the three technologies using two different analyzers and its potential impact on the transfusion decision- making process. *Study design and Methods.* A comparative evaluation was made among the three methods currently available in the Cell-Dyn Saphire (optical, impedance and CD61-ImmunoPLT) and the two methods in Sysmex XE-2100 (Impedance and optical fluorescence). We have measured platelet counts in 300 PB samples from patients with >100 platelets $\times10^9$/L and in 66 PB samples from patients with chemotherapy-induced thrombocytopenia and <100 platelets $\times10^9$/L. We have considered the inmunoPLT method with anti-CD61 our gold-standard. The statistical analysis has been performed with the SPSS software (v15). We have analysed the intraclass correlation among the different parameters. *Results*. PB platelet counts showed a very good correlation among the PLTo, PLTi of both analyzers (intraclass correlation >0.97) when the platelet count is $>100\times10^9$/L. The correlation is good when the count lies between $20\text{-}100\times10^9$/L, especially between the inmunoPLT method and the PLTi used by the Sysmex analyzer. On the contrary, when platelet count is $<20\times10^9$/L the correlation among the CD61 and the optical or impedance counts is very poor. *Conclusions*. Our results suggest that the immunoPLT method should be used with counts $<20\times10^9$/L since the optical and the impedance methods do not provide reliable counts. The use of this method in the transfusion decision-making process could avoid a number of unnecessary platelet transfusions.

0779

CLINICAL OUTCOME AND RISK FACTOR ANALYSES IN THROMBOTIC THROMBOCYTOPENIC PURPURA-HEMOLYTIC UREMIC SYNDROME PATIENTS WHO UNDERWENT THERAPEUTIC PLASMA EXCHANGE

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Background. Plasma exchange (PE) is widely used for treatment of patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome (TTP-HUS). Various responses to PE have been reported according to etiology of TTP-HUS. Factors related to treatment response or survival in these patients need to be elucidated. *Aims.* The objectives of this study were to evaluate clinical outcomes of therapeutic PE according to etiology of TTP-HUS and to analyze factors related to treatment response or survival in TTP-HUS patients who received PE. *Methods*. We analyzed patients who were treated with PE due to TTP-HUS between August 1998 and October 2008 at the Seoul National University Hospital. Response to PE was defined as the achievement of a platelet count 150×10^9/L or more. *Results*. A total of 52 patients (male, 20) were included. Median age was 47 years (range, 17-81). Secondary causes were identified in 38 patients (73.1%): drugs (25.0%), stem cell transplantation (SCT) (19.2%), malignancies (11.5%), pregnancy (5.8%), autoimmune diseases (5.8%), and surgery (1.9%). The other 14 cases (26.9%) were classified as idiopathic TTP-HUS. All patients had thrombocytopenia and microangiopathic hemolytic anemia. Renal dysfunction (serum creatinine >1.4 mg/dL), fever (>38.0'), and neurologic symptoms were observed in 40 patients (76.9%), 23 patients (44.2%), and 32 patients (61.5%), respectively. Neurologic symptoms were as following: motor weakness (17.3%), involuntary movement (1.9%), decreased consciousness (15.4%), seizure (9.6%), disorientation (7.7%), headache (7.7%), and dizziness (1.9%). Patients underwent median 5 sessions of PE (range, 1-32). Twenty seven patients (51.9%) responded to PE and 4 patients (7.7%) relapsed despite initial responses. Response rate in idiopathic and secondary TTP-HUS was 71.4% and 44.7%, respectively. No patients with SCT-related TTP-HUS responded to PE. Median overall survival (OS) was 5.2 months (95% confidence interval (CI), 0-27.3) and estimated 1-year survival rate was 47.8%. Patients with SCT-related TTP-HUS had significantly shorter OS than those with the other etiologies ($p<0.001$). Patients with high baseline serum creatinine (>1.4 mg/dL) had lower response rate (42.1%) than the others (78.6%) in univariate analysis ($p=0.029$) and multivariate analysis including etiology adjustment (relative risk 0.10 (95% CI, 0.02-0.58), $p=0.010$). In addition, patients with fever (>38.0') or neurologic symptoms had significantly shorter OS in univariate analysis ($p=0.027$, $p=0.050$, respectively). In multivariate analysis including etiology adjustment, existence of neurologic symptom was the only significant risk factor for OS (hazard ratio 2.74 (95% CI, 1.11-6.76), $p=0.029$). PE-related adverse events were observed in 18 patients (34.6%): allergic reactions (28.8%), chest discomfort (7.7%), abdominal discomfort (5.8%), transient hypoxemia (3.8%), paresthesia (3.8%), headache (3.8%), and arm pain (1.9%). All adverse events were manageable. There was no fatal event. *Summary and Conclusions*. PE could be an effective treatment in idiopathic TTP-HUS patients. Secondary TTP-HUS patients had various treatment responses according to etiology. High baseline serum creatinine level (>1.4 mg/dL) was predictive factor for poor treatment response and existence of neurologic symptoms was poor prognostic factor. PE-related adverse events were mild and manageable.

0780

AUDIT ASSESSING POSSIBLE RISK FACTORS FOR THROMBOTIC MICROANGIOPATHIES IN A SINGLE CLINICAL APHERESIS UNIT

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Background. Thrombotic microangiopathies (TMA) are a rare, clinically diverse set of conditions sharing the same pathophysiological mechanism of microvascular endothelial injury leading to microthrombi, red cell fragmentation, platelet activation / accelerated consumption and hypoxic damage to multiple organs. It includes the conditions Thrombotic Thrombocytopenic Purpura, Haemolytic Uraemic Syndrome and HELLP syndrome. *Aims*. ADAMTS13 deficiency and E.coli O157 infection alone cannot explain the variable manifestations of TMA. Population studies have revealed sepsis, hypertension and immunosuppression as possible TMA precipitants. We decided to assess risk factors of TMAs in a cohort of patients from a large regional clinical apheresis unit. *Methods.* Analysis of patient records from October 1997 to December 2008 of those receiving therapeutic plasma exchange in a single clinical apheresis unit with the following criteria: 1) Microangiopathic haemolytic anaemia with red cell fragmentation on blood film. 2) Thrombocytopaenia with a platelet count $<150\times10^9$L-1. 3) Elevated lactate dehydrogenase (LDH) >150% of upper limit of local reference range. Data was collected on basic demographics, laboratory variables including platelets, LDH and creatinine, clinical information including presenting features, pre-morbid conditions and previous medications, and clinical outcome at discharge. SPSS version 15.0 was used for statistical testing, significance level 5%. *Results*. 65 patients met our criterion, median age 52 (range 1-81 years) and female to male ratio of 2:1. Outcome at discharge was available from 54 patients, 11 (20%) died, 35 (65%) fully recovered, and 8 (15%) remained impaired, 7 due to dialysis dependent renal failure and one due to cerebrovascular disease (CVA). Only age was related to outcome: older patients were more likely to die ($p=0.018$ on Kruskal-Wallis testing). 39 (60%) patients suffered gastrointestinal symptoms immediately prior to their TMA, of which 27 cases (42%) were due to diarrhoea. 45 patients (69%) either had documented infection on admission or experienced acute GI symptoms before admission. However, E. coli O157 was cultured in just nine cases (14%). Previous to admission 17 patients (26%) were on antihypertensive medication and six patients (9%) were taking calcineurin inhibitors (ciclosporin A or tacrolimus). Nine patients (14%) had a history of autoimmune disease. On admission 24 patients (38%) experienced CNS symptoms; type was significantly associated with age. 11 patients experienced seizures and were younger than those who did not (Median 21 versus 54 years, $p=0.018$, Mann-Whitney testing); six patients experienced a CVA and were older (Mean 72.5 versus 45 years, $p=0.004$, Mann-Whitney testing). We observed a positive correlation between referral platelet count and serum creatinine (r=0.38, $p=0.002$, spearman's

(two tailed) correlation coefficient). *Summary*. This cohort had high rates of infection and diarrhoea immediately before their TMA, and high rates of antihypertensive therapy. These data fit with the hypothesis of acute endothelial damage acting as a trigger in patients predisposed either by genetic susceptibility or by pre-existing anti-ADAMTS13 antibody. Our cohort could be expanded and compared to a large age matched control group to ascertain further risk factors (Figure 1). Limitations of this audit included unavailability of ADAMTS13 testing, and recall bias due to its prospective nature.

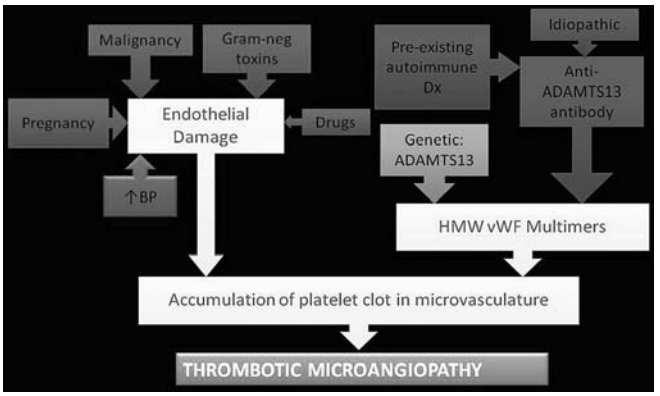

Figure 1. Multifactorial Model for TMA.

0781

ANALYSIS OF CARBONYLATED PROTEINS IN HUMAN BLOOD PLATELETS

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Background. During platelet activation the reactive oxygen and nitrogen species (RONS) are produced. These unstable molecules participate in activation cascade as second messenger and they can also modify other macromolecules (lipids, proteins) in their close vicinity. Generation of new carbonyl groups is one of the first steps in oxidative damage of proteins. Modified proteins can loose their physiologic properties - cooperation in cytoskeleton reorganization during platelet activation. The objective of the study was to propose methods suitable for large-scale oxidative stress studies, e.g. production of platelet's carbonylated proteins. *Aims.* The aim of this work was isolation and identification of carbonylated proteins in human blood platelets both in resting platelets and in platelets activated with various agonists. *Methods*. Platelets were isolated from blood of healthy volunteers who had not taken any antithrombotic drugs. Washed platelets were activated by three different types of agonists: thrombin, collagen and arachidonic acid. Platelet activation was detected photometrically by measuring aggregation response. Carbonylated platelet proteins were derivatized using biotine-hydrazide chemistry with subsequent affinity capture on avidine-agarose. Biotinylated proteins were eluted by biotine and were either analyzed by SDS-PAGE with immunoblotting or digested by trypsine. Resulting peptides were analysed using 2D-HPLC/ESI-MS/MS and carbonylated proteins were identified. The results were compared with 2D-SDS-PAGE of non-biotinylated samples that were subsequently electroblotted, carbonylated proteins were modified by dinitrophenylhydrazine and vizualizated by immunochemical detection. *Results*. The largest amount of carbonylated proteins were found in collagen activated platelets. The data showed that the biotine-based immunocapture of carbonylated proteins as a simple technique can be used to isolation of carbonylated subproteome of (platelet) proteins with minimum false-positive results. The method is suitable for high-throughput screening of carbonylated proteins either using parallel sample processing or due to its easy automation using HPLC systems. *Conclusions.* Human platelets undergoes many oxidative reactions during activation with various agonists, in patients suffering with oxidative stress which is present in different kinds of diseases and also in stored platelet's concentrates. Therefore our results can improve the knowledge of protein processing in platelets both in health and disease.

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0782

PLATELET MICROPARTICLES IN PEDIATRIC IMMUNE THROMBOCYTOPENIC PURPURA

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Background. Immune thrombocytopenic purpura (ITP) is one of the most common hemorrhagic disorders encountered in childhood. Platelet microparticles (PMP) arise in association with platelet activation with procoagulant activity. Elevated PMP were observed in adult ITP and high concentrations of active PMP were reported to be thrombogenic in certain settings. However, the clinical significance of PMP in pediatric ITP has not been studied.Objectives: This study aimed to assess PMP levels in ITP in children and adolescents, and its correlation with clinical status, bleeding score, and response to therapy. *Methods*. Fourty patients with ITP were randomly selected from the Hematology Clinic, Children's Hospital, Ain Shams University,Cairo,Egypt. Twenty patients (14 females and 6 males) with acute ITP (mean age 10.5±3.4 y) and 20 patients (12 females and 8 males) with chronic ITP (mean age 11.1±3.1 y) and thirty sex and age matched healthy controls (20 females and 10 males) with mean age 10.7±4.4 y.Patients were subjected to detailed history, calculation of bleeding score, complete blood count, cytological bone marrow examination and PMP quantitation in peripheral platelets by flow cytometry. *Results*. Compared to controls, acute ITP patients had significant increase in PMP, PMP/platelet count, PMP % (p<0.0001, p<0.0001, p<0.0001; respectively).There was non significant difference in PMP in chronic ITP compared to controls (p>0.05), but highly significant increase in PMP/platelet and PMP % in chronic ITP compared to controls (p=0.0009, p=0.03; respectively). There was significant increase in PMP, PMP/platelet count, PMP % in acute ITP compared to chronic ITP patients (p=0.001, p=0.003, p<0.0001; respectively). No significant correlation was evident between PMP and platelet count in either group (p>0.05). Neither mucosal bleeding, petichiae nor thrombotic manifestations were observed in the studied patients with high PMP. Conclusions:Immune thrombocytopenic purpura in the pediatric age group is associated with elevated PMP which may be protective against severe bleeding events rarely seen in this age group. The role of PMP studies in deciding the management plan of childhood and adolescent ITP needs further evaluation.

0783

INCREASED LEVELS OF PLATELET-LEUCOCYTE COMPLEXES IN BEHCET'S DISEASE PATIENTS WITH MAJOR VASCULAR INVOLVEMENT

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Background. Venous thrombosis, which is quite rarely observed in other vasculitides, is a frequent finding in Behcet's disease (BD). The exact explanation for the thrombotic tendency in BD is unknown. It was suggested that endothelial dysfunction caused by vascular endothelial injury in a prothrombotic milieu might have a role. Nevertheless, activation of platelets and neutrophils might contribute to vascular damage, thereby resulting in hypercoagulability *Aims.* We determined platelet-leucocyte complexes in BD patients with and without major vascular involvement (MVI) and in healthy controls. *Methods*. We included 36 BD patients (22 M, 14 F, mean age: 34.4±8.3 years) and 20 healthy subjects (14M, 6F, mean age: 31.8±4.4 years). All participants gave written informed consent and the protocol was approved by the our university's research ethical committee. BD patients with MVI were taken as a separate group (8 M, 5 F, mean age: 37±8 years). MVI was defined as the presence of pulmonary arterial aneurysm, deep venous thrombosis, vena cava inferior or superior thrombosis, or venous sinus thrombosis. Flow cytometry was used to determine platelet-monocyte complexes (PMC), platelet-neutrophil complexes (PNC), basal and adenosine diphosphate (ADP)-stimulated platelet CD62P expression. *Results*. BD patients with and without MVI and the control group were not significantly different in their ages and sex distribution. Pathergy test was significantly more frequently positive in BD group with major vascular involvement (p=0.01). When active BD patients were compared with inactive BD patients, it was seen that basal and ADP-stimulated CD62P expression, PMC and PNC were not significantly different between the groups (p>0.05). BD patients with MVI had significantly higher PNC (23.3±16.9) than BD patients without MVI (12.8±7.9) and healthy controls (12.8±5.3) (p values <0.01). PMC levels in BD patients with MVI (43±28.8) were signif-

icantly higher than healthy controls (21.1±10.1) (p=0.01); and tended to be nonsignificantly higher than BD patients without MVI (30±20) (p=0.15). The groups were similar in basal and ADP-stimulated platelet CD62P expression (p>0.05). The evaluated parameters were not significantly different in BD patients with and without uveitis, and pathergy-positive and -negative groups. In addition, corticosteroids (8 cases), low-dose aspirin (5 cases), warfarin (4 cases), colchicine (20 cases), azathioprine (7 cases) did not have significant effect on the evaluated parameters. In BD patients, PMC correlated with PNC (r=0.89, p<0.001), CD62P expression correlated with ADP-stimulated CD62P expression (r=0.72, p<0.001). None of these parameters correlated with ESR and CRP. Only in BD patients with MVI, CD62P expression had a negative correlation with CRP level (r=-0.65, p=0.03). *Conclusions*. Our results demonstrate that the formation of platelet-leucocyte complexes might be playing a pathogenetic role in BD patients with MVI. The formation of platelet-leucocyte complexes, independent of an increase in platelet activation parameters, made us consider the role of leucocytes especially neutrophil hyperactivation in MVI of BD.

0784

HELICOBACTER PYLORI ERADICATION IMPROVES PLATELET COUNT IN INFECTED CHILDREN WITH CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Background. Recent reports have suggested, particularly in adults, an association between Helicobacter Pylori infection (HPI) and chronic idiopatic thrombocytopenic purpura (cITP) with improvement of platelet count after eradication therapy. *Aims.* We investigated the association of HPI and cITP and the effect of HP eradication therapy on thrombocytopenia children. *Methods*. Twenty-four children of both sexes mean age 8.0±0.28 years (range 5.4-10.7 yrs), affected by cITP (PLT ≤50×10^9/L) without other causes, lasting more than 6 months, were enrolled. None patient received immunosuppressive therapies at last one year or was splenectomised. HPI was investigated by an enzyme immunoassay for H. pylori antigens in faeces (HpSA). Bacterial eradication was performed by Amoxicillin (50 mg/kg/die), Clarithromicin (15 mg/kg/die) and Omeprazole (20 mg/die) for 7 days. HP status was evaluated before and immediately after eradication and, successively, every 3 months in the first year after treatment. A follow-up of platelet count was performed during 1 year after HpSA. Statistical analysis was performed by t-test. A p value < 0.05 was considered statistically significant. *Results*. Six out 8 patients (75%) had total recovery of platelet count during the first year after bacterium eradication (PLT before therapy 32.5±3.5×10^9/L; after 1 year 275±106.06×10^9/L) with significant increase (p< 0.05), 2 patients (25%) had partial recovery (PLT before therapy 30×10^9/L, after 1 year 103.5×10^9/L) but non significant increase (p>0.05). Non significant differences were found in platelet count between infected and uninfected patients before eradication treatment (PLT 33.0±2.8×10^9/L vs 34.0±5.75×10^9/L) (p>0.05), while significant differences were observed after eradication therapy (PLT 315.0±7.07×10^9/L vs 43.5±2.12×10^9/L) (p<0.05). *Conclusions*. HP assessment should be performed in all cITP patients and eradication therapy should be attempted in positive cases.

0785

DOES ANTI-TNF THERAPY CAUSE ANY CHANGE IN PLATELET ACTIVATION IN ANKYLOSING SPONDYLITIS PATIENTS?

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Background. Inflammation, which has an important role in endothelial dysfunction, plays an important role in the development of atherosclerotic vascular disease. Platelets have a major role in the interplay among inflammation, thrombosis and atherogenesis. Some of their important roles are their interaction with leucocytes and their ability to modify the functions of leucocytes, their adhesion upon the endothelium and ability to activate endothelial cells, their potential to interact with vascular smooth muscle cells. Recently, it has been reported that ankylosing spondylitis (AS) was characterized by endothelial dysfunction and the development of atherosclerotic complications. *Aims.* In our study, we evaluated parameters of platelet activation (platelet-monocyte complexes [PMC] and platelet-neutrophil complexes [PNC], CD62P, sCD40L) and endothelial activation (E-selectin) in AS which is a chronic inflammatory disease. In addition, we searched for the variation in these parameters with disease activation and after anti-TNF therapy. *Methods*. Fiftynine AS patients and 22 healthy controls were included. The clinical features and acute phase parameters were evaluated. All participants gave written informed consent and the protocol was approved by the our university's research ethical committee. In all patients and healthy controls, platelet-monocyte complexes (PMC), platelet-neutrophil complexes (PNC), basal and ADP-stimulated P-selectin (CD62P) expression were determined by flow cytometry; soluble E-selectin (sE-selectin) and soluble CD40L (sCD40L) were determined by ELISA. AS patients were divided into two groups as active and inactive by using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). In 15 AS patients, the evaluated parameters were assessed before and after 12 weeks of anti-TNF therapy. *Results*. The mean age of AS patients (36.7±9.1 years) was not significantly different from mean age of the healthy control group (33.1±6.3 years) (p=0.1). ESR and CRP were significantly higher in AS patients than in controls (p<0.001). PMC in AS patients (31.7±22.3) were significantly higher than in the control group (22.3±10.6) (p=0.013). PNC tended to be higher in the former group (p=0.079). Basal and ADP-stimulated CD62P-positive platelets were similar between AS and control groups (p>0.05). sCD40L level in AS group (1.5±0.7) was significantly higher than in the control group (1.1±0.4) (p=0.016). sE-selectin level in AS patients (22.6±7.8) tended to be lower than in the control group (27.2±9.5) (p=0.064). The evaluated parameters were similar in active and inactive AS groups (all p>0.05). After TNF-blocker therapy, there were significant decreases in mean ESR (p=0.018), CRP (p=0.039), and BASDAI (p=0.002) in the AS group; however, there were no significant changes in other parameters (p>0.05). In AS group, PMC correlated with PNC (r=0.832, p<0.001), CRP (r=0.313, p=0.017) and platelet count (r=0.428, p=0.001). PNC correlated with platelet count (r=0.272, p=0.039). There was a positive correlation between sCD40L level and platelet count (r=0.349, p=0.04). *Conclusions*. In our study, we found that PMC, PNC, and sCD40L were higher in AS patients than in controls. This might be contributing to endothelial dysfunction and atherosclerosis which are said to be present in AS in association with chronic inflammation. Nevertheless, disease activity did not seem to be related to these parameters. In addition, there has been no change in any of the parameters after anti-TNF therapy.

0786

IDIOPATHIC THROMBOCYTOPENIC PURPURA AND AUTOIMMUNE HEMOLYTIC ANEMIA IN THE PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY

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Background. Common Variable Immunodeficiency (CVID) is a heterogeneous group of disorders characterized by hypogammaglobulinemia and an increased susceptibility to recurrent infections as well as autoimmunity and malignancies. Idiopathic Thrombocytopenic Purpura (ITP) and Autoimmune Hemolytic Anemia (AIHA) are two autoimmune disorders which may be seen in association with CVID. *Aims*. To describe the clinical features and outcome of ITP and/or AIHA among patients with CVID, we report clinical and laboratory characteristics in 7 CVID patients who manifested autoimmune hematologic disorders. *Methods*. A two-page questionnaire was designed to collect all necessary information from studied patients, including: past medical histories, the onset of CVID, onset and episodes of ITP and/or AIHA, other associated complications of CVID and the required treatments. *Results*. Among 85 CVID patients, seven cases had ITP and/or AIHA (8%). Four of these patients had one or more episodes of ITP, one patient had AIHA, and two patients had both ITP and AIHA (Evans syndrome). Almost, all patients experienced chronic and recurrent infections mostly in respiratory and gastrointestinal systems during the course of the disease. Among the seven patients, five presented their underlying disease with recurrent respiratory and/or gastrointestinal tract infections, while in two remaining patients, CVID was presented with ITP. Three patients died until now; two because of hepatic failure and one due to pulmonary hemorrhage. *Conclusions*. As CVID is prone to autoimmune disorders, it should be considered as a differential diagnosis of adult-onset ITP and possibly in children. Chronic and recurrent ITP, especially in the presence of propensity to respiratory and gastrointestinal infections mandate the evaluation for an underlying immune dysregulation such as CVID.

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ABNORMAL HEPATOBILIARY LABORATORY VALUES AMONG PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Introduction. Idiopathic thrombocytopenic purpura (ITP) is a disease caused by inadequate platelet production as well as increased platelet destruction. Several ITP therapies may cause abnormal hepatobiliary laboratory values, but the epidemiology of these abnormalities is unknown in this patient population. *Objective*. To estimate the prevalence, incidence rates, and the relative risk of hepatobiliary laboratory abnormalities (HBLA) among a cohort of patients with ITP. *Methodology*. This was a retrospective database analysis using eligibility and medical claims data from a large U.S. health plan affiliated with i3 Drug Safety. Chronic ITP patients were identified using the following criteria: a) at least two physician claims separated by at least six months with ICD-9 CM diagnosis code 287.3 for primary thrombocytopenia, b) at least 12 months of continuous enrollment prior to the date of the diagnosis code eligibility, and c) at least 18 years of age between January 1, 2000 and December 31, 2006 with follow up through September 30, 2007. Study outcomes were elevated levels of alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin, and alkaline phosphatase (ALP) based on laboratory database results. Serious HBLA was defined as >3x ULN for ALT and/or AST and >1.5x for bilirubin and/or ALP. *Results*. Within the chronic ITP cohort (n=3.244), 805 (25%) patients had at least one hepatobiliary laboratory test result during the 12-month baseline period. After excluding patients with recorded evidence of HBLA at baseline, 2.557 (79%) patients were eligible for the incidence sub-cohort. The baseline prevalence of ALT and AST > 3x ULN was 4.6% and 3.7%, respectively. The baseline prevalence of total bilirubin and ALP >1.5x ULN was 4.2% and 3.2%, respectively. The incidence of new HBLA in this cohort was 1.24/1,000 person-years (95% CI: 0.52-2.56) for ALT >3x ULN and 0.41/1,000 person-years (95% CI: 0.08-1.32) for AST >3x ULN. These incidence rates for total bilirubin >1.5x ULN and ALP >1.5x ULN were 2.69/1,000 person-years (95% CI: 1.51-4.47) and 1.03/1,000 person-years (95% CI: 0.39-2.26), respectively. Factors significantly associated with new or repeat occurrence of ALT > 3x ULN were male gender, history of diabetes, history of liver disease, history of other comorbidities related to secondary immune mediated thrombocytopenia such as lupus erythematosis, malignant neoplasm of lymphatic or hematopoetic tissue, HIV infection, history of alcohol use requiring medical attention, high use of systemic corticosteroids, and treatment with interferon or cyclosporine. Factors significantly associated with new or repeat occurrence of total bilirubin > 1.5x ULN were age, male gender, history of congestive heart failure, history of liver disease, history of alcohol use requiring medical attention, and treatment with interferon. *Conclusions*. The prevalence of HBLA among the chronic ITP population is relatively high compared to other disease populations. Abnormal hepatobiliary laboratory test results were significantly associated with male gender, liver disease and several other comorbidities, thus making identifying drug-induced liver injury in this population more challenging.

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CHRONIC AND RECURRENT IDIOPATHIC THROMBOCYTOPENIC PURPURA IN CHILDHOOD - SINGLE INSTITUTION EXPERIENCE

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Background. Idiopathic thrombocytopenic purpura (ITP) is the most frequent hemorrhagic disease in children. There are three forms of ITP: acute, chronic and intermittent. In chronic forms the platelet count remains low for six months after diagnosis, and in recurrent forms the drop in platelet count appears after a period of normality. *Aims.* To asses treatment response and outcome in children with chronic and recurrent ITP admitted at a single pediatric medical center. *Methods and Results*. A retrospective chart review was made for all children ages 2 months-15 years diagnosed with ITP and treated at the University Children's Hospital in Skopje between 1997 and 2006. Follow up period was 1-11 years. Tow hundred and twenty five patients with diagnosis ITP were identified. Chronic ITP (persistence > 6 months) was noted in 24 patients (11%), and recurrent ITP in 13 patients (5.8%). Chronic patients presented at an older age; median age at diagnosis was 8,8 years (range from 3-15 years), with females being affected more frequently than males (f:m=1,6:1). Bone marrow aspiration confirmed the diagnosis in all cases. The risk of chronic ITP was partially predicted by presenting platelet count > 30×10^9/L at 11 patients (45,8%) and age >10 years at 10 patients (41,6%), but not by both. Just 5 patients (20,8%) presenting by these two features developed chronic course Treatment consisted of: 1. glucocorticosteroids (GS) in 13 patients (6 responded with CR, 6 had persistent mild thrombocytopenia and 1 patient with ICH died. 2. GS + Immunoglobulins (IVIG) in 4 patients (3 are in CR). 3 GS + Splenectomy in 5 patients: 2 are in CR, 3 in PR (two of them without treatment). 4. GS + IVIG + Splenectomy in 1 patient with CR. 5. GS + IVIG + Immuran + Splenectomy in 1 patient with CR. Among the patients with recurrent disease-13 patients (5,8%) from 225 with ITP, 8 ware males. All of them had platelet count <30×10^9/L at the presentation of the disease and almost all 12 (92.3%) presented with mild to moderate hemorrhagic manifestations at the onset of the disease and during the recurrence (just 1 girl had serious bleeding-gross metrorrhagio). The median time to recurrence was 11,8 months (range 4-84 months). All of them achieved CR (11 with GS, 1 with IVIG and 1 with both GS and IVIG) as a first treatment and 6 with GS, 1 with IVIG and 6 with no treatment at the recurrence of the disease. *Conclusions*. Chronic and recurrent ITP in childhood run a benign course in most cases and the therapeutic intervention has to be individualized.

0789

ITP AND CELIAC DISEASE: A NON SPORADIC ASSOCIATION

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Background. Idiopathic thrombocytopenic purpura (ITP) is an autoimmune condition caused by platelet auto antibodies, that leads to increased platelets destruction. The typical presentation in childhood consists in sudden onset of petechiae, bruising and bleeding manifestation in patients otherwise in good health. The estimated incidence of ITP ranges between 10-125 new cases/one million (adults and children) year. ITP is often associated with other autoimmune disorders. Celiac Disease (CD) is a very frequent autoimmune condition due an intolerance of the gluten (estimated incidence in Italy is 1/100-150 people) whose more common clinical manifestations are gastrointestinal symptoms, and/or sideropenic anaemia, and/or failure to thrive. Several published studies has shown associations between ITP and CD with a median range of 1/100. *Aims.* The aim of this investigation is to evaluate and verify the real incidence of autoimmune disease and especially of CD in a group of new diagnosed ITP patients. Patients and *Methods*. Since December 2007 till December 2008 have been enrolled 17 children (9 M and 8 F) mean age 5yrs, ranged between 8 mts-14 yrs with new diagnosed ITP. All patients were screened for CD determining the anti transglutaminase antibodies (TTG) and anti endomisial antibodies (EMA). All patients were evaluated also 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 and anti tireoperooxhidase antibodies (AbTPO) and anti tireoglobulin antibodies (AbTg), Direct Coombs test. Serologically positive patients to EMA and TTG were submitted to upper endoscopy with jejunal biopsy. *Results*. 2 patients of 17 (1/8.5) were positive to EMA and TTG. Jejunal biopsy was positive for CD (grade 3rd-4th Marsh Classification), confirming diagnosis of CD. Both patients had no gastrointestinal manifestations and only one of two had a mild sideropenic anaemia (Hb 10.2 gr/dL, MCV 72 fL, iron serum levels 24 mcg/dL, ferritin serum levels 10 mgL).Gluten free diet gave only partial remission of ITP and both patients needed treatment with oral corticosteroids.1/2 still had low platelet count after 1 year of diet (PLt>50.000) but didn't required other treatment than gluten free diet. *Summary and Conclusions*. Our investigation has shown a frequency of the association between ITP and CD in our patients significantly superior compared with previous literature reports (1/8.5 vs. 1/100) and that gluten free diet alone would not conduce to a complete remission of ITP. We suggest to introduce the CD screening as a first step procedure in ITP patients. Otherwise further studies are needed to establish the real incidence of the ITP/CD association, considering the short period of the investigation and few number of patients and a possible bias of population.

0790

CLINICAL AND RADIOLOGIC EVALUATION OF CYTOMEGALOVIRUS-INDUCED THROMBOCYTOPENIA IN INFANTS BETWEEN 1 MONTH AND 6 MONTHS OF AGE

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Background and Aims. Neonates with congenital or perinatal infection with cytomegalovirus (CMV) are asymptomatic in up to 90% of case. However, 10~15% of them develop sequelae such as sensorineural hearing loss, microcephaly, mental retardation, and chorioretinitis lateron. Infants with CMV infection may also have thrombocytopenia, which recently gains attention due to its association with neurological sequelae. However, little is known about the CMV infection-associated thrombocytopenia after the neonatal period. In this regard, the authors investigated the clinical findings of a series of infants diagnosed with CMV infection and with thrombocytopenia. Method. From July 2005 and July 2008, infants with thrombocytopenia less than 6 months of age were screened for CMV infection by CMV IgM. Those who were positive for CMV IgM were further tested for CMV IgG and polymerase chain reaction (PCR) for CMV, CMV pp65 Ag, and urine culture, along with brain magnetic resonance imaging (MRI) and otologic and ophthalmologic evaluations. *Results*. A total of 21 patients aged between 1 month to 6 months (11 boys and 10 girls) were admitted because of thrombocytopenia and were tested for CMV infection. Six among the patients (28.6%) were positive for CMV IgM: four were further positive for CMV IgG, CMV PCR, pp65 Ag, and urine culture, and two were positive for all those test except for pp65 Ag. The median gestational period was 39 weeks (range, 32.3~40.0), and the median age of the patients at diagnosis was 2 months (1.8~4) The median count of platelets at admission was 6,500/μL (range, 2,000~105,000). Except for one patient with a platelet count 105,000/μL, all patients had a platelet count lower than 10,000/μL and were treated with intravenous immunoglobulin at 2 g/kg. All patients recovered a normal platelet count; no patient progressed to chronic thrombocytopenia. One patient (16.7%) was diagnosed with Evan's syndrome and had microcephaly and calcifications on brain MRI. One patient was born at preterm and had a small nodular GRE-dark signal intensity lesion in the caudothalamic notch on the left side, which was thought to be due to intracerebral bleeding. One patient had unilateral sensorineural hearing loss. Conclusions. CMV infection needs to be considered in patients with thrombocytopenia aged between 1 to 6 months, and close follow-up is needed for neurodevlopmental sequeale.

0791

NATIONAL SURVEY ON PREVALENCE AND TREATMENT OF IMMUNE THOMBOCYTOPENIC PURPURA IN POLAND - PLATE QUESTIONNAIRE

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Forty-one treatment centers in Poland participated in the study and responded the PLATE questionnaire concerning prevalence, clinical picture and treatment policies of immune thombocytopenic purpura (ITP) in the previous 12 months. The study was conducted from October 2007 till September 2008. Retrospective data on 3238 patients with ITP were collected, 1331 of whom were new cases (3.5/100,000 per year). The patients were referred to the specialist mostly by primary care physicians (69% of cases) and presented with asymptomatic thrombocytopenia (52,7%) or bleeding symptoms: easy bruising following minor trauma (16,6%), spontaneous bruising (21,9%), mucosal bleeds (17,5%), hematuria (2%) and severe bleeding episodes (internal bleeding: 0,5%, intracranial bleeding 0,2%). Of all 3238 patients participating in the study 41% were untreated, 32% treated with glycocorticosteroids (GCS) only, 11% unsuccessfully treated with GCS before splenectomy and 16% after splenectomy (either untreated or receiving GCS or refractory to GCS). Of all patients diagnosed with ITP who initially do not require treatment a mean of 31% (median 23%) progress and need to start therapy. Initial therapy with GCS lasted on average 14 weeks (median 12 weeks, minimally 4 weeks, maximally 55 weeks). As the most common cause of GCS cessation half of the centers named "therapy failure", 10 centers "complete remission", 10 centers "improvement" and only one center "GCS side effects". Danazol was administered to a total of 320 patients,174 patients received intravenous immunoglobulins (IVIG), 27 patients rituximab and 13 patients anti-RhD immunoglobulin (not available for routine use in ITP patients in Poland). At all centers during the previous 12 months a total of 197 patients (5%) required splenectomy. The most common indication for this procedure (55%) was refractoriness to GCS. Splenectomy proved unsuccessful in 29% of cases (mean; median 30%) *Conclusions*. A national registry of ITP cases would be helpful in surveillance and optimizing of treatment strategies.

0792

DYNAMICS OF PLATELET MORPHOFUNCTIONAL STATUS IN ANTICOAGULANT THERAPY

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Background. In patients with chronic renal failure (CRF), treated with hemodialysis (HD), platelet hemostasis, the regulation of coagulation and fibrinolytic systems can be disturbed, thus contributing to either thrombotic or bleeding complications. The conventional management of thrombotic disorders is based on the use of heparin, oral anticoagulants and aspirin. Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) are a routine anticoagulant in HD. Despite progress in the sciences, these drugs still remain a challenge and mystery. The purpose of this study was to investigate the influence of UFH and LMWHs (nadroparin) on morphofunctional status of living platelets, spontaneous platelet aggregation; adenosine diphosphate (ADPH), collagen, ristomycin induced platelet aggregation before and after HD in patients with CRF. *Methods*. 30 healthy volunteers (the control group) and 45 patients with CRF before and after carrying out HD procedures have been examined. Morphofunctional status of platelet peripheral blood we determined by method of vital computer morphometry with using computer phase-interference microscope (CPM) "Cytoscan" (Moscow, Russia). We measured and compared spontaneous platelet aggregation; adenosine diphosphate (ADPH), collagen, ristomycin and adrenaline induced platelet aggregation. *Results*. The results of studies showed that in patients with CRF who use UFH mean metric platelets parameters (diameter, perimeter, high, area and volume) were constituted 3,1±0,8 mkm; 9,2±2,8 mkm; 0,96±0,3 mkm; 5,7±3,2 mkm^2; 2,2±1,4 mkm^3 (p<0,05). We identified 4 platelet forms that have different morphological features and different parameters of size distribution. In the cell population we distinguished 4 morphologic forms of platelet with according to various activation levels: I - resting platelets, II - platelets with low activation level, III - platelets with high activation level and IV degenerate functionally incomplete platelets. The proportion of different morphological cell types were 52.3%; 36.6%; 10.3%; 0.8%, respectively. In patients with CRF who use nandroparin consisted 2,7±0,6 mkm; 8.1±2.0 mkm; 1.1±0.4 mkm; 4.3±2.0 mkm^2; 1.9±1.1 mkm3 (p<0.05); morphological forms were I - 57.2%; II - 33.5%; III - 9.3%; IV - 0.5%. In patients with CRF who used UFH spontaneous and adrenaline induced platelet aggregation were increased by 15-50% in according to control group; ADPH and collagen induced platelet aggregation were decreased by 10-16,8%; ristomycin induced platelet aggregation was in norm. As to nadroparin in patients with CRF spontaneous and adrenaline, ADPH induced platelet aggregation were in norm; ristomycin induced platelet aggregation was increased not significantly; collagen induced platelet aggregation increased almost by 20%. *Conclusions*. The computer morphometry of living platelets is guarantied rapid and objective analysis of the platelet hemostasis, showing early appearances of platelet complications in patients with CRF. Morphofunctional status of living platelets can be as markers of intravascular thrombogenesis during HD. Single bolus of nadroparin ensures efficient and convenient anti-thrombotic protection during HD procedure. In spite of UFH remains the gold standard of treatment in patients with CRF, our results demonstrated that LMWHs are as safe and efficient compared to UFH. However, due to their renal elimination, they have to be monitored by measuring hemostasis parameters.

Myelodysplastic syndromes II

0793

PROMOTER HYPERMETHYLATION STATUS OF THE MEG3 AND SNRPN IMPRINTED GENES IN MYELODYSPLASTIC SYNDROMES

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Background. MEG3 gene (located on chromosome 14q32) and SNRPN gene (located on chromosome 15q11-q13) belong to the family of imprinted genes and are involved in the pathogenesis of chromosome 14 uniparental disomy and Prader-Willi syndrome respectively. *Aims.* As both genes have been demonstrated to act as tumor suppressor genes, we sought to investigate whether promoter hypermethylation of the differentially methylated region (DMR) occurs in patients with myelodysplastic syndromes (MDS). *Methods*. We studied 43 patients, 31 males and 12 females, with previously untreated MDS.Patients were classified according to WHO and WPSS risk stratification systems and according to cytogenetic analysis in good, intermediate and poor prognosis karyotypes. Median age was 73 years (range 16-92). DNA methylation pattern was determined by methylation-specific PCR of bone marrow samples previously subjected to bisulphite modification, according to standard protocols. Twelve subjects investigated for borderline thrombocytopenia, that were proven to have no malignancy, uniparental disomy 14 or Prader-Willi syndrome served as controls. *Results*. The normal pattern of the MEG3 gene consists of 2 alleles, one corresponding to the methylated paternal allele, and one corresponding to the unmethylated maternal allele, while the normal pattern of the SNRPN gene consists of a band deriving from the methylated maternal chromosome and deriving from the unmethylated paternal chromosome. No abnormal methylation pattern was observed in any of the control group subjects. We found that alterations of the DMR (presence of the methylated allele only) of the MEG3 and SNRPN genes were present in 15 (34.88%) of the patients studied, while 8 patients (18.6%) presented abnormal methylation pattern of both genes. There was a trend in patients with abnormal MEG3 methylation for reduced overall survival (HR 2.15; p=0.072,). No association was found between methylation of SNRPN and overall survival (HR=1.08; p=0.85). However, patients who presented with abnormal methylation of both genes had poorer overall survival (HR=2.14; p=0.147). We observed no significant association between WPSS risk group, cytogenetic risk group and MEG3 or SNRPN aberrant methylation. However, patients with RAEB-I disease were 20 fold more likely to have the MEG3 gene methylated (OR=20, p=0.027). *Summary and Conclusions*. MEG3 and SNRPN promoter hypermethylation occurs in MDS patients, and may be implicated in disease's pathogenesis. The impact of the methylation status of these genes on overall survival warrants further investigation.

0794

SERUM ERYTHROPOIETIN AT DIAGNOSIS IN LOW GRADE MYELODYSPLASTIC SYNDROME CORRELATES WITH BOTH RED CELL ZINC PROTOPORPHYRIN AND SERUM LDH AND MAY REFLECT SEVERITY OF INEFFECTIVE ERYTHROPOIESIS

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Background. Anemia in low grade myelodysplastic syndromes (MDS) (refractory anemia (RA), refractory cytopenia with multi-lineage dysplasia (RCMD), refractory anemia with ring sideroblasts (RARS) and refractory cytopenia with multi-lineage dysplasia and ringed sideroblasts (RCMD-RS)) is characterised by ineffective erythropoiesis due to increased proliferation and increased apoptosis of erythroid precursors. Previously, we detected elevated percentages of hypochromic red cells (%HRC) in many low grade MDS cases, as well as a strong correlation between %HRC and red cell zinc protoporyphyrin (ZnPP) levels, suggesting that functional iron deficiency can contribute to ineffective erythropoiesis, probably as a result of mitochondrial dysfunction with abnormal transport and incorporation of iron into heme. A recent meta-analysis suggests that recombinant erythropoietin therapy (R-EPO) use in MDS results in better hemoglobin (Hb) responses in patients with lower baseline serum EPO levels. *Aims*. We wished to investigate for correlations between serum EPO levels at diagnosis and other baseline characteristics in low grade MDS. *Methods*. We measured complete blood count (CBC), reticulocyte count, serum LDH (normal range 0-500 IU/L), serum iron and percentage transferring saturation, serum ferritin, red cell ZnPP (normal range 0-3 ug/g Hb) and serum EPO (normal range 5.7-19.4 IU/L) in 22 untreated patients (15 male, 7 female, mean age 67) with *de novo* low grade MDS at diagnosis (16 RCMD, 6 RCMD-RS). All patients had normal/increased iron stores on bone marrow aspirate. *Results*. Mean±SD for Hb, serum LDH, serum EPO and red cell ZnPP (results available for 17 patients) were 11.17±1.5 g/dL, 414.36±90.84 IU/L, 77.63±87.13 IU/L and 4.57±4.12 ug/g Hb respectively. We found statistically significant correlations between serum EPO and red cell ZnPP levels (Pearson correlation coefficient, r=0.647, p=0.005), between serum LDH and red cell ZnPP (r=0.571, p=0.013) and between serum EPO and serum LDH levels (r=0.45, p=0.04). However, there were no significant correlations between either serum EPO, serum LDH or red cell ZnPP levels and serum iron, percentage transferring saturation, serum ferritin or Hb concentration. *Conclusions*. We detected statistically significant correlations between serum EPO and red cell ZnPP, between serum LDH and red cell ZnPP and between serum EPO and serum LDH. One possible interpretation is that red cell ZnPP and serum LDH reflect the degree of ineffective erythropoiesis in low grade MDS and that there may be a compensatory increase in EPO production in proportion to the degree of ineffective erythropoiesis, possibly due to impaired oxygen carrying capacity of red cells with increased red cell ZnPP. A correlation between ineffective erythropoiesis and serum EPO in low grade MDS could partially explain why R-EPO therapy may be less effective in patients with higher baseline serum EPO levels. However, other possible effects, by heme oxygenase/heat shock protein 32 inhibition or otherwise, of chronically elevated red cell ZnPP levels within the myelodysplastic bone marrow microenvironment remain unknown and a possible area of future research.

0795

ANALYSIS OF EXPRESSION OF THE WNT-ANTAGONIST SFRP1 AND QUANTIFICATION OF ITS PROMOTER METHYLATION BY PYROSEQUENCING IN BONE MARROW MONONUCLEAR CELLS FROM PATIENTS WITH DIFFERENT RISK-TYPES OF MDS, ALL, AND AML

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Secreted frizzled related proteins (SFRPs) belong to a family of extracellular antagonists of Wnt-dependent signaling. The loss or decrease of SFRPs leads to activation of the Wnt pathway and may play an important role in the pathogenesis of solid tumours and hematopoietic malignancies. Epigenetic alterations of SFRP genes have been previously examined in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). However, in myelodysplastic syndrome (MDS), the role of SFRPs in dyserythropoiesis has not been investigated. SFRP1 is one of five known secreted frizzled related proteins whose expression was recently observed to be down-regulated due to hypermethylation in various malignancies. Therefore we have examined whether the RNA expression of this gene is correlated with the promoter methylation of SFRP1 in bone marrow cells derived from patients with MDS, AML and ALL as compared to healthy individuals. RNA and DNA was isolated from bone marrow mononuclear cells from patients with different subtypes of MDS (low risk, n=16; int-1, n=24; int-2, n=14; high risk, n=13), ALL (n=20) and AML (n=24) at the time of initial diagnosis. Bone marrow mononuclear cells from 24 healthy individuals served as normal controls. RNA expression of SFRP1 was analyzed by real time reverse transcription PCR. Promoter methylation was quantified by DNA-pyrosequencing. A transcriptional down-regulation was observed in 13 (86%) low risk, 21 (87%) int-1, 14 (82%) int-2 and 11 (84%) high risk MDS samples. Twenty-two (91%) of AML and 17 (85%) of ALL samples showed marginal expression of SFRP1. The down-regulation compared to normal controls was as follows: 3.2 fold for low risk, 2.9 fold for int-1, 8.3 fold for int-2, 10.7 fold for high risk MDS, 16 fold for AML and 19.3 fold for ALL. In all 4 MDS risk types a low promoter methylation for SFRP1 was detected. In AML and ALL samples, we confirmed as previously reported, a significant correlation between transcriptional down-regulation and quantitative promoter hypermethylation. Independent of the risk type, all MDS samples showed a significant lower expression in comparison to healthy controls (n=19) but no aberrant promoter methylation could be observed. In summary, epigenetic inactivation of SFRP1 caused by hypermethylation may

be one underlying mechanism in AML and ALL but can not be applied in our MDS cohort. Therefore, the regulation of SFRP1 seems to be different in MDS as compared to acute leukemia. Current studies explore other regulatory mechanisms of SFRP1 including its role in Wnt-dependent signaling in myelodysplastic syndrome.

0796

A NOVEL PEDIGREE WITH HETEROZYGOUS GERMLINE RUNX1 MUTATION CAUSING FAMILIAL MDS-RELATED AML - CAN THESE FAMILIES SERVE AS A MULTISTEP MODEL FOR LEUKAEMIC TRANSFORMATION IN MDS AND AML?

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Background. Heterozygous germline mutations in RUNX1 are causative genetic alterations in familial platelet disorder with a propensity to myeloid malignancy (FPD/MM). To discuss the potential use of FPD/MM as a multistep model for leukaemic transformation in MDS and AML, we report here on a father and daughter with familial MDS-related AML (MDR-AML) and differing clinical course caused by a heterozygous germline mutation in RUNX1 and different secondary chromosomal aberrations. *Subjects*. Following a brief history of symptomatic anaemia, the female index patient presented with MDS-related AML (MDR-AML) at age 13. While her twin brother, her one younger and one older brother, and her mother are clinically healthy, MDR-AML was diagnosed in her 47-year-old father six months earlier. No history of thrombocytopenia or platelet defects was reported in the family. *Results*. In both patients, a heterozygous nonsense mutation was detected in RUNX1 (NM_001001890.2) by direct sequencing. The mutation c.520C>T (p.Arg174X) causes a premature truncation at the end of the runt homology domain (RHD). Chromosome analysis of bone marrow cells from the index patient showed a deletion of 5q (del(5q)) and a structural aberration of 2q. In addition, array-based comparative genomic hybridization (aCGH) confirmed the del(5q) and pointed to an unbalanced translocation t(2;6)(q36;q23), finally confirmed by fluorescence *in situ* hybridization using a specific probe for MYB. In contrast, the only aberration identified in bone marrow cells of the diseased father was a loss of the Y chromosome (-Y). *Conclusions.* Currently less than 30 pedigrees with FPD/MM are known. While most RUNX1 mutations in FPD/MM cluster in RHD and are unique to individual pedigrees, a broad range of intra and interfamilial clinical variability characterizes FPD/MM. Whereas -Y is a typical chromosome aberration of adult MDS associated with a good prognosis, del(5q) is rarely seen in childhood MDS and usually occurs within complex clones associated with a more unfavourable prognosis. Notably, del(5q) is frequently accompanied by aberrations of chromosome 6. In the index patient, the gain of 6q led to an additional copy of the proto-oncogene MYB, an essential transcription factor in haematopoietic cells. While heterozygous mutations in RUNX1 are not sufficient for leukaemogenesis, somatically acquired secondary events may promote transformation leading to overt MDS and AML. The recruitment of different secondary alterations may partially explain the variable penetrance and clinical heterogeneity seen in FPD/MM. Consequently, rare FPD/MM-related myeloid malignancies may serve as a model for multistep leukaemogenesis in MDS/ AML and, as illustrated here, aCGH may lead to the identification of candidate genes involved in malignant transformation in familial and sporadic myeloid malignancies.

0797

DECREASE IN INTRA- AND EXTRA-CELLULAR FREE IRON SPECIES AND OXIDATIVE STRESS PARAMETERS AND INCREASE IN SERUM AND URINARY HEPCIDIN DURING TREATMENT WITH DEFERASIROX IN IRON-LOADED PATIENTS WITH MDS

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Background. Chronically transfused patients with acquired or congenital haemolytic anaemias, including myelodysplastic syndromes (MDS), eventually develop iron overload. The major criteria used to quantify the degree of iron overload are the number of transfusions and serum ferritin levels >1000 ng/mL. Recently, increased levels of intra- and extra-cellular free iron species, which catalyze the generation of reactive oxygen species (ROS) and consequently induce oxidative stress, have been identified in iron-overloaded patients. These include non-transferrin-bound iron and the redox-active fractions of labile plasma iron (LPI) pool and cellular labile iron pool (LIP) [Cytometry 73:22, 2008], both susceptible to iron chelation. *Aims*. To measure LPI, LIP, serum ferritin, and serum and urinary hepcidin, in iron-overloaded patients with low-risk MDS following treatment with the once-daily oral iron chelator, deferasirox. Changes in parameters of oxidative stress, such as ROS, reduced glutathione (GSH) and membrane lipid peroxidation, were also determined. *Methods*. Nineteen patients (7 males, 12 females, mean age 68±13 years) with low-risk MDS (IPSS 0-1) who received a mean of 62 packed cell (PC) units were treated with deferasirox 20 mg/kg/day for a mean of 95 (63-163) days. Mean baseline serum ferritin level was 3008±1797 ng/mL. ROS, GSH, lipid peroxidation and LIP were measured using flow cytometry techniques in red blood cells (RBCs), platelets and polymorphonuclear leukocytes (PMN) [Eur J Haematol 79:463, 2008]. LPI was measured using fluorescence-based methods [Blood 102:2670, 2003], and serum and urinary hepcidin levels were measured by enzyme-linked immunoassay [Blood 112:3922, 2008]. *Results*. In RBCs, there were significant decreases in mean ROS (18%, p=0.01) and lipid peroxidation (72%, p=0.002), with concomitant increases in mean GSH in RBCs (108%, p=0.0001), platelets (53%, p=0.003) and PMN (66%, p<0.02). LIP in RBCs and platelets decreased from 21±2 to 15±2 and 26±13 to 19±10 MFC (mean fluorescence channel; p<0.02), respectively [Blood 112:924a, 2008]. In 16 patients with available data, LPI (normal ≤0.4 U) decreased from 0.39±0.43 to 0.11±0.45 U (p=0.02). RBC indices, serum ferritin and haemoglobin levels did not change throughout the study. Five and 10 patients with normal mean baseline serum (178±65 ng/mL) and urinary hepcidin (929±529 ng/mL), respectively, received a mean of 51 PC units. Fourteen and nine patients with high mean baseline serum (634±193 ng/mL) and urinary hepcidin (5840±4112 ng/mL), respectively, received a mean of 68 PC units. After treatment, hepcidin increases of 28% and 60% were found in patients with high baseline serum and urinary hepcidin, respectively. *Conclusions*. These data suggest that treatment with deferasirox for a mean of 95 days reduced toxic free iron species (LPI and LIP) and parameters of oxidative stress in low-risk, iron-overloaded patients with MDS. The novel methodologies applied in this study may be useful for evaluating the severity of iron overload and for monitoring iron chelation efficacy. The different baseline hepcidin levels were correlated with the number of transfusions received prior to treatment, effecting erythropoietic activity. ROS mediates hypoxic suppression of hepcidin [BBRC 356:312, 2007], therefore amelioration of oxidative stress resulted in an increase in serum and urinary hepcidin levels.

0798

COMPARISON OF ALTERNATIVE SPLICING OF THE RETROVIRAL RECEPTOR/HEME EXPORTER FLVCR1 IN MYELODYSPLASTIC SYNDROMES AND NORMAL ERYTHROPOIESIS WITH DIAMOND BLACKFAN ANEMIA

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Background. Recent observations suggest enhanced alternative splicing of transcripts encoding feline leukemia virus subgroup C cellular receptor (FLVCR1) and subsequent FLVCR1 insufficiency may be a contributing factor to the erythropoietic defect observed in Diamond Blackfan anemia (DBA) primary cells (Rey MA *et al.*, Haematologica November 2008 93:1617.) Because there are parallels between the erythropoietic defects in DBA and myelodysplastic syndromes (MDS), including generation of a 5q- syndrome phenocopy by RNA-mediated interference of expression of ribosomal protein subunit RPS14 (Ebert BL *et al.* Nature 2008; 451:335), we studied FLVCR1 mRNA splicing patterns in primary cells from MDS patients. *Methods*. We examined density centrifugation-isolated peripheral blood or bone marrow mononuclear cells from 15 normal donors and 20 patients with various forms of MDS lacking del5q31. In a subset of samples, CD71+ (enriched for erythroid precursors) and CD71- cell populations were separated immunomagnetically and compared. RNA was isolated, and cDNA generated and amplified by PCR using primers complementary to the FLVCR1 transmembrane 1 and 12-encoding sequences. Splicing isoforms identified by gel or on-chip electrophoresis were then cloned into competent cells using TOPO cloning, and sequenced. *Results*. We identified 5 different FLVCR1 iso-

forms: the full-length, 10-exon, 2008 bp transcript encoding a 555 aa protein ("FL"), and 4 variants lacking exons 2 (E2-), 6 (E6-), and combinations thereof (lacking exons 2 and 6 (E2-6-); and a novel isoform lacking exons 2,3, and 6 (E2-3-6-)). We did not identify variants lacking exon 3 alone (E3-) or exons 3 and 6 (E3-6-), previously reported in a single DBA sample. Patterns in CD71+ and CD71- cells in the same sample were identical. The E2, E2-6-, and E2-3-6- isoforms include a premature termination codon, whereas E3- and E3-6- encode variant FLVCR1 that renders transfected cells only weakly susceptible to subgroup C feline leukemia virus. A previous analysis (Rey MA *et al.* Haematologica 2008) had reported FL and E6- in healthy cells and in DBA cells, but observed E2-, E2-6-, E3-, and E3-6- exclusively in patients with DBA. In contrast, we detected the E2-6- FLVCR1 isoforms in 6/15 healthy controls and 9/20 MDS; E2- and E2-3-6- were MDS-restricted (1 patient each). Notably, 4 MDS patients lacked full-length FLVCR1 transcripts and exhibited only shorter transcripts, predicted to encode proteins with diminished function. *Conclusions*. Variant transcripts of FLVCR1 generated by alternative pre-mRNA splicing identical to those recently detected in DBA are observed in MDS, and may contribute to the defective erythropoiesis seen in those acquired disorders. However, one FLVCR1 transcript previously reported to be restricted to DBA was observed in multiple healthy control samples.

0799

CD34 CELLS ARE UNDER OXIDATIVE STRESS - POSSIBLE IMPLICATION IN MDS PATHOGENESIS

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Myelodysplastic syndromes (MDS) are a heterogeneous bone marrow disorders characterized by hemopoietic insufficiency associated with cytopenias and an additional risk of leukemic transformation. The aetiology and pathogenesis of MDS remain poorly characterized and a therapeutic impasse persists because of the disease's multifactorial pathogenetic features, heterogeneous stages, and the late onset (patients aged 65 and older). Reactive oxygen species (ROS) are highly reactive oxygen derivatives produced mainly as byproducts of aerobic respiration and reduced glutathione (GSH), an antioxidant defense, reacts with them to prevent oxidative damage to intracellular molecules (DNA, proteins, and lipids). Oxidative stress, resulting from an imbalance between ROS production and antioxidant defenses, contributes to cell damage, apoptosis and ineffective hematopoiesis, and may be involved in MDS aetiology and pathogenesis. Indirect evidences observed by other authors suggest a role for oxidative stress in MDS pathogenesis. The elevated plasma levels of malondialdehyde, the lipid peroxidation product, the higher expression of the antioxidant enzymes glutathione peroxidase 1 (GPx1) and manganese superoxide dismutase (MnSOD), and the *in vitro* stimulation of MDS progenitor cells by thiol antioxidants, such as N-acetyl cysteine, provide some support for this hypotesis. With this study we hope to contribute to the clarification of the involvement of oxidative stress and mitochondrial dysfunction in the pathogenesis of MDS and correlate these mechanisms with risk prognostic groups, prognosis, namely the evolution to acute leukemia. For this purpose we have examined the expression levels of ROS, peroxide and superoxide, GSH and mitochondrial membrane potential, in CD34 bone marrow cells populations collected at diagnosis from 22 patients with *de novo* MDS and in 6 Immune thrombocytopenic purpura (ITP) (non-malignant controls). The expression of these oxidative stress parameters was evaluated by flow cytometry using the fluorescent probes, H2DCF-DA, DHE, mercury orange and JC1, respectively. The results are expressed as mean intensity fluorescent (MIF) arbitrary units. The patient group median age was 77 years (33-84), gender M/F=12/11, WHO subtypes: RCMD (n=9), RA (n=5), RAEB-1 (n=1), RAEB-2 (n=6), 5q- syndrome (n=1) and IPSS: low (n=9), intermediate-1 (n=9) and intermediate-2 (n=4). Our results show that CD34 MDS cells have higher peroxides levels expression (519±626 MIF), although a lower statistically significative superoxide (205±98 MIF) and GSH (170±148 MIF) expression levels, when compared with controls (334±187 MIF, 332±170 MIF, 171±70 MIF, respectively). However, we observed that ROS and GSH expression is MDS subtype-dependent. The higher peroxide and superoxide levels were observed in RA patients (1459±373 MIF and 252±126 MIF, respectively), and the lower GSH levels were observed in RAEB-2 patients (32±15 MIF). On the other hand, we observed that RCMD patients have lower mitochondrial membrane potential (0,947±0,530 JC1 M/A) than controls and others subtypes (0,643±0,448 MIF, 0,601±0,319 MIF, 0,516±0,356 MIF, respectively). Our study suggests the involvement of oxidative stress and mitochondrial dysfunction in pathogenesis of MDS, mainly in subtypes RA and RAEB-2.

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0800

JAK2 V617F MUTATION IN PRIMARY MYELODYSPLASTIC SYNDROMES: A MULTICENTER RETROSPECTIVE STUDY

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Introduction and Aims. The JAK2 V617 mutation, a molecular hallmark of typical myeloproliferative disorders (MPDs) has been reported to occur in myelodysplastic syndrome (MDS), yet its clinical significance remains unclear. Furthermore, there is a paucity of data on the prevalence of JAK2 V617F among MDS cases with deletion of the long arm of chromosome 5 [del(5q)]cytogenetic abnormality. *Methods*. We retrospectively reviewed the case files of the participating hematology departments based in tertiary-level hospitals to identify primary MDS,that fulfill the WHO 2001 diagnostic criteria. We studied 265 primary MDS cases representative of the MDS population referred to the collaborating hospitals. To establish the frequency of JAK2 V617F among MDS cases with the del(5q), we screened an additional cohort of 65 MDS cases known to harbor this abnormality by conventional G-banded cytogenetics. We also screened 22 CMML cases, 6 RARS-T cases and 10 MDS/MPD-unclassified (MDS/MPD-U) cases. Clinical and laboratory characteristics of JAK2 V617F positive cases were compared to JAK2 V617F negative cases DNA isolated from peripheral blood or bone marrow fresh samples or from archived bone marrow smears. The JAK2 V617F mutation was detected using a tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) assay with a sensitivity of 1%. *Results*. Overall, we identified 12 cases carrying the JAK2 V617F mutation within the primary MDS cases. With respect to MDS subtype, one had the del(5q) syndrome, 2 had RA, 3 had RARS, and 6 RAEB. Among the 265 MDS cases representing the general MDS population, 9 patients (3.4%; 95% CI 1.6% to 6.3%) carried the mutation. Among del(5q) MDS patients, the JAK2 V617F mutation was more common (8%; 95% CI 3% to 16.6%). Among JAK2 V617F positive cases the del(5q) (p=0.016) was more common Patients carrying the V617F mutation also had higher neutrophil (p=0.039) and platelet counts (p=0.017). There were no differences between JAK2 V617F positive and wild-type patients regarding age, International Prognostic Scoring System (IPSS) grouping, or hemoglobin values. In multivariable analysis, only platelet count at diagnosis was associated with JAK2 V617F positivity (p=0.015). The JAK2 V617F mutation was not associated with decreased transfusion requirements or recombinant erythropoietin use and did not appear to influence the odds of transformation to acute leukemia. Among, MDS/MPD cases, 3 CMML cases, 5 RARS-T cases and 2 MDS/MPD-U carried the mutation. *Conclusions*. JAK2 V617F mutation is a rare event in primary MDS, occurs commonly in association with del(5q) and correlates with increased platelet counts at diagnosis. The mutation does not appear to carry prognostic significance or modify the clinical course of the disease. JAK2 V617F is common among MDS/MPD overlap cases.

0801

SUPPRESSION OF THE DNA DAMAGE RESPONSE IN ACUTE MYELOID LEUKEMIA VERSUS MYELODYSPLASTIC SYNDROME

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Background. The molecular mechanisms responsible for the evolution from the preleukemic entities of low-risk myelodysplastic syndrome (MDS) to the less favorable forms of high-risk MDS, as well as those enabling transformation to acute myeloid leukemia (AML), are still incompletely understood. Evidence from solid tumors demonstrates that preneoplastic lesions activate signaling pathways of a DNA damage response (DDR), which functions as an "anti-cancer barrier" hindering tumorigenesis. *Aims and Methods*. To test the hypothesis that alteration of the DDR underlies evolution/transformation of MDS, we assessed markers of DDR (phosphorylation of ATM, Chk-1, Chk-2 and H2AX, apoptosis; G2/M arrest) in cell lines representing different entities of MDS (P39, MOLM-13) and AML (MV4-11, KG-1) before and after γ-irradiation. In addition, the expression pattern of DDR proteins in *ex vivo* patient material was determined by immunohistochemistry. Thus bone marrow biopsies of normal controls (n=2), MDS (n=27; IPSS: Int1: n=7, Int2: n=14, High: n=6) and AML (n=5) patients (pts) were stained for expression of P-ATM-Ser1981, P-Chk-1-Ser317, P-Chk-2-Ser68, and γ-H2AX. *Results*. While γ-irradiation induced apoptosis and G2/M arrest and a concomitant increase in the phosphorylation of ATM, Chk-1 and H2AX in MDS-derived cell lines, this radiation-response was attenuated in the AML-derived cell lines. Noteworthy, KG-1 but not P39 cells exhibit signs of an endogenous activation of the DDR. In addition, immunohistochemical assessment of BM biopsies provides evidence that i) normal controls exhibit <5% P-ATM+, γ-H2AX+, P-Chk-1+ and P-Chk-2+ cells; ii) the percentage of P-ATM+ cells increases in samples from AML pts (median: 75% P-ATM+) as compared to high-risk MDS samples (IPSS high: median 42% P-ATM+, Int-2: median: 26% P-ATM+) and significantly correlates with the percentage of BM blasts (p<0.01); iii) the frequency of γ-H2AX+ cells is heterogeneous in all subgroups of AML and MDS and is not correlated with the percentage of BM blasts; iv) whereas intermediate-1 MDS samples contain as little P-Chk-1+ and P-Chk-2+ as healthy controls, staining for checkpoint kinases increases in intermediate-2 and high-risk MDS (median P-Chk-1+: 32%, median P-Chk-2+: 51%) yet declines to near-to-background levels in AML samples (median P-Chk-1+: 2%, median P-Chk-2+: 8%). P-Chk-1+ and P-Chk-2+ expression are not correlated with the percentage of BM blasts. Nevertheless P-Chk-1+ expression significantly correlates with the P-Chk-2+ expression (p<0.001). Conclusions: Thus the activation of Chk-1 and Chk-2 behaves in accord with the paradigm established for solid tumors, while ATM is activated during and beyond transformation. We hereby demonstrate the heterogeneity of the DDR response in MDS and AML and provide evidence for its selective suppression in AML due to the uncoupling between activated ATM and inactive checkpoint kinases.

0802

IMMUNE EFFECTORS AND REGULATORY T CELLS IN MYELODYSPLASTIC SYNDROMES (MDS)

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Background. Primary MDS identify a heterogeneous group of hematological clonal disorders characterized by ineffective and dysplastic hemopoiesis with a varying degree of cytopenias and a substantial risk of progression to acute leukemia. Relevance of immune response dysregulation in the pathogenesis of BM failure conditions has been suggested; several studies in MDS patients have described occurrence of oligoclonal $CD8^+$ T cell repertoire, ability of $CD8^+$ T lymphocytes to impair *in vitro* development of the erythroid and granulocytic progenitors, primary defects of tolerance control as well as targeting of myelodysplastic BM precursors by Cytotoxic T lymphocytes (CTL). *Aims*. Our study focuses on the immunological networks that characterize the first stages of MDS. Here we describe bone marrow (BM) and peripheral blood (PB) distribution of immune effectors and their activation status in order to identify more homogeneous groups of MDS patients in which the pathogenetic involvement of immune-mediated mechanisms might be inferred. *Methods*. We examined whole BM and PB specimens of 51 MDS patients that were categorized according to WHO classification and IPSS score: 27 belonged to the Low Risk Group (9 RA, 1 RAS, 16 RCMD, 1 MDSU), 17 to the Intermediate-1 (Int-1) Risk Group (3 RA, 12 RCMD, 1 RAEB 1, 1 MDSU), 3 to the Intermediate-2 (Int-2) Risk Group (1RAEB 1 and 2 RAEB 2)and 4 to the High Risk Group (4 RAEB 2). Analysis of immune effectors and $CD4^+CD25^{high}Foxp3^+$ (T reg) were performed by using immune fluorescence and flow cytometry. Mann-Withney test, Wilcoxon matched-pairs signed-rank test were used when appropriate. In order to evaluate the hypothesis that in the Low and Int-1 category the immunological variables could have a no homogeneous distribution, a multistep cluster analysis algorithm was applied. *Results*. This study reveales an altered distribution of adaptive immune effectors in BM versus PB of Low and Int-1 Risk MDS patients, with increased BM Treg levels in the Int-2/High Risk Group. More intriguingly, BM Treg cells cluster a Low Risk sub-group characterized by significant BM recruitment of CD8 effectors, while a clustered distribution of CD54 expression on BM CD8 T lymphocytes and of CD4 cells are observed in Int-1 Risk Group. *Conclusions*. The clustered distribution of immunological variables in MDS patients highlights the relevance of BM analysis for a more homogeneous grouping of MDS patients. Taken in all our data point to the involvement of immune-mediated mechanisms in the first stages of the disease. We suggest that the clustered BM distribution of immunological parameters could represent an useful criterion for a more homogeneous grouping of MDS patients. This approach could shed light over the intra-group heterogeneity of current MDS classification also representing a potential tool to improve the clinical management of patients.

0803

NANOG MRNA IS HIGHLY EXPRESSED IN MDS CELLS AND DURING ERYTHROID DIFFERENTIATION

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Background. Myelodysplastic syndrome (MDS) is a group of hematopoietic disorders characterized by abnormal differentiation and by a risk of progression towards acute myeloid leukemia (AML). In MDS, NFκB activation is responsible for the progressive suppression of cell apoptosis, contributing to the progression towards AML. The family of NFκB proteins is essential in many physiologic responses such as immunity, differentiation and development. NFκB is repressed by NANOG binding in the cytoplasm, resulting in inhibition of the transcriptional activity of NFκB proteins. This process cooperates with Stat3 to maintain pluripotency. *Aims*. Verify NANOG expression in bone marrow cells and during $CD34^+$ erythroid differentiation of MDS patients. *Methods*. Bone marrow samples were collected from 12 MDS (9 RA, 2 RARS and 1 RAEBt) patients, 8 AML and 7 healthy donors. The erythrocytes were lysed and the RNA was extracted for relative analysis by Real Time PCR, using the gene ABL as endogenous control. CD34+ cells were isolated from bone marrow of 4 patients with MDS and 3 healthy donors for erythroid differentiation. Briefly, CD34 cells were cultured in methylcellulose with SCF(50ng/mL), IL3 (30UI/mL) and EPO (3U/mL) for 6 days. The colonies were then washed and the cells incubated in a liquid culture medium α-MEM with EPO (2U/mL), 30% of FBS, 8% of BSA, 300 mg/mL of holotransferrin and 10-5M of ß-mercaptoethanol. The cells were kept in culture for 9 more days. The cells were collected on days 0, 3 and 6 of this second phase for RNA extraction.

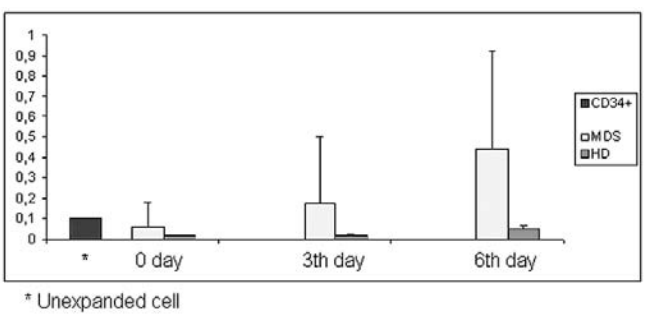

Figure 1. NANOG mRNA expression during erythroid differentiation of $CD34^+$ cells from MDS patients and healthy controls (HD). Results are presented as mean± SD, of cells collected on days 0, 3 and 6 of second phase of erythroid differentiation. NANOG expression of unexpanded $CD34^+$ sample obtained from one healthy donor is showed for comparison.

Results. NANOG was overexpressed in MDS cells, as compared to AML patients and healthy donors [median (min-max); MDS = 3.16 (0.93 -14.11); AML = 1.23 (0.27-4.88); healthy donors = 1.16 (0.16-3.24); MDS vs AML p=0.0082; MDS vs normal, p= 0.0141]. NANOG overexpression in MDS was also higher throughout all phases of CD34$^+$ erythroid differentiation when compared to healthy donor cells. The higher expression of NANOG was observed in MDS patients, on the 6th day of culture, being at least 4 times higher than in unexpanded CD34$^+$ cells from healthy donors. These results are presented in the Figure 1 as mean ± SD. *Conclusions*. The reduced expression of NANOG in AML cells compared to MDS may be related to the increased activity of NFκB during MDS progression. It is known that the inhibition of PI3K results in both NANOG mRNA and protein decrease. Taken together these results lead us to suggest that the increased transcription of NANOG during the erythroid differentiation may be related to PI3K activation.

0804

EXPRESSION PATTERNS OF CELL CYCLE AND DNA REPLICATION GENES DISCRIMINATE EARLY AND ADVANCED MDS

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Background. MDS is a heterogeneous group of clonal disorders characterized by ineffective hematopoiesis resulting in peripheral cytopenia. MDS pathogenesis is still uncertain and identification of deregulated genes may contribute to detection of pathogenetic mechanisms involved in the disease at the molecular level. *Aims*. We studied differential gene expression in various MDS subtypes to identify possible molecular mechanisms participating on MDS pathogenesis and/or progression towards advanced stages or AML. Due to frequent transformation of MDS into AML, we also included patients with AML with multilineage dysplasia (MLD) evolved from MDS. *Methods*. Our study comprised 51 bone marrow samples from patients with primary MDS, AML with MLD and 7 controls. To determine MDS subtype specific gene expression pattern, we performed gene expression profiling using Illumina BeadChips on CD34$^+$ haematopoietic progenitors. *Results*. In an attempt to distinguish controls from patient samples, we identified set of 825 genes and based on this gene set hierarchical clustering clearly separated controls from patients. Several pathways were identified to be deregulated in MDS: B cell receptor signaling pathway, chronic myeloid leukemia, small cell lung cancer and adipocytokine signaling pathway for down-regulated genes, while basal transcription factors and toll-like receptor signaling pathway were found for over-expressed genes. Highly down-regulated genes were involved in haematopoietic cell differentiation and maturation (VPREB1, VPREB3, EBF1, NR4A2) or have been reported to be associated with tumour progression and invasion (NPY, CTGF). On the contrary, genes being highly up-regulated in MDS participate in haemoglobin synthesis (HBG1, HBG2), cell growth and proliferation (MYC) or angiogenesis (ANGPT1). To determine expression differences among MDS subtypes, we grouped the patients into 6 categories according their diagnosis. Hierarchical clustering performed on the set of 241 significant genes grouped early MDS with RAEB-1 while RAEB-2 were clustered to AML with MLD patients, suggesting high difference between RAEB-1 and RAEB-2 diagnosis with respect to gene expression profiling. Further, clustering analysis resulted in identification of 5 distinct gene clusters - for each cluster functional annotation by DAVID database was done. Genes involved in cluster 3 and 5 showed inverse expression pattern between early MDS and RAEB-2 or AML with MLD patients. Within cluster 3, we found genes involved in mitotic cell cycle, DNA replication and chromosome segregation up-regulated in early MDS. Up-regulation of these genes may reflect increased proliferative activity of bone marrow. Since annotation of genes from cluster 5 was not successful (p<0.05) we focused on the particular genes showing the highest up-regulation in RAEB-2 and AML with MLD patients and we identified genes involved in the regulation of gene expression (BMI1), signal transduction (EMR1, proto-oncogene MERTK), anti-apoptosis (VNN1). This finding may reflect defective proliferation and decreased rate of apoptosis in advanced MDS. *Summary*. To conclude, we defined set of genes differentially expressed between controls and MDS or AML with MLD samples. Furthermore, our data demonstrate distinct expression patterns of early and advanced stages of MDS, where RAEB-1 patients showed more similarity to early MDS in contrast to RAEB-2 with profiles corresponding with AML with MLD.

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0805

LEUKEMIC TRANSFORMATION OF MDS PATIENTS DYSPLAYING ABNORMAL CYTOGENTICS WITHOUT DEFINITE DYSPLASIA IN BONE MARROW CELLS

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Background. In 2008, the WHO classified patients with cytopenia that had cytogenetic abnormalities without dysplasia of bone marrow cells as myelodysplastic syndrome, unclassifiable (MDS-U). Because of the rarity of this disease category, its clinical course has not been fully studied. *Aims*. We performed a retrospective study to determine the prognosis and clinical course of this disease category. *Methods*. Thirty one patients with the characteristics of this category of MDS at the time of the initial diagnosis were studied. The bone marrow of these patients initially showed no definite dysplasia and less than 1% of blasts. The risk of leukemic transformation and leukemic transformation-free survival of the patients were analyzed by the Kaplan-Meier survival analysis. Allogeneic stem cell transplantation was considered as a censoring event. *Results*. The most common cytogenetic abnormality of the patients was trisomy 8 (n=18), followed by monosomy 7/deletion 7q (n= 5) and deletion 1q (n=5). Other chromosome abnormalities included isochromosome 17q (n=1), trisomy 15 (n=1), and monosomy 21 (n=1). The initial diagnoses of the patients were aplastic anemia (n=26), Behcet's disease (n=2), paroxysmal nocturnal hemoglobinuria (PNH) (n=1), systemic lupus erythematosus (n=1), and drug related cytopenia (n=1). During the follow-up period, we observed three cases of typical MDS and three cases of AML transformation. The time to AML evolution, from the initial diagnosis, was 2.4, 2.7, and 3.3 years, respectively. The 5-year cumulative leukemic transformation risk was 0% in the trisomy 8 group of patients and 37.5% for the other abnormal chromosome findings (p= 0.15). The 5-year probability of leukemic transformation-free survival was 92.9% in the trisomy 8 group and 52.1% for all other chromosome abnormalities (p=0.014). *Conclusions*. The results of this study support the 2008 WHO classification where cytopenia that have cytogenetic abnormalities without dysplasia of bone marrow cells is a subtype of MDS and associated with a risk for AML transformation. In addition, the presence of trisomy 8 as the sole cytogenetic abnormality, in the absence of bone marrow dysplasia, was not associated with the diagnosis of MDS.

0806

THE USE OF IRON CHELATION THERAPY FOR TRANSFUSION DEPENDENT MYELODYSPLASTIC SYNDROME PATIENTS: A CROSS-SECTIONAL STUDY IN BELGIUM

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Background. MDS is a hematological disorder of older adults, with a median age at diagnosis of approximately 70 years.[1] Patients with isolated erythroid lineage involvement have a good prognosis; however, more than 90% of patients with MDS suffer from anemia, with 50-60% of patients having a hemoglobin level below 10 g/dL. Almost all patients with MDS are likely to receive red blood cell (RBC) transfusions at some time, and in 40% this is in reality the sole therapeutic option that can be offered. Irrespective of the strategy adopted to determine which patients with MDS should receive transfusions, transfusion dependency significantly worsens the survival. There is a lack of information on patient properties and treatment patterns in Belgium. *Aims*. To describe patients in terms of MDS subtypes, prognostic scores, selected laboratory values, treatment modalities, transfusion needs, and iron chelation therapy and to determine the percent of transfusion dependent MDS patients with serum ferritin levels ≥1000 μg/L. *Methods*. A cross-sectional observational study was conducted with the aim of assessing treatment patterns of transfusion-dependent MDS in Belgium. Though not validated, it was assumed that the majority of MDS patients in Belgium are seen in hematology centers, hence patients for the study were recruited in 30 large hematology centers. Patients were identified from patient files and current active case load. Transfusion dependence was defined as having received at least one RBC transfusion every 8 weeks over a period of 4

months prior to inclusion. *Results*. During the 2 month recruitment period we have identified a cohort of 193 transfusion dependent MDS patients in 30 participating centers. The median age of the patients was 76 yrs (range 44-103) and the median age at diagnosis was 74 yrs (range 39-95). Half of the patients received growth factor support for a median duration of 9 months. The average number of RBC units patients received in the past 4 months was 12 units (range 2-40). Transfusion need was higher in patients with higher IPSS scores than patients with lower IPSS scores. From the 176 patients whose serum ferritin values were available, 126 (65%) of these had levels above 1000 µg/L. 90 patients (47%) received iron chelation therapy with either Deferoxamine or Deferasirox. No difference in age was reported between chelated and non-chelated patients. 56 of the 92 non-chelated patients with available serum ferritin values reported values above 1000 µg/L. 74% of these 56 patients had an IPSS score of Low to Intermediate 1, making the majority of them potential candidates for iron chelation therapy, according to international recommendations.[2] *Summary and Conclusions*. This Belgian cross-sectional study identified a cohort of 193 transfusion dependent MDS patients and provides valuable information concerning treatment patterns. Many patients did not receive iron chelation therapy, although they were suitable candidates. Additional data on the reasons for not initiating iron chelation therapy will be presented at the meeting.

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0807

LENALIDOMIDE TREATMENT MODULATES CELL SURFACE MOLECULE EXPRESSION AND CYTOKINE SECRETION OF HUMAN MESENCHYMAL STROMAL CELLS FROM HEALTHY DONORS AND MDS PATIENTS

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Background. Recent studies in patients with MDS have clearly demonstrated the clinical efficacy of lenalidomide. However, its exact mechanisms of action have not been elucidated yet. Myelosuppression is the most common adverse event and seems to dependent on dose as well MDS subtype, being rather infrequent in patients other than del5q. *Aims*. The aim of this study was to investigate whether lenalidomide affects the bone marrow microenvironment. Therefore, we analyzed properties of isolated mesenchymal stromal cells (MSCs) from MDS patients and from healthy controls. *Methods*. Bone marrow samples were collected from healthy donors (n=5) and patients with MDS (n=10; del5q MDS n=5, RA n=2, RAEB1/2 n=3). After density centrifugation MSCs were isolated according to the standard adhesion protocol and cultured in the presence or absence of lenalidomide (50 µM). We examined its effects on cell growth and proliferation; the expression of cell surface molecules, cytokine secretion as well as the potential for osteogenic differentiation. *Results*. Lenalidomide treatment of MSCs caused no phenotypical changes but proliferation was slightly increased. Interestingly, lenalidomide provoked an upregulation of mean fluorescence intensity of CD29 by 17.8±4.4% and of CD73 by 24±5.7%. Furthermore, the secretion of IL-6 and IL-8 was increased in lenalidomide treated cells whereas VEGF levels remained unchanged. We found osteoblastic differentiation to start as early as on day 5 with lenalidomide whereas in the control cells first calcium deposits were visible after 7 days. All described effects were observed in both normal and MDS MSCs. *Summary*. In conclusion, lenalidomide influences the expression of distinct cell surface molecules, cytokine secretion as well as the potential for osteogenic differentiation of MSCs by a yet unknown mechanism. Whether these in-vitro effects are associated with the clinical efficacy of this compound in patients with MDS remains to be investigated.

0808

INCREASE IN BONE MARROW IMMATURE NON-LYMPHOID EARLY PRECURSORS PREDICTS A SHORT SURVIVAL IN MYELODYSPLASTIC SYNDROMES

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Background. Bone marrow (BM) $CD34^+$ cell population is composed mainly by myeloblasts and B-lymphoid precursors, besides minor populations and non-classifiable early precursors. Their increase is associated with disease progression. *Aims*. to compare the influence on survival of SSCint/CD34+/CD13-/117- (early) progenitors measured by flow cytometry at diagnosis with established risk factors. *Methods*. in newly diagnosed patients BM was examined by flow cytometry. Monoclonal antibody combinations: CD15/CD34/CD45/CD117, CD16/CD34/CD45/CD13 and CD3/CD34/CD45/CD19. In the CD34/SSC plot the subsets: SSC low/$CD34^+CD19^+$ cells, SSC int/$CD34^+/CD13^+$ and SSCint/$CD34^+CD19^-/CD13^-$ were separated. The influence on overall survival of age, PB counts and IPSS and subsets of $CD34^+$ cells were analyzed with the Cox model. *Results*. 35 cases were studied: 11 RA, 2 RARS, 12 RCMD, 10 RAEB. IPSS: 14 cases were low risk, 12 intermediate-1, 3 of intermediate-2 and 6 high risk. Median follow-up time: 17 months. The number of early progenitors was correlated with the total number of $CD34^+$ cells (r=0.58; p=0.0005), with myeloblasts (r=0.68; p<0.0005), with $CD34^+/CD117^-$ cells (r=0.93; p<0.0005) and with $CD34^+/CD117^+$ cells (r=0.75; p<0.0001). In univariate Cox regressions, patients with a high percentage of early progenitors (p=0.01), an elevated percentage of $CD34^+$ cells (p=0.0005) and a reduced platelet count (p=0.04) had shorter overall survival. In the multivariate Cox regression only the percentage of early progenitors (p=0.001) and platelet count (p=0.03) were independent risk factors. *Conclusions*. the increase in early progenitors may indicate the presence of a maturation block in the abnormal clone and predict a poor survival of the patients.

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0809

LINK BETWEEN OCCUPATIONAL EXPOSURE TO HEAVY METALS AND HIGH RISK MYELODYSPLASTIC SYNDROMES

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Background. Secondary myelodysplastic syndromes (MDS) occur upon cancer therapy (chemotherapy and radiotherapy) or occupational/environmental exposure to toxics (benzene and radiations). They represent 20% of MDS and are characterized by high-frequency chromosomal abnormalities. The link between exposure to other solvents, heavy metals, pesticides, cigarette smoking and secondary MDS remains unclear. *Aims*. To evaluate the relationship between occupational exposure to these toxics and MDS cytogenetics. *Methods*. History of occupational activities was obtained for 261 MDS patients referred to our clinical department over a two-year period. Two French employment status classifications (encoded NAF and PCS, respectively) were used to quantify occupational exposure to solvents, heavy metals, pesticides and cigarette smoking. MDS characteristics were recorded (bone marrow evaluation, karyotype, IPSS) and data were analyzed using the stata9.2.analysis software. *Results*. Exhaustive data were obtained for 184 patients (119 men; 65 women); with median age at diagnosis of 73 years. Patients without cytogenetic data (n=36), therapy-related MDS (n=17), incomplete professional history (n=12) or CMML (n=12), were excluded. Distribution of MDS categories according to WHO classification was as follows: RA (18.5%), RARS (26.1%), RCMD (15.2%), 5q-syndrome (5.5%), RAEB1 (27.7%), and RAEB2 (5.5%). Abnormal karyotype was observed in 34.8% of patients, and consisted mainly of del5q (8.7%), trisomy 8 (3.8%), del20q (3.8%), and complex karyotype (3.8%). High-risk MDS represented 13.6%. Occupational activities were mainly related to farming and agriculture (20%), building (14.1%) and textile (7%) industries. Eleven men (9%) and 36 women (55%) were exempted from any toxic exposure. Exposure to solvents, heavy metals and cigarette smoking was significantly higher in men than women, while no difference in exposure to pesticides was found according to sex. Bivariate analysis did not reveal any association in relation to exposure to either solvent, or cigarette smoking, or pesticide. On the contrary, expo-

sure to heavy metals (20 men; no women) was significantly linked to high-risk MDS (OR= 8.18 [2.36; 29.09]; $p<0.001$). Within this group, RAEB2 represented the main target population (OR= 8.00 [1.20; 58.21]; $p=0.015$). Among the karyotype abnormalities, the presence of del20q or abnormal 11 was associated to metal exposure ($p<0.04$). *Conclusions*. Our data revealed a potential link between exposure to heavy metals and chromosomal abnormalities. Larger cohorts and case-control studies are required to confirm our results. Using job-exposure matrix, the precise level of exposure to each toxic should be determined and provide further knowledge regarding the developmental conditions and process of MDS.

0810

DIFFERENCES IN EXPRESSION OF GENES INVOLVED IN NUCLEAR FACTOR KAPPA B(NFKB) PATHWAY AFTER ANALYSIS BY QUANTITATIVE POLYMERASE CHAIN REACTION ARRAYS IN MYELODYSPLASTIC SYNDROMES (MDS) PATIENTS

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Background. Nuclear factor-kappaB (NFkB) is a transcription factor used by most eukaryotic cells as gene regulator over cell proliferation and cell survival. It is believed that persistent NFkB activation promotes oncogenesis and enhances metastasis, invasion and angiogenesis of tumor cells. According to myelodysplastic syndromes (MDS) pathogenesis, as the disease progresses a survival advantage of the neoplastic clone takes place changing the original pattern from increased apoptosis and ineffective haemopoiesis to accelerating clonal proliferation and leukemic transformation. *Aims*. The aim of the present study was to investigate whether NFkB pathway contributes in MDS pathogenesis and to determine the pathway's activation status in terms of MDS grade. *Meethods*. Eight MDS patients (2 males, 6 females) were included in the study. Four of them suffered from low grade MDS (RA -RARS) and the rest from high grade MDS (RAEB / RAEB-T). Our control group consisted of 4 healthy, age matched individuals. Informed consent was obtained from all participants. RNA was extracted from peripheral blood by Total RNA Blood purification kit (Invitrogen) according to manufacturer's instructions. A second purification was conducted using Array Grade Total RNA isolation kit (SuperArray).Reverse transcription was performed using RT2 First Strand kit (SuperArray).Real time quantitative polymerase chain reaction (qPCR) followed using the RT2 Profiler PCR Array system of SAbiosciences. In this method 84 genes of NFkB pathway were analyzed simultaneously. The Ct values were compared according to ΔΔCt method. *Results*. In general we found fold up-regulation of the NFkB relevant genes pathway in low grade MDS compared to control group while the opposite was observed in high grade MDS. IRAK1, TLR7, NLRP12 genes were found up regulated in early MDS ($p<0.05$) while IL1B and TNFSF10 were down regulated in advanced MDS ($p<0.05$).Statistical analysis revealed significant differences in most of the genes examined when we compared the MDS subgroups. The following genes (49/84)AGT,ATF1,BCL3,CFB,CASP8,CCL2,CD40,CHUK, CSF2,CSF3,EDARADD,EDG2,ELK1,F2R,FADD,FASLG,HMOX1,HTR2 B,IFNA1,IFNB1,IKBKE,IKBKG,IL10,IL1A,IL1B,IL1R1,IL6,IRAK2,LTA,LT BR,MALT1,NLRP12,NFKB1,NFKB2,RELB,RIPK1,TBK1,TICAM2,TLR4, TLR6,TLR7,TLR9,TNF,TNFRSF10A,TNFRSF10B,TNFRSF1A,TNFSF14,T RADD,TICAM1 were down regulated in high grade MDS compared to low ($p<0.05$),while the comparison of all the remaining examined genes showed tendency to significance. *Conclusions*. We used peripheral blood as our study material. The high expression of most examined genes involved in NFkB pathway in early MDS may be in part explained by an attempt to balance the established increased apoptosis of MDS marrow cells. We may speculate the opposite for advanced MDS. The above findings are in contrast with what is reported so far for $CD34^+$ marrow MDS cells. Our results indicate that probably peripheral blood cells behave differently from progenitors although they may belong to the same MDS clone. These findings suggest that NFkB activation does not contribute much in MDS progression and thus may not be an attractive therapeutic target.

0811

INFLUENCE OF SERUM FERRITIN LEVELS ON OVERALL SURVIVAL IN PATIENTS WITH MYELODISPLASTIC SYNDROME

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Background. Recent studies have suggested that iron overload may be independently associated with a poor survival in patients diagnosed of myelodisplastic syndrome (MDS). Whether iron overload is a surrogate marker of the severity of the disease or if it affects outcome independently by end organ damage, is still controversial. *Aims*. To determine if high levels of ferritin are independently associated with a poor survival and to determine which variables at diagnosis can identify those patients at risk for iron overload. *Patients and Methods*. Between January 1994 and September 2008, 248 patients were diagnosed with MDS. We excluded patients treated with iron chelation, erythropoietin, or any chemotherapy regimen that could affect survival or transfusion requirements. We included patients with, at least, a ferritin determination during follow-up. One hundred and seventy six patients remained for analysis. Variables included as potential prognostic factors were: age, gender, IPSS score, platelet mass, LDH levels, transfusion requirement at diagnosis and maximum ferritin level reached thorough follow up. *Results*. Median follow-up was 22 months (range 1-171). Ninety eight patients (56%) were male and 78 (44%) female. Of these patients, 28 (16%) transformed to acute myelogenous leukemia, and 73 (41%) remained alive. Median ferritin level was 438 ng/mL (interquartile range 176-1131). By univariate analysis, characteristics associated with worse survival ($p<0.05$) were low platelet mass, older age, high LDH level, transfusion requirement at diagnosis and a high IPSS score. Higher ferritin ($p=0.08$) levels were not significantly associated with worse survival in univariate analysis although a trend was noted. On multivariate analysis, age [OR 1.1 (1.05-1.7); $p<0.001$], IPSS stage [OR 1.9 (1.63-2.34); $p<0.001$], and transfusion requirement at diagnosis [OR 1.6 (1.35-1.9); $p=0.035$] were all independently associated with a poor prognosis. An intermediate IPSS score ($p=0.038$) and transfusion requirement at diagnosis ($p=0.02$) were independently correlated with a combined endpoint of maximum ferritin higher than 1500 ng/mL and/or more than 20 red blood cell units transfused. *Conclusions*. Our data show that iron overload, a variable associated with transfusion requirements at diagnosis and with intermediate IPSS score, is not independently correlated with a worse survival in the whole group of MDS. Further trials in highly transfused patients are expected to help us establish the real impact of ferritin levels on prognosis of MDS.

0812

PHOSPHOINOSITIDE-PHOSPHOLIPASE C (PI-PLC) BETA1 SIGNALLING IS INVOLVED IN ERYTHROPOIETIN RESPONSE OF LOW RISK MDS PATIENTS

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Background. The phosphoinositide 3 kinase (PI3K)/Akt signaling and phosphoinositide-phospholipase C (PI-PLC) beta1 pathways are involved in many different cellular processes, including proliferation, differentiation, and apoptosis. An impaired regulation of the PI3K/Akt axis has often been associated with hematologic malignancies, including acute and chronic human leukemias. Our group previously demonstrated not only that Akt is activated in high-risk MDS patients, but also that there is an inverse correlation between PI-PLCbeta1 expression and Akt activation, in that low levels of PI-PLCβ1 were correlated to high levels of activated Akt and vice versa (Follo MY *et al.*, Leukemia 2008). Moreover, recent results proved that PI-PLCbeta1 mono-allelic deletion is associated with a higher risk of evolution into AML in MDS patients (Follo MY *et al.* JCO 2009). Erythropoietin (EPO) treatment is effective in correcting anemia in a variable portion (40-60%) of low risk MDS patients. However, little is known about the molecular mechanisms underlying the effect of this drug in MDS, and the reasons why some patients do not respond to EPO, or subsequently lose response. The EPO signalling, and particularly the activation of the EPO receptor, has been linked to the activation of the PI3K/Akt axis, which in turn is connected with both PI-PLCβ1 and PI-PLCgamma1 signalling. As a consequence, EPO could affect the survival and apoptotic pathways of MDS cells. *Aims*. In this study we further investigated the role of PI-PLCbeta1 and Akt in MDS, focusing on patients at lower risk of evolution into AML,

treated by EPO. Methods. We studied 16 patients, with IPSS risk low or intermediate-1: 8/16 patients (50%) showed a favourable response to EPO. We also studied Akt and PI-PLCbeta1 expression. Firstly, we analyzed the presence of PI-PLCbeta1 mono-allelic deletion in the MDS patients by FISH anayses. Subsequently, we quantified the expression of both PI-PLCbeta1 mRNAs. Finally, we investigated the degree of Akt activation, as well as PI-PLCbeta1 protein expression, before and during the EPO treatment. *Results*. Our data show not only that 31% (5/16) of our low risk MDS patients displayed the PI-PLCbeta1 mono-allelic deletion, but also that 3 of the patients bearing the deletion were refractory to EPO treatments. Moreover, in responder patients we observed an increase in activated Akt levels. *Summary and Conclusions*. Our data confirm the hypothesis of a possible involvement of inositides in the EPO signalling, in that the patients who showed the mono-allelic deletion of PI-PLCβ1 did not respond to EPO treatment, whilst responder patients showed an activation of Akt. Therefore, our findings indicate that MDS patients bearing the PI-PLCbeta1 mono-allelic deletion, even if they belong to the low or intermediate-1 IPSS risk group, are characterized by a worse prognosis, as they show a higher risk of AML evolution and a lower probability to respond to EPO treatment.

0813

EFFECT OF AZACITIDINE (AZA) ON TRANSFUSION INDEPENDENCE (TI) AND OVERALL SURVIVAL IN PATIENTS (PTS) WITH HIGHER-RISK MYELODYSPLASTIC SYNDROMES

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Background. The international, multicenter AZA-001 trial established that AZA significantly improves overall survival (OS) in pts with higher-risk myelodysplastic syndromes (MDS) compared with conventional care regimens (CCR) (Fenaux, Lancet Oncol 2009). Red blood cell (RBC) transfusion requirements have been shown to be predictive of OS in pts with MDS. This descriptive analysis examined OS in AZA pts in AZA-001 relative to their transfusion status. *Methods*. 179 pts with higher-risk MDS (FAB: RAEB, RAEB-t, or CMML, and IPSS: Int-2 or High) were randomized to AZA (75 mg/m^2/d SC x 7d q 28d). Randomization was stratified on FAB and IPSS. Erythropoietic stimulating agents were not allowed. Transfusion independence (TI), RBC or platelet, was defined as a transfusion-free period of ≥56 consecutive days. Baseline (BL) transfusion dependence was defined as requiring ≥1 transfusion during the 28-day pretreatment period. Survival curves were plotted using the Kaplan-Meier (KM) method for groups based on BL transfusion status. *Results*. Overall, 50/111 (45.0%, 95% CI: 35.6, 54.8) BL RBC transfusion-dependent pts in the AZA group achieved RBC TI and 16/38 (42.1%) BL platelet-dependent pts achieved platelet TI. Pts who were TI at some point during treatment regardless of BL transfusion status had a longer duration of AZA therapy and longer OS (Table). Median OS was not reached (NR) in pts who were BL RBC dependent and achieved RBC TI and in pts who were BL platelet dependent and who achieved platelet TI during AZA treatment. *Conclusions*. Pts who became, or remained, transfusion independent received more AZA treatment cycles and had prolonged OS compared with pts who were transfusion dependent.

Table 1.

BL Transfusion status	On Treatment Transfusion Status	N patients (ITT Population)	Median Number of Treatment Cycles (range)	Median OS months (95% CI)
RBC Dependent	Independent	50	14.0 (3-29)	NR (25.1, NR)
	Dependent	61	4.0 (1-13)	7.3 (4.8, 10.5)
RBC Independent	Independent	58	11.0 (2-39)	NR (26.3, NR)
	Dependent	10	2.0 (1-12)	9.3 (1.4, 17.2)
Platelet Dependent	Independent	16	11.0 (4-26)	NR (15.9, NR)
	Dependent	22	3.0 (1-11)	4.6 (1.9, 10.6)
Platelet Independent	Independent	126	10.0 (2-39)	34.7 (25.1, NR)
	Dependent	15	2.0 (1-4)	2.0 (1.2, 3.9)
NR = Not reached				

0814

EVALUATION OF APOPTOTIC MOLECULAR MARKERS AND GENE METHYLATION STATUS IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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The apparent discrepancy between cellular marrow and peripheral blood cytopenias in MDS may be related with programmed cell death(apoptosis), which occurs with increased frequency in MDS marrow. Apoptotic cell death may be triggered by several mechanisms involving death receptors and/or mitochondria where Bcl-2 family proteins have an important role. Although increased apoptosis in MDS may occur as an early rescue or compensatory response to deregulated clonal proliferation, it may also represent a pathophysiologic consequence of the epigenetic changes, particularly altered DNA methylation. Global hypomethylation is believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes as those involved in cell cycle regulation, DNA repair, growth signaling, angiogenesis, and apoptosis. Although the cause of altered patterns of DNA methylation in cancer remains unknown, it is believed that serum folate/vitaminB12 concentrations might strongly influence DNA methylation patterns. We hope to contribute for the identification of molecular markers involved in apoptotic signalling pathways and the role of folate status and epigenetic gene modulation in MDS, its interference with prognosis, evolution to AML and therapeutic approach. We have examined the expression levels of proteins involved in apoptotic pathways(Bax, Bcl-2, p53, Fas/FasL, TRAIL/TRAIL-Rs, cytochrome c, survivin) by flow cytometry using monoclonal antibodies, the methylation status of the cell cycle regulators p15/p16 using a methylation specific PCR and the serum folate/vitaminB12 concentrations in CD34 BM cells populations collected at diagnosis from 23 patients with MDS and in 8 controls(ITP). The median age was 77 years(33-84), gender M/F=12/11, WHO subtypes: RCMD(n=9), RA(n=5), RAEB-1(n=3), RAEB-2(n=5), 5q-syndrome(n=1) and IPSS: low(n=6), intermediate-1(n=12) and intermediate-2(n=5). Six of the patients(RAEB and IPSS intermediate-1/2) evolve to AML. Our preliminary results show that CD34 MDS cells patient's have higher Bax, survivin(p0.0048), p53, Fas-L, cit c, TRAIL-Rs expression levels and Bax/Bcl-2 ratio, and lower levels of Bcl-2, TRAIL, Fas and R1+R2/R3+R4 ratio compared with controls. CRDM patients have the higher survivin levels compared with the others subtypes (p0.03) and higher R3 levels compared to RA patients(p0.03). This subtype of patients(RA) also shows higher R3 levels when compared to RAEB-2(p0.03). We found 74% of cases with at least one methylated gene promoter: p15 methylation occurred in 37%, while p16 methylation occurred in 68% of MDS patients. p15 methylation was present in all the RA and 5q- syndrome patients and in 25% of RCDM patients. P16 methylation was observed in all subtypes except RAEB-2(75%-RA and RCDM; 100%-5q-syndrome and RAEB-1).The highest quintiles of serum B12 had 100% p16 methylation. All the quintiles of serum folate had similar p16 methylation. However, the p15 methylation increased progressively in the second to the fifth quintile. P15 and p16 seem to be an event in the MDS development and high concentrations of serum folate/vitaminB12 might be associated with the risk of promoter methylation in tumor-specific genes, especially p16 gene. Apoptosis deregulation may be implicated in MDS. In RA and RCDM subtypes an increase in apoptosis predominates while in RAEB the resistance to apoptosis may contribute to a less favourable prognosis.

Acute myeloid leukemia - Biology III

0815

REPRESSION OF THE MYST2 ACETYLTRANSFERASE IN LEUKEMOGENESIS AND ITS ASSOCIATION WITH HEMATOPOIETIC CELL GROWTH

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The histone acetyltransferase Hbo1/Myst2 is a member of the MYST family of acetyltransferases. Several members of this family, e.g. Tip60, Moz and Morf play important roles in leukemia development. Myst2 is the main acetyltransferase for histone H4 and plays an important role in replication licensing. Its involvement in leukemogenesis is unknown. In a ChIP-Chip based approach we recently identified Myst2 as a transcriptionally repressed target of PML-RARα. The aim of the current study was to elucidate its potential role in leukemic cells and leukemia development. We used ChIP-Chip to identify PML-RARα target genes. To further validate the role of Myst2 in leukemia we analyzed Hbo1/Myst2 expression in primary patient samples. We used overexpression of Myst2 in murine bone marrow cells as well as shRNA mediated repression of Myst2 in a cell line model to further show the role of this protein in the growth regulation of leukemic cells. The Myst2 promoter was directly bound by PML-RARα. The mRNA expression of Myst2 was significantly reduced in APL as well as other AML patients compared to normal bone marrow and $CD34^+$ progenitor cells. Myst2 was induced during monocytic differentiation in U937 cells and repressed during granulocytic differentiation in HL60 cells. Total histone 4 acetylation levels at lysines 5 and 8 were altered accordingly. PML-RARα repressed the differentiation-dependent induction of Myst2 with a concomitant decrease in histone H4 lysine 5 and lysine 8 acetylation in PML-RARα expressing cells. In functional experiments, Myst2 expression in Lineage negative murine bone marrow cells resulted in decreased colony formation and proliferation. On the other hand, shRNA mediated repression of Myst2 in the leukemic cell line Kcl22 enhanced growth and colony formation compared to cells containing scrambled shRNA sequences. These data establish that Myst2 is a direct target of PML-RARα. Its widespread repression in AML suggests other regulatory mechanisms as well. Its regulation and growth suppressive functions suggest a potential role in leukemogenesis.

0816

RADIOIMMUNOTHERAPY USING ANTI-CD33 ANTIBODIES RADIOLABELED WITH THORIUM-226 OR BISMUTH-213 OVERCOME CHEMO- AND RADIORESISTANCE IN MYELOID LEUKEMIA CELLS

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Background. One of the primary causes for therapeutic failure in radiotherapy and chemotherapy of leukemia and solid tumors is the resistance to radiation and chemotherapeutic drugs. Attempts to improve the results of chemotherapy and radiotherapy by increasing the total radiation absorbed dose, by increasing the concentration of chemotherapeutic drugs, or by changing chemotherapeutic drugs have only partly been successfull. Targeted radiotherapy using monoclonal antibodies radiolabeled with α-particles such as Bi-213 is a promising treatment approach for high-risk leukemia. The therapeutic efficacy of the anti-CD33 mAb HuM195 labeled with the single α emitter Bi-213 is currently under study in a phase I/II trial of leukemia. The α-particle emitter Th-226 is a novel therapeutic nuclide emitting a rapid cascade of four α particles in its decay chain. *Aims*. In the present study we compared cell death and activation of apoptosis pathways induced by bismuth-213 bound to anti-CD33 ([Bi-213]anti-CD33) with thorium-226 bound to anti-CD33 ([Th-226]anti-CD33) in sensitive and chemo- and radioresistant CD33 positive myeloid leukemia cells. *Methods*. The human myeloid sensitive cell line HL-60 (CD33 +/+) and the HL-60 (CD33 +/+) cell lines resistant to radiation and chemotherapy and the human lymphoblastic leukemia T-cell line CEM (CD33 -/-) were treated with 225, 75, 22.5, 7.5 and 2.25 kBq/mL of [Bi-213]anti-CD33 or [Th-226]anti-CD33, using comparable specific activities. 24, 48 and 72h after irradiation cell death and apoptosis were measured by flowcytometry and activation of apoptosis pathways were determined by Western Blot analyses. *Results*. We found that [Bi-213]anti-CD33 and [Th-226]anti-CD33 induced cell death and activated apoptosis pathways with different efficiencies in CD33 positive myeloid leukemia HL-60 cells. In contrast, CD33 negative lymphoblastic leukemia CEM cells were not killed using anti-CD33 antibodies radiolabelled with Th-226 or Bi-213. [Th-226]anti-CD33 triggered higher apoptosis rates in myeloid leukemia HL-60 cells in comparison to [Bi-213]anti-CD33 at equal activity concentrations using comparable specific activities. The differences in cell death induction were found between 35 and 85% depending on activity concentrations and time points. [Th-226]anti-CD33 or [Bi-213]anti-CD33 activated caspase-3 and cleaved PARP in myeloid leukemia HL-60 cells. In addition, after treatment with [Th-226]anti-CD33 or [Bi-213]anti-CD33 mitochondria were activated resulting in caspase-9 activation. Bcl-xL, a death-inhibiting protein of the Bcl-2 family, was down regulated after treatment with [Th-226]anti-CD33 or [Bi-213]anti-CD33. This indicates that both [Th-226]anti-CD33 and [Bi-213]anti-CD33 activate the intrinsic mitochondrial pathway. However, [Th-226]anti-CD33 activated the apoptosis pathway earlier and with higher efficiencies than [Bi-213]anti-CD33. Furthermore, both [Th-226]anti-CD33 and [Bi-213]anti-CD33 killed chemo- and radioresistant myeloid leukemia cells. *Summary and Conclusions*. Our findings demonstrate that both [Th-226]anti-CD33 and [Bi-213]anti-CD33 induce apoptosis by specific activation of the intrinsic mitochondrial pathway and break chemo-and radioresistance specifically in CD33 positive myeloid leukemia cells. Notably, anti-CD33 labeled with the α cascade emitter Th-226 induces cell death and activates apoptosis pathways with higher efficiencies in comparison to anti-CD33 labeled with Bi-213 emitting one α particle per decay. This suggests that Th-226 seems to be a more potent α-particle emitter for leukemia therapies than Bi-213.

0817

CBL MUTATIONS IN ACUTE MYELOID LEUKEMIA AND MYELOPROLIFERATIVE NEOPLASMS

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Background. Deregulation of tyrosine kinase (TK) activity is one hallmark in the pathogenesis of acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPN). In both, AML and MPN mutational activation of receptor TK (RTK) genes such as FLT3, KIT or JAK2 has been shown to play a key role in leukemic transformation and progression of the disease. In addition, activation of RTK can occur independently from the mutation; as an example more than 80% of adult AML cases show high levels of FLT3 expression. Recent data suggest that FLT3, KIT, JAK2 or other RTKs can be active in cells containing inactivating mutations within the linker region of the proto-oncogene Casitas B-lineage lymphoma gene (CBL). CBL functions as E3 ligase that ubiquitinates and regulates activated RTKs, such as FLT3. First studies on CBL mutations in AML and MPN showed that these mutations occur with a low frequency. However, recent studies demonstrated that CBL mutations are highly associated with 11q acquired uniparental disomy (UPD) in MPN. *Aims*. To evaluate the frequencies of CBL mutations in myeloid leukemias. *Methods*. A large cohort of adult AML [n=287; normal karyotype (CN), n=119; various, n=5; core-binding factor (CBF) leukemia, n=162; karyotype not evaluable, n=1] and MPN patients (pts) [n=95; essential thrombocythemia (ET), n=29; polycythemia vera (PV), n=37; primary myelofibrosis (MF), n=17; post-ET-MF, n=2; post-PV-MF, n=7; MPN not classified, n=3] were analyzed by using a DNA-based PCR assay for amplification of exons 8 (including introns 7 and 8) and 9 (including intron 9) followed by direct sequencing. *Results*. Mutation analysis revealed 14 CBL mutations (exon 8, n=11; exon 9, n=3) in 13 AML cases and 3 mutations in the 95 MPN pts [exon 9, n=3). In AML, CBL mutations affected intron 7 in five cases, intron 8 in one case, exon 8 in five cases, whereas two mutations occurred in intron 9 and one in exon 9 of the gene. All three MPN associated mutations were located in exon 9. Most of the mutations have already been described and the majority of them were substitutions as well as frameshift mutations. Of note, 10 of the 14 CBL mutations were detected in CBF leukemias [t(8;21), n=3; inv(16), n=6)], including one inv(16)-positive AML having two CBL-mutations. The other four mutations occurred in CN-AML. Interestingly, all 3 CBL-mutated MPN pts (one ET, two PV) also had a JAK2V617F mutation. In addition, SNP array data were available in 26/119 CN-AML cases as well as in 40/95 MPN cases revealing one case with 11qUPD in each group.

However, both cases showed CBL wildtype. *Summary*. In this large cohort of myeloid leukemias, CBL mutations occurred at low frequencies. In AML, CBL mutations were associated with CBF leukemias, a finding that is in accordance with a previous study. Therefore, it will be of interest to further investigate the potential cooperative activity of mutant CBL with the CBF-related fusion proteins CBFB-MYH11 and RUNX1-RUNX1T1 as well as with other CBF-associated gene mutations (e.g. FLT3, KIT).

0818

CYTOPLASMIC NPM1 DETECTION BY FLOW CYTOMETRY AS SURROGATE FOR THE PRESENCE OF MUTATIONS IN THE NPM1 GENE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. In about 30% of patients with AML the predominantly nucleolar protein nucleophosmin (NPM1) is dislocated into the cytoplasm (NPM1c), caused by mutations in exon 12 of the NPM1 gene. NPM1-mutations are associated with a specific subgroup of AML with particular clinical, cytogenetic, and prognostic characteristics. AML with NPM1-mutations are part of the new WHO classification, making the detection an important diagnostic aspect. Thus far, NPM1 mutations can be detected either using molecular techniques or immunocyto- or -histochemistry. We reasoned that by the cytoplasmic dislocation, the protein might be also accessible for rapid diagnostics using intracellular flow cytometry. *Aims*. The aim of the present work was to evaluate this procedure *in vitro* and prospectively in newly diagnosed patients with AML. *Methods*. Therefore, cell lines and bone marrow (BM) of 23 healthy donors as well as 254 newly diagnosed AML patients, enrolled in two different treatment protocols, were prospectively investigated by a lyse-wash flow cytometric procedure including an anti-NPM1 monoclonal antibody labelled with Zenon technology. NPMc was measured after fixation and permeabilisation. Thereafter, mean fluorescence intensity (MFI) was evaluated. All patient samples were routinely analyzed for NPM1-exon 12 mutations using PCR followed by high resolution fragment analysis. *Results*. Cell lines with known mutational status (mutant: OCI-AML3 and wt: MV4-11) showed a clear separation (MFI: 44.6 vs. 3.7). In healthy BM the median MFI for the NPMc expression was 3.8±1.7. Thus, the threshold MFI for NPM positivity was set to 8.0. Overall in 95% of samples (243/254) flow cytometry and PCR showed concordant results - positivity in 32% (mNPM1c=82) and negativity in 63% (wtNPM1-=161) of patients. Thus, the MFI was significantly higher in mNPM1c cases than in PCR negative patients and BM donors (MFI: 16.7 vs. 3.1 vs. 3.8; $p<0.0001$). The mNPM1c group presented with significantly lower CD34, HLA-DR, and without aberrant CD2 and CD19 expression (17% vs. 75%, $p<0.0001$; 78% vs. 89%, $p=0.0192$; 0% vs. 10%, $p=0.0014$); 0% vs. 7%, $p=0.0178$), but a comparable expression of other myeloid and aberrant antigens. FLT3 mutations were detectable in significantly more mNPM1c patients (57% vs. 14%, $p<0.0001$). Interestingly, the few mNPM1c patients with CD34 expression (14/82) showed distinct diagnostic features compared to the CD34neg patients, such as lower MPO expression ($p=0.0528$), presence of FLT3-ITD in almost all patients ($p=0.0028$), and a significantly lower proportion of type A mutations ($p=0.0075$). Taking PCR results for exon 12 as reference, discrepant results regarding flow cytometric NPM1c detection occurred in 5% as 5 false negative and 6 false positive cases. Interestingly, two patients with wtNPM1 in exon 12 but NPM1c by flow cytometry showed truncating mutations in exon 10 and 11, respectively. *Conclusions*. Flow cytometry allows a reliable and rapid detection of NPMc expression as surrogate for the presence of NPM1 mutations and could thus guide further molecular analyses. It might help to identify patients having mutations outside the mutational hotspot in exon 12.

0819

EXTENSIVE MUTATIONAL STATUS OF GENES AND CLINICAL OUTCOME IN PEDIATRIC ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous but aggressive disease. Although AML is a relatively rare malignancy in the pediatric population, representing only 15 to 20% of the acute leukemias in this age group, it remains a challenging disease with an inferior treatment outcome as compared to pediatric acute lymphoblastic leukemia. Several factors are known to predict the long-term survival in both childhood and adult AML. Among these, cytogenetic findings are considered to be the most powerful single prognostic factor in AML. Most patients belong to the category with standard-risk disease and the stratification of this heterogeneous group appears to be insufficient. New molecular markers are continuously being defined and not only have prognostic significance but also and more importantly, offer the potential for biologically targeted therapy. Very few studies have been performed in pediatric populations. *Aims*. The main aims of the study were to evaluate the prognostic impact of eight gene mutations including the Wilms' tumor 1 (WT1) mutation in pediatric acute myeloid leukemia and to determine whether these molecular lesions could be used to improve the molecular risk stratification to better predict the response to therapy. *Methods*. We studied the mutational status of the FMS-like tyrosine kinase 3 (FLT3), kit proto-oncogene, RAS, CCAAT/enhancer binding protein α (CEBPα), nucleophosmin (NPM1) and WT1 genes in 76 cases of *de novo* AML. Patients were treated between 1995 and 2002 and enrolled in the Leucémie Aiguë Myéloblastique de l'Enfant (LAME) 89/91 and LAME 99/01 pilot study protocols. All mutations were screened by direct sequencing. *Results*. At diagnosis 8%, 6.5%, 4%, 9%, 5%, 10.5%, 1.3% and 8% of the patients had FLT3-ITD, FLT3-TKD, c-KIT, CEBPα, NPM1, N-RAS, K-RAS and WT1 mutations, respectively. Overall 40 mutations were detected in 34 cases with a prevalence of 45%. With a median follow-up of 72 months, event-free survival (EFS) was 59±6% and overall survival 67±5%. Patients with NPM1, FLT3-TKD or c-KIT mutations were all alive at 5 years without any events. In contrast, patients with FLT3-ITD and/or WT1 mutations displayed a significant reduction in 5-year EFS (17% vs. 63% for the wild-type, $p=0.004$ and 0.01). In a multivariate analysis, the presence of a WT1 mutation remained an independent prognostic factor for risk of relapse ($p=0.004$). *Conclusions*. We report an exhaustive study of the mutational status of genes in pediatric AML and provide evidence that WT1 mutations predict an extremely high risk of relapse. NPM1 and WT1 mutations should be prospectively researched at diagnosis in pediatric AML and could be suitable for treatment stratification.

0820

'CUP-LIKE' ACUTE MYELOID LEUKEMIA: A REPORT FROM A SINGLE CENTER

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Introduction. "Cup-like" nuclei in *de novo* acute myeloid (AML) blasts is a morphologic finding that has recently been associated with a normal karyotype, a characteristic immunophenotype and nucleophosmin (NPM) mutations.We investigated cup-like nuclear morphology of both *de novo* and secondary AML blasts and its association with hematologic findings, immunophenotype, cytogenetic data and cytoplasmic expression of NPM (NPMc). *Material and Methods*. Bone marrow and peripheral blood slides from 116 randomly selected AML patients (83 *de novo* of all FAB subtypes

and 33 secondary to myelodysplastic syndromes or therapy-related) were investigated at diagnosis for cup-like nuclei by 3 independent morphologists. Cup-like positive AML was defined as >5% of blasts with a cup-like nuclear invagination spanning at least 25% of the nuclear diameter. Immunocytochemistry (APAAP method) was performed with the monoclonal antibodies NPMAb-1 and C23 used as control (Thermo Scientific), in order to look for cytoplasmic (mutated) NPM. Immunophenotypic data by flow cytometry and karyotypes were recorded at diagnosis. Statistical analysis was performed with the Fischer's exact test. *Results*. Cup-like nuclear morphology was found in 24/83 *de novo* AMLs (29%), classified as: 2M0, 5M1, 7M2, 7M4, 1M5b,1M6(pure erythroid) and 1 mixed-lineage and in 4/33 secondary AML cases (12%). A normal karyotype was found in 70% of the cup-like(+) AMLs (vs 39.5% of the cup-like negative cohort, p=0.004). Cytoplasmic NPM was found in 76.4% of the cup-like(+) AMLs (vs 44.2%, p= 0.02). Cup-like (+) AMLs presented with a significantly higher white blood cell count (median WBC=102,8×10^9/lt vs 26,7×10^9/lt, p<0.0001). Cup-like(+) blasts were associated with low or absent CD34 expression (64,3% vs 21.5%, p<0.0001), as well as with low or absent HLA-DR (33,3% vs 6,45%, p=0.0019).Interestingly, 6 cup-like (+) AML cases with agranular blasts had been initially misdiagnosed as acute promyelocytic leukemias by immunophenotype, but AML-M3 was later excluded by karyotype. *Conclusions*. Cup-like morphology of AML blasts was observed in 24% of the AML cases studied and it was more frequent in *de novo* than in secondary AML, especially in the presence of hyperleukocytosis. It was not specific to any FAB subtype. This morphologic finding predicted a normal karyotpe and NPM1 mutations. Morphologic and immunophenotypic similarities of cup-like(+) AML with variant types of APL should be kept in mind when a quick differential diagnosis is mandatory.

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DEFINING T(15;17)(Q22;21) TRANSLOCATION MECHANISM IN THERAPY RELATED AND *DE NOVO* ACUTE PROMYELOCYTIC LEUKEMIA

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Background and Aims. Most of the proposed mechanisms mediating chromosomal rearrangements in *de novo* leukemias are sequence-dependent and it has been suggested that various recombination-promoting sequences play a role in the genesis of translocations. Therapy-related acute myeloid leukemias (t-AMLs) provide an extraordinary opportunity to investigate key mechanisms of leukemogenesis by relating specific genetic abnormalities to the biologic effects of chemotherapeutic agents. We and others (Hasan *et al.* Blood 2008, Mistry *et al.* NEJM 2005) have shown that mitoxantrone induced topoisomerase II (topoII) mediated DNA damage at the PML and RARA breakpoint loci and their subsequent repair via error prone non homologous end joining (NHEJ) pathway lead to the formation of the t(15;17)(q22;q21) in therapy-related acute promyelocytic leukemia (t-APL). Here we characterized genomic breakpoints in 9 previously unreported cases of t-APL to investigate the sequence specific distribution of translocation breakpoints and their relationship to therapy given for the primary disorder. *Patients and methods*. Nine patients with t-APL developing after treatment of breast cancer (n=1), corpus uteri carcinoma (n=1), Hodgkin lymphoma (n=1) and multiple sclerosis (n=6) were studied, of whom 7 had received topo II targeted therapy. A long-range nested PCR strategy followed by direct sequencing was adopted to identify PML and RARA breakpoints at the DNA level. A web based tool (http://transpogene.tau.ac.il/cgibin/tg/alugene/transpogene.pl) was used for the identification of Alu recombination sequences in the vicinity of the breakpoint regions. *Results*. Genomic analysis revealed the occurrence of breakpoints within the mitoxantrone targeted hotspot DNA region in two out of seven cases with breakpoints in PML intron 6. Chromosome 17 translocation breakpoints in 7 topoII inhibitor related cases were concentrated at the 3'end of RARA intron 2, whereas for patients who did not receive topoII targeted therapy RARA breakpoints were observed to fall at the opposite end of the intron, which is ~17kb in length (Figure 1). The 5' region of RARA intron 2 containing the *de novo* APL breakpoints included 9 Alu repeat elements. V(D)J hepta- and nonamer consensus sequences were detected near 2 of the 9 analysed RARA breakpoints (matching sequences were 4 per heptamer and 7 per nonamer). In one patient the RARA breakpoint was located within a recombination signal sequence between the last nucleotide of a 23 bp spacer and the first base of a nonamer. *Conclusions*. This study suggests a different distribution of breakpoints in the RARA locus in *de novo* and therapy-related APL implying differences in t(15;17) translocation mechanism according to disease context. While there is evidence supporting a role for recombination mechanisms underlying *de novo* APL, cases arising following exposure to topoII targeted agents involve DNA damage followed by repair mediated by the error prone NHEJ pathway.

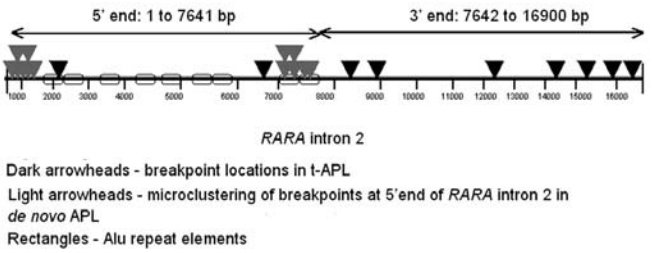

Figure 1. Distribution of RARA breakpoints

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SPECIFIC ACTIVITY OF ANTI-CD33 ANTIBODIES RADIOLABELED WITH THORIUM-226 OR BISMUTH-213 PLAYS A CRITICAL ROLE IN EFFICIENT KILLING OF LEUKEMIA CELLS DURING RADIOIMMUNOTHERAPY

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Background. Targeted radioimmunotherapy of bone marrow using monoclonal antibodies radiolabelled with α-particles is a promising treatment approach for high-risk leukemias and lymphomas. Different specific activities are used during radioimmunotherapy. However, the effect on cell killing using different specific activities were not well examined *Aims*. In the present study we examined the effect of different specific activities (MBq/µg antibodies) on induction of cell death and activation of apoptosis pathways in CD33 positive myeloid leukemia cells using the anti-CD33 antibody HuM195 radiolabeled with the single α emitter bismuth-213 ([Bi-213]anti-CD33) or with the α cascade emitter thorium-226 ([Th-226]anti-CD33). *Methods*. The human myeloid sensitive cell line HL-60 (CD33 +/+) was treated with 225, 75, 22.5, 7.5 and 2.25 kBq/mL of [Bi-213]anti-CD33 or [Th-226]anti-CD33, using different specific activities (0.023, 0.09, 0.23 and 0.9 MBq/µg anti-CD33). 24, 48 and 72h after treatment induction of cell death, induction of apoptosis, cell cycle, caspase activation and activation of apoptosis pathways were determined using flowcytometry and Western Blot analyses. *Results*. Our results demonstrate that specific activities of anti-CD33 antibodies radiolabeled with Bi-213 or Th-226 play a critical role in induction of cell death, apoptosis and in activation of apoptosis pathways in CD33 positive myeloid leukemia cells. The efficacy of cell death induction depends on both dose and specific activity. At a specific activity of 0.23 MBq/µg anti-CD33, it was possible to kill 80% of leukemia cells at a very low activity concentration of 7,5 kBq/mL of Th-226 after 72h. Further decreasing the specific activity to 0.09 MBq/µg anti-CD33 of Th-226 reduced cell killing to 40% and decreasing the specific activity to 0.023 MBq/µg anti-CD33 of Th-226 made it impossible to kill leukemia cells at comparable activity concentrations. Similar results were observed for Bi-213. However, in contrast to Th-226 higher activity concentrations of Bi-213 were needed. Only at specific activity of 0.9 MBq/µg anti-CD33, it was possible to kill 80% of myeloid leukemia cells at activity concentration of 7.5 kBq/mL of Bi-213. At specific activity of 0.23 MBq/µg anti-CD33 only 15% were eradicated at comparable activity concentrations of Bi-213. Similar results were also found after examination of the effect of different specific activities of radiolabeled anti-CD33 antibodies with Th-226 or Bi-213 in activation of apoptosis pathway at comparable concentrations. Only at a specific activity of 0.23 MBq/µg anti-CD33 for Th-226 and a specific activity of 0.9 MBq/µg anti-CD33 for Bi-213 at the activity concentration of 7.5 kBq/mL a strong activation of caspase-3, caspase-2, caspase-10, caspase-9 and

PARP cleavage were found. Activation of apoptosis pathway were strongly decreased at lower specific activities. *Summary and Conclusions*. Our findings suggest that not only the activity concentrations of anti-CD33 antibodies radiolabeled with Th-226 or Bi-213 play an important role in killing leukemia cells and in activation of apoptosis pathways but also the specific activities are crucial. These findings have important implications for leukemia therapies using radiolabeled antibodies with different α-particle emitters such as Th-226 and Bi-213 during radioimmunotherapy.

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ANGIOGENESIS IN ACUTE PROMYELOCYTIC LEUKEMIA IS MEDIATED BY THE HEPATOCYTE GROWTH FACTOR

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Background. Angiogenesis is critical in the pathogenesis of different hematological malignancies. An increased angiogenesis in the bone marrow of patients with acute promyelocytic leukemia (APL) has been demonstrated in previous studies and VEGF is believed to be the major positive mediator of angiogenesis. Gene expression profiling has demonstrated that the hepatocyte growth factor (HGF), another potent angiogenic factor, is highly expressed in APL, while its expression is absent in all remaining acute myeloid leukemias. *Aims*. 1) To investigate the involvement of HGF as a mediator of angiogenesis in APL. 2) To explore the effect of all-trans retinoic acid (ATRA) on HGF expression. *Methods*. Functional assays were performed using the human promyelocytic cell line NB4, which was cultured in RPMI 1640 with L-glutamine and supplemented with antibiotics and 10% fetal bovine serum. The secretion of HGF and VEGF was measured using ELISA kits (Quantikine, R&D Systems). Ten APL samples were cultured for HGF quantification in the media. Neutralizing antibody for HGF (R&D Systems) was used at 5 microg/mL concentration. Migration of HUVECs was assessed by the CytoSelect Cell Migration kit (Cell Biolabs). Transmigration was quantified by counting the migrated cells using an inverted microscope and by measuring absortion at 560 nm. All the experiments were repeated three times with similar results. *Results*. The overexpression of HGF observed at mRNA level in APL was correlated with the quantification of HGF content in the media of cell cultures from 10 APL samples as well as in the conditioned media from NB4. To explore the angiogenic potential of the NB4 cells, the conditioned media was tested in the endothelial cell migration assay. After incubation for 6 hours, endothelial cell migration was significantly stimulated compared to control. When neutralizing antibodies to HGF were added to the conditioned media a strong inhibition of endothelial cell migration was observed. In order to know if the antiangiogenic effect of ATRA was related to a putative effect of this drug on HGF production, NB4 cells were treated with ATRA (1 microM) during 5 days. Quantitative ELISA assay showed that ATRA treatment induced a significant reduction of HGF production by the NB4 cells at 72 hours (more than 80% of control production), whereas a similar decrease of VEGF was reached after 120 hours, indicating that the inhibition of angiogenic factors by ATRA in APL occurs significantly earlier for HGF. *Conclusions*. The HGF produced by promyelocytic blast cells promotes endothelial cell migration. The inhibition of HGF secretion by ATRA represents a new mechanism by which ATRA abrogates angiogenesis.

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0824

ANALYSIS OF SUBCELLULAR COMPARTMENT EXPRESSION OF SURVIVIN WILD-TYPE AND SPLICING VARIANTS 2B AND DELTAEX3 IN PATIENTS DIAGNOSED WITH ACUTE MYELOID LEUKEMIA: IMPACT ON CLINICAL OUTCOME

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Background and Aims. Suppression of apoptosis is a major contributing factor to oncogenesis in acute myeloid leukemia (AML). Survivin wild-type (WT) (142aa) is a member of inhibitor of apoptosis protein family (IAP) regulating both apoptosis and cell cycle progression. Survivin binds to several structural components of the mitotic apparatus and can block apoptosis by inhibiting caspases 9, 3 and 7. Recently, different splicing variants have been described such as Survivin-2B (165aa) and Survivin-DeltaEx3 (137aa). Survivin WT and 2B can localize to both nuclear and cytoplasmic compartments whereas DeltaEx3 localizes only in the nucleus. Survivin is undetectable in differentiated adult tissue but is over expressed in most cancers. In this study we analyzed the subcellular compartment distribution of Survivin WT and splicing variants in AML, the correlation with proliferative status and the impact on outcome. *Methods*. We included 68 AML consecutive patients. Median age was 62 years (range 8-89). Median leukocyte count was 10.3×10^9/L (range 0.8-285). FAB subtypes were: M0=8, M1=20, M2=13, M3=8, M4=11, M5=7 and M6=1 with the following cytogenetic findings: t(15;17)=8, t(8;21)=2, complex karyotype=8, del7=1, 11p23=2, normal karyotype=16 and others= 9. There were 11.3% patients with FLT3-ITD and 16.9% with NPM1 mutation. Bone marrow samples were obtained at diagnosis. Nuclear and cytoplasmic proteins were harvested with Q-proteome cell compartment (Qiagen) and protein concentration assayed using Protein Assay Kit (Bio-Rad). Survivin protein was detected by Western Blot Protein with Primary antibody for human-Survivin and specificity of each isoform was assessed by blocking peptide experiments. We used beta-actine and Laminin A (Cell Signaling Tecnology) to test compartment specificity. Proteins were visualized by enhanced chemiluminescence (ECL-Plus, GE Healthcare) in Chemigenius-2 and quantified using GeneTools software. Proliferative status was measured by total Akt and Akt-pSer473 and cell cycle analysis was assessed by double Hoechst 33342-Pyronin Y staining and flow cytometry. *Results*. In cytoplasmic protein extracts, Survivin WT and 2B were detected in 45% and 45.2% of patients respectively but DeltaEx3 was never detected. Nuclear isoforms were detected in 46.7%, 30% and 28.3% of cases for WT, 2B and DeltaEx3 respectively. Considering all isoforms, 23.3% of samples expressed Survivin in both nucleus and cytoplasm. 21.7% only in nucleus and 23.3% only cytoplasmic. Interestingly, there was strong statistical correlation between the levels of p-Ser473 Akt (detected in 56% in marrow samples with high levels in 27%) with Survivin-WT in cytoplasm (p=0.01) and inverse correlation between p-ser473 Akt with nuclear Survivin-WT (p=0.002). Meaningfully, lack of cytoplasmic Survivin WT was associated with an increased proportion of cells in Go cell cycle phase (11.1% vs. 3.6%, p=0.04). Finally, cytoplasmic Survivin WT localization and high p-Ser473 Akt levels, were both significantly correlated with CR achievement with one induction cycle (p<0.01), less relapse rate (p=0.01) and less mortality rate. By contrast, nuclear Survivin localization was associated with a significant increased relapse rate (p=0.03). *Conclusions*. Cytoplasmic localization of WT and 2B Survivin is associated to an increased proliferative status with constitutive activation of PI3k/Akt and more favourable outcome. However, nuclear localization of Survivin WT and isoforms could represent an adverse finding.

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WILMS' TUMOR 1 GENE MUTATIONS ARE ASSOCIATED WITH A HIGHER RISK OF RELAPSE IN YOUNG ADULTS WITH ACUTE MYELOID LEUKEMIA: A STUDY FROM THE ACUTE LEUKEMIA FRENCH ASSOCIATION

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Background. Wilms' Tumor 1 (WT1) is a transcription factor overexpressed in most cases of acute myeloid leukemia (AML). Recently, several groups reported that WT1 mutations occur in approximately 10% of normal karyotype AML (CN-AML) and cluster to exons 7 and 9, and that these mutations independently predict poor outcome in patients with CN-AML. *Aims*. The objectives of this study were to evaluate the frequency, the main clinical and biological associated features and the prognostic significance of WT1 mutations in a retrospective cohort of young adults with AML, excluding acute promyelocytic leukemia. *Methods*. We studied a cohort of 268 young adults (15-50 years) with AML uniformly treated in the Acute Leukemia French Association-9802 (ALFA-9802) trial. WT1 exon 7 and 9 mutations were retrospectively screened by PCR and direct sequencing. The patients were also assessed for the presence of FLT3 internal tandem duplication (FLT3-ITD), FLT3-

D835/I836, NPM1 and CEBPA mutations. *Results*. 14 out of 268 AML patients (5%) harbored a WT1 mutation. Among the 106 patients with CN-AML, WT1 mutations were detected in 9 cases (8.5%). All WT1 mutations detected within this cohort of patients were heterozygous frameshift mutations in exon 7. WT1 mutations were associated with a younger age (p=0.02) and a FLT3-ITD (p=0.03). No association was found between the presence of WT1 mutations and FAB subtype or white blood cells (WBC) count at diagnosis. The karyotype of WT1 mutated AML was predominantly normal (9/14, 64%). None of these cases was found in the favorable cytogenetic risk group defined by the presence of either a t(8;21)(q22;q22) or an inv(16)/t(16;16)(p13;q22). Out of 268 patients, 243 (91%) achieved complete remission (CR) after induction chemotherapy. The CR rate was lower in WT1 mutated patients compared with wild-type cases, but without reaching significance (79% versus 92%, p=0.13). The median follow-up for patients who remained alive was 3.8 years. Patients with WT1 mutations were found to have a shorter overall survival (OS at 4 years: 22% versus 56%, p=0.01) and a higher risk of relapse (RR at 4 years: 82% versus 46%, p=0.0008) compared with wild-type cases. Within the subgroup of patients with CN-AML (n=106), the presence of a WT1 mutation was also associated with an increased risk of relapse (p=0.0006, 4y-RR: 86% versus 40%). In multivariate analysis considering WBC count, molecular risk group (NPM1 mutated/FLT3-ITD negative or CEBPA mutated/FLT3-ITD negative versus other genotypes), and WT1 mutational status as covariates, the presence of a WT1 mutation remained an independent adverse prognostic factor for the risk of relapse in CN-AML. Even after adjustment on other covariates, WT1 mutation was not predictive of OS. *Conclusions*. We showed that WT1 mutations represent an adverse prognostic factor in young adults with AML. Prospective trials should confirm the clinical relevance of WT1 mutations in relation to other prognostic factors in AML.

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EVI1 OVEREXPRESSION IS ASSOCIATED WITH UNFAVORABLE SUBTYPES IN PEDIATRIC ACUTE MYELOID LEUKEMIA

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Background. The EVI1 (ecotropic virus integration-1) gene plays an important role in hematopoiesis especially in megakaryocyte development. The MDS1 gene is located upstream of EVI1, and its function is currently unknown. Normally the MDS1/EVI1 intergenic splice variant is co-expressed with EVI1. In adult acute myeloid leukemia (AML) overexpression of EVI1 (EVI1+) can be found in patients with chromosome 3q26-rearrangements. Often, these patients do not co-express MDS1/EVI1. Recently EVI1+ was also discovered in a separate subgroup of patients that did not have 3q26-rearrangements. Occasionally, they did not show overexpression of MDS1/EVI1. In these patients cryptic inversions of chromosome 3 were identified with fluorescence *in situ* hybridization (FISH). Of interest, EVI1+ was found to be an independent poor prognostic marker in adult AML (Lugthart *et al.*, Blood 2008). *Aim and Methods*. In pediatric AML, 3q26-rearrangements are rare and the role of EVI1 is unknown. In this study, we investigated the frequency and clinical relevance of EVI1+ in pediatric AML. EVI1 expression was analyzed in 233 pediatric AML patients, of whom microarray gene expression profiling data were available. *Results*. EVI1+ was found in 25 pediatric AML patients (11%), and confirmed with real-time quantitative PCR. This included 13/49 (26%) patients with MLL-rearranged AML: 5/22 (23%) cases with t(9;11); and all (n=4) cases with t(6;11). Moreover, EVI1+ was found in 4/7 (57%) cases with AML M7; in 2/3 (66%) cases with AML M6; in both cases with monosomy 7; in 1/43 (2%) cases with normal cytogenetics; in 2 patients with random cytogenetics, and in 1 patient with a cytogenetic failure. EVI1+ was not found in the t(8;21), inv(16) and t(15;17) subgroups. 3/25 EVI1+ patients lacked the MDS/EVI1 transcript, but no cryptic 3q26-rearrangements were detected with FISH. Molecular analysis showed that one patient had a CEBPα mutation; one patient had an FLT3-ITD; and 3 patients showed a mutation in the RAS oncogene. EVI1+ was not correlated with sex or white blood cell count. Survival analysis was restricted to the subset of patients who were treated using uniform DCOG and BFM treatment protocols (n=204). In this cohort, EVI1+ patients had a worse 5-years event-free survival (pEFS) compared to patients without EVI1+ (30 vs. 43%, p=0.02). However, multivariate analysis, including cytogenetics, age and WBC, showed that EVI1+ was not an independent prognostic factor for survival. Moreover, within the unfavorable/normal cytogenetic subgroup, there was no difference in outcome between patients with and without EVI1+. *Summary and Conclusions*. We conclude that EVI1+ is found in ~10% of pediatric AML, and highly correlated with specific cytogenetic (MLL-rearrangements) and morphologic (FAB M6/7) subtypes. In contrast to adult AML, no 3q26-rearrangements or cryptic inversions were found, and EVI1+ was not an independent prognostic factor. This difference in prognostic relevance may be due to differences in treatment. Alternatively, these results may indicate that EVI1 plays a different role in disease biology between adult and pediatric AML. This is at least suggested by the lack of 3q26 aberrations in pediatric AML.

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EXPRESSION OF ANGIOTENSIN-CONVERTING ENZYME (ACE, CD143) AND SHAPERONS (BIP, CALNEXIN, CALRETICULIN) BY LEUKEMIC DENRITIC CELLS IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS

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Background. The dendritic cells (DC) play a key role in the induction of primary and adoptive immune response. AML blast cells can be induced to differentiate into leukemic dendritic cells (LDC), which are quite similar to monocytes derived DC by the expression profile of integrins and co-stimulatory molecules. The only difference between LDC and DC is absence of surface ACE expression on LDC in contrast to high level of ACE on DC. *Aims*. We propose that the absence of surface ACE expression in LDC is due to the block of normal ACE transport to the cell surface, normally controlled by shaperons. To confirm this hypothesis we quantified the level of intracellular and surface ACE and Bip. At the same time mRNA expression levels of ACE and shaperons Bip, Calnexin, Calreticulin in DC and LDC were measured. *Methods*. Blood samples were collected from 12 AML patients at diagnosis before induction chemotherapy and 10 healthy donors. Mononuclear cells were isolated using gradient centrifugation with Ficoll-Paque and were differentiated into dendritic cells by culturing with 180 ng/mL calcium ionophore for 4 days at 37°C and 33°C (activated factor for shaperons' induction). DC were stained for surface and intracellular ACE (two mAbs - clones1D8 and 9B9) and Bip (mAb KDEL) analyzed by flow cytometry, simultaneously the mRNA expression levels of ACE and shaperons Bip, Calreticulin, Calnexin were evaluated with RT PCR. *Results*. The surface ACE expression on LDC cultured at 37°C was 3±2% of positive cells (mAb 9B9), 2±1% (mAb1D8), surface Bip expression was 6±1% and were increased up to 45 ±10% (mAb 9B9), 57±8% (mAb1D8) and 63±7% for Bip with lowering culture temperature to 33°C. The surface ACE expression on DC cultured at 37°C was 46±9% (mAb 9B9) and 5±2% (mAb 1D8), surface Bip expression was 36±4%, at 33°C - 34±3% (mAb 9'9), 6±2% (mAb 1D8), 21±4% for Bip. The intracellular ACE expression in LDC cultured at 37°C was 71±9% (mAb 9'9), 52±8% (mAb1D8), intracellular Bip expression was 92±7%, and were lowered to 6±2% (mAb 9B9), 5±2% (mAb 1D8), 52±6% for Bip with lowering culture temperature to 33°C. The intracellular ACE expression in DC cultured at 37°C was 50±10% (mAb 9B9), 27±7% (mAb 1D8), intracellular Bip expression was 69±7%, at 33°C - 40±10.3% for 9B9, 33±7% for 1D8, 88±4% for Bip. Gene expression had been measured by real time PCR using TaqMan procedure and calculated in relative units using GapDH expression for normalization. Expression of ACE was increased in 57,1% cases of DC cultured at 33°C compared to DC cultured at 37°C and in 89% cases of LDC cultured at 33°C compared to LDC cultured at 37°C. Expression of Bip, was increased in 87,5% cases of DC and in 55,5% cases of LDC. For Calnexin appropriate measures showed 62,5% for DC and 55,5% for LDC; for Calreticulin 28,5% for DC and 66,6% for LDC respectively. *Conclusions*. The data demonstrate the block of ACE transport to the cell surface of LDC at normal human temperature 37°C and thus provide the evidence of the altered expression of normal antigens by LDC. We confirmed not only differences of LDC and DC discovered by standard cultivation but the different reactions of these cells on the stress conditions. Hence, this LDC behaviour may be responsible for inadequate antitumor immune response.

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CHARACTERISATION OF A NEW MONOCLONAL ANTIBODY RAISED AGAINST THE MUTANT A OF THE NPM1 PROTEIN: ITS APPLICATION IN THE DIAGNOSIS OF THE NPMC+AML

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Background. Nucleophosmin (NPM) gene mutations occur in up to 35% of the human AML cases (60% of cases with otherwise normal karyotype). Twenty-two different mutations have been described, six of which, including type A (75-80% of all cases), encode for the same protein. Mutations introduce a nuclear export signal, which relocalises NPM to the cytoplasm (NPMc+AML). Methods used for the detection of NPM mutations include: DNA sequencing, real-time Q-PCR, denaturing high-performance liquid chromatography, capillary electrophoresis, and allele-specific (ASO)-RT-PCR assay. Detection of cytoplasmic NPM dislocation by immunofluorescence (IF) or immunohistochemistry (IHC) can also be utilised. To date there is no well-functioning mutant-specific monoclonal antibody (MAb) for the diagnosis. *Aims*. The aim of the study was the production and characterisation of a NPM mutation-specific MAb for the diagnosis and follow-up of minimal residual disease, as well as mutant identification in molecular complexes and the studies of its in-cell dynamics. *Methods*. Standard mouse immunisation/hybridoma screening method was used to raise a MAb against a 19-mer polypeptide (CQEAIQDLCLAVEEVSLRK) corresponding to the C-terminal part of the NPM mutant A. The antibody's efficacy was evaluated by ELISA, Western blotting (WB), immunoprecipitation (IP), IF and IHC. A series of 95 *de novo* AML patients was studied by capillary electrophoresis and ASO-RT-PCR and, in parallel with our antibody, by IF on bone marrow smears and/or peripheral blood films; marrow/blood samples of 41 of these patients were also tested in parallel by flow cytometry. *Results*. The antibody is specific for the mutated NPM, does not cross-react with the wtNPM, as seen in a series of human and murine cells, or with any other unrelated protein. It works well in ELISA, WB, IP, IF, IHC and flow cytometry. It allows for the detection of as few as 0.01% of positive cells as revealed by flow cytometry analysis performed mixing mutant-positive and negative cell lines. Of the 95 consecutive *de novo* AML cases 38(40%) were found to be positive for the presence of the mutant by IF; by flow cytometry 15/41(37%) patients showed positivity. There were no discrepancies with the results obtained by capillary electrophoresis and ASO-RT-PCR. The percentage of positive cells within the blast gate varied between 2-88%(mean 28%). Moreover, 3/38(8%) positive patients were non-A mutations: two D (amino acid sequence identical to A) and one B (differing by one amino acid from mutant A). *Summary and Conclusions*. NPM mutant is usually detected by its cytoplasmic dislocation as a surrogate marker of the mutation using anti-NPM antibodies and confirmed by different molecular biology tests. Here, we present a mutant-specific MAb that can be applied in IF and flow cytometry for the diagnosis of NPMc+AML. Although, it was raised against the mutant A, it recognised also mutants B and D that together with type A are by far the most frequent of all mutations identified so far. This is the first time that a leukaemia-associated mutant has been used for the establishment of a specific diagnostic marker, as the presence of a positive staining is an unequivocal sign of the mutation.

0829

SURVIVIN EXPRESSION IMPACTS ON SURVIVAL IN ACUTE MYELOID LEUKEMIA PATIENTS

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Survivin, coded by the gene BIRC5 (baculoviral IAP repeat-containing 5), represents an important tumor-associated antigen that is involved in critical cellular pathways like control of cell proliferation and viability. Of particular note, survivin is a key player of the survivin-Borealin-INCENP core complex that regulates important proteins involved in cell division like aurora B kinase or polo-like kinase 1. However, little is known of the role of survivin in acute leukemia. In order to further address the impact of survivin expression in acute myeloid leukemia (AML), we evaluated its expression in two large independent microarray data sets comprising 618 AML cases. Both data sets showed no correlation of BIRC5 expression levels and age, BM blasts, LDH, preceding malignancy or overall survival. However, with regard to overall survival we found a negative prognostic impact of high BIRC5 expression levels in inv(16) core binding factor AML: in CBF AML cases lower BIRC5 expression was associated with better clinical outcome (p=0.004). Notably, this was mainly due to inv(16) cases with low BIRC5 expression (p=0.007). For AML cases with t(8;21) we found no significant difference, despite the fact that survivin seems to be a critical regulator of AML1/ETO-induced oncogenicity in AML. Furthermore, we found expression of BIRC5 correlated with a number of genes involved in cell cycle regulation. In addition, both data sets showed a correlation of survivin with aurora B kinase (AURKB) expression underlining the recent findings that survivin regulates the expression of this protein via the survivin-Borealin-INCENP core complex. Several clinical studies are ongoing to inhibit AURKB in an effort to target genes involved in the survivin cancer network. This strong correlation between BIRC5 expression and aurora B kinase (AURKB) and polo-like kinase 1 (PLK1) suggested that a combination of survivin and AURKB and/or PLK1 inhibition might prove to be valuable, especially in AML cases with an inv(16). In addition, we observed a strong association between BIRC5 and other leukemia-associated antigens (LAA) like SSX2IP (SSX2 interacting protein) and HMMR (hyaluronan-mediated motility receptor, also known as RHAMM) expression (p<0.001), thereby suggesting that survivin might also provide an effective immunological target in therapy of AML. In conclusion, in patients with CBF AML, and particularly patients with inv(16), high BIRC5 expression is a negative prognostic factor and an interesting structure for a targeted strategy against survivin expressing leukemic cells. Down-regulation of this complex system involved in tumorigenesis might be an important target for tumor cell control in acute leukemias.

0830

HUMAN MYELOID LEUKEMIA BLASTS ARE HIGHLY SENSITIVE TO IRON DEPRIVATION

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Objectives. Iron is a cofactor critical for energy metabolism and cell proliferation. Targeting iron homeostasis induces cell cycle arrest and apoptosis particularly in cancer cells which have been shown to be more sensitive to iron depletion than their normal counterparts. We sought to evaluate the efficacy of iron homeostasis targeting therapies in human acute myeloid leukemia (AML). For this purpose, we investigated the sensitivity of AML blasts or leukemic cell lines to iron chelators (deferoxamine and deferasirox) and A24, an anti-transferrin receptor (TfR) monoclonal antibody. A24 binds TfR and competes with Fe-Tf for TfR binding. In addition, it impairs cell surface receptor expression by inducing TfR endocytosis and sequestration in lysosomal compartments. Therefore, A24-targeted cells are profoundly impaired in iron uptake and undergo apoptosis. *Material and Methods.* A total of 15 AML patients peripheral blood were obtained from Necker and Saint Louis Hospital (Paris, France). They were collected at the initial diagnostic assessment and previously to treatment administration, after informed consent. Mononuclear cells were separated by Ficoll-hypaque density centrifugation and cultured in IMDM medium supplemented with 15% FCS, Stem Cell Factor, IL3 and FLT3-L with or without A24 (0.02 to 20 µg/mL) or iron chelators (deferoxamine: 0.07 to 50 µM and deferasirox: 0.04 to 10 µM) Cell proliferation was assessed by 3H-thymidine incorporation and cell death was evaluated by the annexinV/propidium iodide staining. For *in vivo* experiments, leukemia cells were subcutaneously xenografted in nude mice. *Results*. Iron deprivation inhibited cell proliferation and induced apoptosis in all AML subtypes. Nevertheless, myeloid cells seemed to be more sensitive than monocytic cells to iron-deprivation. These results were confirmed using the two leukemic cell lines HL60 and U937. Leukemic cell apoptosis was dependent on mitogen-activated protein kinases (MAPK) activation and inhibitors of JUNK and p38 prevented apoptosis induced by iron-chelating agents. *In vivo*, a single injection of A24 completely prevented tumor development in xenografted mice, whereas iron-chelators delayed tumor growth. *Discussion and Conclusions*. Acute myeloid leukemia are aggressive hemopathies and patients prognostic is poor because of a high degree of relapse after conventional chemotherapy. We show here that that iron deprivation impairs AML cell proliferation and induces apoptosis through the activation of MAPK pathway. *In vivo* experiments validated the efficacy of iron-chelating therapies. Altogether these findings indicate iron metabolism is a target in AML therapies which could be combined with chemotherapy opening new therapeutic alternatives in AML treatment.

Acute myeloid leukemia - Clinical II

0831

COMBINATION OF IDARUBICIN, HIGH DOSE CYTARABINE AND SORAFENIB IS HIGHLY EFFECTIVE IN ACHIEVING REMISSION IN NEWLY DIAGNOSED, FLT3-MUTATED ACUTE MYELOID LEUKEMIA

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Background. Sorafenib is an orally active multi-kinase inhibitor with potent activity against FLT3 kinase and the ability to induce apoptosis in FLT3-mutant human acute myeloid leukemia (AML) cell lines at nM concentrations. It has less plasma protein binding than several other FLT3 inhibitors. In a phase I study of single agent sorafenib in patients (pts) with AML, escalating doses were well tolerated with no myelosuppression and with significant clinical activity predominantly (but not exclusively) in FLT3 mutated pts. *Aims*. This is a phase I/II study to determine the clinical activity and tolerability of combination of sorafenib with standard AML chemotherapy. *Methods*. Cytarabine 1.5 g/m^2 over 24 hrs daily x 4 (x 3 for pts over 60) and idarubicin 12 mg/m^2 daily x 3 are administered with sorafenib. In the phase I portion of study, pts with relapsed AML were treated with escalating doses of sorafenib (400 mg qod, 400 mg daily and 400 mg bid) for 7 days during induction. Pts achieving CR receive up to 5 courses of consolidation with idarubicin 8 mg/m^2 daily x 2 and cytarabine 0.75 g/m^2 daily x 3 in addition to continuous sorafenib 400 mg bid for up to 28 days per cycle. Cycles are repeated every 4 to 6 weeks. Maintenance with sorafenib 400 mg bid would continue for up to a year after consolidation. *Results*. Ten pts (median age 34, range 21-58) with relapsed AML (median prior therapy 2, range 1-6) were treated on the phase I portion. Seven were FLT3-ITD positive and 4 achieved CR; sorafenib 400 mg bid was established as a safe dose in induction. In the phase II portion 48 pts (12 with FLT3-ITD and 2 with FLT3-TKD) have been treated. Median age is 53 (range 18 - 66). Cytogenetics were diploid in 20, +8 in 5, -5/-7 in 5, t(9;11) in 3, miscellaneous in 11, and unavailable in 4. The median presentation WBC was 5.2×10^9/L (range 0.6 - 122.7×10^9/L). FLT3 mutation burden was low in blasts from 4 pts, and high in 10. 7 pts were FLT3-ITD+/NPM1- and 1 was FLT3-D835+/NPM1-. 45 pts are evaluable for response (3 too early) and 38 (84%) have achieved CR (n=34), or CRp (n=4), including 14 of 14 FLT3 mutated patients. 3 died during induction from pneumonia, 4 were resistant. The regimen is well tolerated with grade 3 and higher adverse events possibly related to the addition of sorafenib during induction including hyperbilirubinemia (6), elevation of transaminases (3), diarrhea and colitis (4), rash (3), pancreatitis (1), pericarditis (1), elevated creatinine (1), and cardiac/hypertension (3). With a median follow-up of 28 weeks (range, 1 - 59 weeks), the probability of survival at 6 months is 85%; 9 pts have relapsed with a median CR duration of 8 months (range, 0.5+ - 12+ months). Among the patients with FLT3 mutation, 6 have relapsed and 8 remain in CR. Peripheral blood samples from 8 pts were evaluated prior to and 24-48 hrs post sorafenib administration, and prior to chemotherapy. In six pts (75%), sorafenib alone induced apoptosis (determined by flow cytometry) in blasts and in CD33/CD34 positive leukemia progenitor cells. Expression of phospho-ERK (pERK) was detectable by flow cytometry in 5 of 7 samples tested at baseline; 24-hour exposure to sorafenib resulted in >50% downregulation of pERK in 3 of the 5 samples. Plasma inhibitory assay was performed using day 7 samples from 10 pts; mutant FLT3 was suppressed by all samples with 5-fold more potent suppression against mutant versus wild-type FLT3. *Summary*. Sorafenib can be safely combined with idarubicin and cytarabine; it has a high CR rate particularly in patients with FLT3 mutations. Correlative studies confirm potent activity of sorafenib against ERK and FLT3 signaling.

0832

IMPROVED OUTCOME BY ADDITION OF LOMUSTINE (CCNU) TO IDARUBICIN AND CYTARABINE IN ELDERLY PATIENTS WITH *DE NOVO* ACUTE MYELOID LEUKEMIA. A REPORT FROM THE GOELAMS GROUP

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In elderly patients with acute myeloid leukemia (AML) treated intensively, no improvement has been shown in the last 20 years. We performed a retrospective study in 847 patients over 60 years old, prospectively enrolled in 3 trials conducted in France between 1995 and 2005, with the aim to investigate prognostic factors for complete remission (CR) achievement and survival. Induction therapy consisted in the association of Idarubicin 8 mg/m^2 d1-5 and Cytarabine 100 mg/m^2 d1-7 (Group I, 339 patients) or the same drugs with the addition of lomustine (10mg\m^2 orally at day 1)(Group II, 508 patients). Consolidation therapy consisted of anthracycline and cytarabine courses at lower doses, preceded or not by a first course with intermediate dose cytarabine. The patients' characteristics were similar between the two groups concerning sex, WBC count, ECOG, and cytogenetics, yet patients were older in Group II versus Group I (55% versus 45% over 69 years of age, p<0.0001).The CR rate was significantly higher for patients in Group II compared to Group I (67% vs. 57%, p=0.002). The toxic death rate was not different between groups. In multivariate analysis, three good prognostic factors emerged for achieving complete remission: good or intermediate cytogenetics (p<0.0001), ECOG < 2 (p<0.0001), and adjunction of lomustine to induction chemotherapy (p=0.002). The median overall-survival was significantly improved for patients treated with lomustine (12.7±2.2 months vs 8.7±2.7 months, p=0.004). In multivariate analysis, five prognostic factors affected positively overall survival: adjunction of lomustine to induction chemotherapy (p<0.0001), age <69 years (p=0.001), ECOG <2 (p=0.001), FAB other than AML0,6 or 7 (p=0.004) and good or intermediate cytogenetics(p=0.007). The median event-free-survival was also improved for patients treated with lomustine (10.7± 2.2 months vs 7± 2.7 months, p=0.002). Event-free survival was affected by the same prognostic factors as overall survival. We conclude that lomustine might be added in standard induction therapy as it allowed to obtain both better CR rate and survival in this retrospective study.

0833

PROLONGED EXPOSURE TO LINTUZUMAB MONOTHERAPY IN ACUTE MYELOID LEUKEMIA AND MYELODYSPLASTIC SYNDROMES - RESULTS OF A PHASE 1 TRIAL

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Background. Lintuzumab (SGN-33, HuM195) is a humanized antibody that binds CD33, a sialoglycan protein expressed on the majority of myeloid malignancies and on normal myeloid and monocytic progenitors. *In vitro*, binding of CD33 by lintuzumab induces antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and antibody-dependent cellular phagocytosis. In prior clinical testing at lower dose levels, lintuzumab monotherapy elicited objective responses in patients with relapsed and refractory acute myeloid leukemia (AML) without dose-limiting toxicity. *Aims*. A phase 1, single-arm, dose-escalation trial was performed to assess the safety, immunogenicity, pharmacokinetics, and antileukemic activity of lintuzumab at higher doses and increased dosing frequency than previously studied. *Methods*. Cohorts of

3-6 patients with advanced myeloid malignancies and CD33 expression on >50% of marrow blasts received intravenous lintuzumab as outpatients at weekly doses of 1.5 to 8 mg/kg for 5 weeks (Cycle 1); the 8 mg/kg dose was used to treat a planned expansion cohort of 50 patients with AML or myelodysplastic syndrome (MDS). Outpatient infusions were continued every other week for patients who demonstrated clinical benefit. Informed consent was obtained prior to initiating therapy. *Results*. A total of 82 patients [AML (59), MDS (19), and chronic monomyelocytic leukemia (4)] with a median age of 74 years (range 50-89) were treated at 9 study centers. Dosing cohorts included 1.5 (6), 2.5 (4), 4 (4), and 8 mg/kg (68). Significant toxicity was not observed and a maximum tolerated dose was not established. The most common adverse events (AEs) were chills (37%), fatigue (35%), pyrexia (30%), and nausea (27%). Serious AEs related to lintuzumab in more than 1 patient were febrile neutropenia (3 patients) and rectal hemorrhage (2 patients). No anti-therapeutic antibodies were detected in any patient. Lintuzumab accumulation occurred during weekly dosing and Cmin/Cmax values increased with dose level. Among 59 patients with AML, 4 achieved complete remissions (CRs), 3 had partial remissions, and 2 were classified in morphologic leukemia-free state. Five of these 9 objective responses were at the 8 mg/kg dose level. In addition, 7 patients with AML who did not achieve objective response at the 8 mg/kg dose level had ≥50% reduction of marrow blasts. Twelve patients with AML (20%) received lintuzumab treatment for over 16 weeks. One of these patients achieved and maintained a CR while receiving lintuzumab treatment for over 1 year. In patients with MDS, 14 of 19 had a best response of stable disease; of these, 1 patient at the 8 mg/kg dose level had major hematologic improvement. Five of 19 patients with MDS achieved marrow blast percentage reductions ≥50%. Overall, 42 patients (51%) received more than 1 cycle of therapy. *Summary and Conclusions*. Lintuzumab was reasonably well tolerated at doses up to 8 mg/kg/week, achieving serum lintuzumab exposures approximately 20 times higher than in prior studies. There was evidence of antitumor activity in both AML and MDS patients. Further studies of lintuzumab in combination with low-intensity chemotherapy in AML and MDS are ongoing.

0834

AS1411 IN COMBINATION WITH CYTARABINE IN RELAPSED AND REFRACTORY ACUTE MYELOID LEUKEMIA

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Background. AS1411 is the first nucleic acid aptamer to be tested in oncology clinical trials. It is an aptamer that binds to nucleolin, which is upregulated in the cytoplasm and on the surface of cancer cells. AS1411 exerts its cytostatic, followed by cytotoxic, action on the cell upon translocation into the cell by nucleolin, a multi-faceted protein that can interact with DNA, RNA and proteins and ultimately promotes cell proliferation. AS1411 has been shown to kill a wide variety of cancer cell lines. Acute myeloid leukemia (AML) cell lines KG-1 and MV4-11 have an IC50 of approximately 2 μM in the presence of AS1411. MV4-11 cells have been shown to upregulate nucleolin on the cell surface, and also to localise AS1411-FITC to the nucleus. The sensitivity of AML cells to AS1411 has also been seen in *ex vivo* patient cell blasts treated with AS1411. When AS1411 is combined with cytarabine, a standard treatment for AML, a synergistic effect is produced against MV4-11 cells. A synergistic effect has also been seen *in vivo* with AS1411 in combination with cytarabine in an MV4-11 subcutaneous xenograft model. AS1411 has been studied in a dose-escalating phase I study in solid tumours, where objective responses were seen and the safety profile was good. *Methods*. This randomized, multi-centre phase II trial compares AS1411 plus high-dose cytarabine (HiDAC) to HiDAC alone as treatment for relapsed or refractory AML. Patients in cohort I were randomized 2:1 to receive AS1411 10 mg/kg/day as continuous IV on days 1-7 + HiDAC 1.5 g/m^2/ twice daily on days 4-7 or HiDAC alone for 4 days. Following safety assessment, a second cohort was randomized to receive AS1411 40 mg/kg/day + HiDAC or HiDAC alone. All patients randomised to receive HiDAC alone who did not achieve remission could cross-over to receive HiDAC + AS1411 at the dose at which they were randomised. Study objectives were comparison of response rates (CR+CRp), safety and tolerability between treatment arms. *Results*. Accrual to the study has been completed with 71 patients randomized: 22 to AS1411 10 mg/kg/day + HiDAC (AS1411-10), 26 to AS1411 40 mg/kg/day + HiDAC (AS1411-40) and 23 to HiDAC alone (control). Safety findings are currently available for 44 patients (AS1411-10, 21; AS1411-40, 9; control, 14). The main grade 3 and 4 toxicities were hematological, notably febrile neutropaenia, neutropaenia and thrombocytopaenia; and infections. Safety findings were similar across groups, except that grade 3 hypokalaemia was more frequent with AS1411-40. Deaths within 28 days of treatment were: AS1411-10, 1/21; AS1411-40, 1/9; and control, 2/14. Response data are currently available for 39 patients; response rates (CR+CRp) were: AS1411-10, 16% (3/19); AS1411-40, 14% (1/7); and control, 0% (0/13). Full response data for the study will be presented. *Conclusions*. These data provide preliminary evidence suggesting that AS1411 in combination with HiDAC has antileukaemic activity and an acceptable safety profile in patients with relapsed and refractory AML.

0835

CLASSIC II: UPDATED REMISSION DURATION AND SURVIVAL RESULTS OF SINGLE AGENT CLOFARABINE IN PREVIOUSLY UNTREATED OLDER ADULT PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AND AT LEAST ONE UNFAVORABLE BASELINE PROGNOSTIC FACTOR

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Background. The CLASSIC II trial has previously reported an independently confirmed overall remission rate of 45.5% (37.5% CR and 8.0% CRp) and 30- and 60-day mortality rates of 9.8% and 16.1%, respectively (Blood 112: 558, 2008). We now report updated duration of remission (DOR), disease-free survival (DFS), and overall survival (OS). *Methods*. Single-arm, multi-center, Phase II, open-label, 2-stage study of patients with untreated acute myeloid leukemia (AML), ≥60 years old, and at least one unfavorable prognostic factor: age ≥70 years, antecedent hematologic disorder (AHD), PS=2, and/or intermediate/unfavorable risk myeloblast karyotype. Clofarabine (CLO) administered days 1-5 at 30 mg/m^2 during induction and 20 mg/m^2 during re-induction/consolidation for maximum 6 cycles. *Results*. 116 patients enrolled, 112 in full analysis set. Median age 71 years. Median duration of follow-up from first dose for all patients was 36 weeks (range, 1-85). Median DOR (censored at alternative therapy) for CR/CRp was 56 weeks (95% CI, 33 weeks - not yet estimable [n/e]). Median DFS (not censored at alternative therapy) for CR/CRp was 37 weeks (95% CI, 26-56 weeks). Median OS for all patients was 41 weeks (95% CI 28 - 53 weeks), for CR/CRp 59 weeks (95% CI, 50 weeks - n/e), and for CR was 72 weeks (95% CI, 53 weeks - n/e) Thirty-day mortality was 9.8% for all patients with 4.7% and 13.0% for age <70 and age ≥70 years, respectively. *Conclusions*. These data expand on the previously reported efficacy and safety data of single agent CLO in adult AML. Complete remissions appear durable, and DFS and OS compare favorably to historical experience with other regimens, particularly in patients with these unfavorable prognostic factors. These results suggest that single agent CLO is an effective option for older adult patients with untreated AML and one or more unfavorable baseline prognostic factor(s). Patients continue in long-term follow-up, with 42 alive at the most recent data cut-off (Nov. 2008).

0836

PROGNOSIS OF PATIENTS WITH AML CARRYING 11Q23/MLL RECIPROCAL TRANSLOCATIONS: A RETROSPECTIVE STUDY OF 191 CASES FROM THE FRENCH AML-INTERGROUP

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Background. Acute myeloid leukemias (AMLs) carrying 11q23/MLL reciprocal translocations are usually associated with a bad prognosis. However, this AML subtype constitutes a heterogeneous group and the major factor influencing prognosis seems to be the MLL partner involved in the translocations. *Aims*. To determine the impact of AML with 11q23/MLL abnormalities according to the partner chromosome, to investigate another prognosis factors for outcome and compare the results of allogeneic SCT with chemotherapy alone. *Methods*. We thus performed a large retrospective study in 191 patients, including 102 adult and 89 children, prospectively enrolled in 11 different trials conducted in France between 1987 and 2002 (BGMT 87, BGMT 91, BGMT 95, LAM 2001, LAM-SA-2002, Alfa 9801, Alfa 9802, Alfa 9000, Goelam02, LAME89-91, LAME2000). Patients with MLL were not included. All patients received induction treatment with cytarabine and an anthracycline followed by an intensive consolidation with either high-dose cytarabine (HDAC) based chemotherapy or autologous or allogeneic stem cell transplantation. *Results*. In adult population: Median age was 37,7 years. The majority of patients (56%) had AML-M5. 38% carried t(9;11), 20% carried t(6;11), 20% carried t(11;19), 7% carried t(10;11) and 15% carried other reciprocal translocations. Overall CR rate was 86%. At 5-year, DFS and OS were 20% and 25%. Only patients with t(11;19) indicated a relatively favorable outcome with a 5-year DFS and OS of 42% and 50%. This outcome was significantly better than for patients with any other translocation (p=0.001). In patients with t(9;11) or other various translocation, 5-year DFS and OS were 20% and 25%. In patients with t(6; 11), 5-year DFS and OS were 0%. Results in pediatric population: Median age was 1,8 years. The majority of children (56%) had AML-M5. 58% carried t(9;11), 15% carried t(10;11), 9% carried t(11;19), 7% carried t(1;11) and 11% carried other translocations including t(11; 17), t(4; 11) or t(6; 11). Overall CR rate was 89%. At 5 years, estimated DFS and OS were 60% and 65%. In children with a t(9;11) translocation, 5-year DFS and OS were 72% and 78%. This outcome was significantly better than for patients with any other translocation (p=0.013). In children with t(10;11) or t(11;19), 5-year DFS and OS were 47% and 50%. In those with other various translocations, 5-year DFS and OS were 18% and 13%. In adults and children, the prognosis was not modified by a presentation with complex karyotype or by the type of consolidation received (HDAC, autologous or allogeneic transplantation). *Conclusions*. 1) AML with 11q23/MLL constitutes a heterogeneous group and the major factor influencing prognosis is the MLL partner involved in the translocation. For adults, we showed a better outcome with t(11;19) and this translocation might be considered as intermediate cytogenetic risk. For children the good prognosis of t(9;11) is confirmed. 2) In adults and children, prognosis of these translocations is no modified by complex karyotype or by the type of post-remission treatment received. 3) The t(9;11) translocation is the most common recurrent one but with a very different prognosis in adults as compared to children.

0837

IMPROVED OUTCOME WITH ANDROGENS AS MAINTENANCE TREATMENT IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA AFTER ICL REGIMEN AS INDUCTION THERAPY: RESULTS OF THE GOELAMS SA-2002 MULTICENTER PHASE III RANDOMIZED OPEN TRIAL

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The French group, GOELAMS, performed a multicenter phase III randomized open trial to evaluate whether adding androgens to post-remission induction therapy was associated with an improved outcome of elderly patients with *de novo* AML. All patients received the ICL induction protocol (idarubicin 8mg/m^2 day1-5, cytarabine 100mg/m^2 day1-7 & lomustine 200 mg/m^2 day1). Patients in complete or partial remission received maintenance therapy consisting in 6 reinduction courses (idarubicin 8 mg/m^2 day1, cytarabine 100 mg/m^2 day1-5.), once every 3 months, with a continuous regimen of methotrexate and 6-mercaptopurine in-between these. At diagnosis, patients were randomized to receive (AndroG arm) or not (Control arm) norethandrolone 10 to 20 mg per day, according to body weight. Norethandrolone was started after recovery from aplasia, between day-20 and day-30 following induction chemotherapy, and was to be continued during the 2-year maintenance therapy period if a complete or partial remission was achieved following induction chemotherapy. The two randomized arms were well-balanced for known prognostic factors such as cytogenetics. Between June 2002 and January 2005, 330 patients with *de novo* AML and age ≥60 y at diagnosis (median 70, range 60-86) were included. For pts alive at last news, median follow-up from induction chemotherapy start was 3.6 and 1.1 y for the overall group. Complete remission incidence, by day-40 and day-80, was 56% vs 52%, and 83% vs 79% in the Control arm and the AndroG arm, respectively (p=0.52, Gray test). Fine & Gray competing-risk model was used to estimate relapse incidence and the effect of norethandrolone. As for the previous endpoint, the potential benefit of norethandrolone on 5-year leukemia-free survival (LFS), event-free survival (EFS), and overall survival (OS) was analyzed on an intention-to-treat basis, using univariate and multivariate adjusted models. S-Plus2000Pro software was used for all analyses.

Tables 1-2.

Table 1	Full time period from diagnosis				
Endpoints	Control	AndroG	Gray test p-val	HR; 95%CI	Cox p-val
Relapse	66%	54%	0.21	--	--
LFS	23%	33%	--	0.79; 0.58-1.09	0.15
EFS	16%	22%	--	0.95; 0.75-1.22	0.69
OS	19%	26%	--	0.96; 0.74-1.23	0.72

Table 2	Time period starting 1 year from diagnosis				
Endpoints	Contro l	Andro G	Gray test p-val	HR; 95%CI	Cox p-val
Relapse	55%	33%	< 0.01	--	--
LFS	37%	54%	--	0.56; 0.33-0.94	0.028
EFS	32%	52%	--	0.57; 0.36-0.89	0.013
OS	37%	60%	--	0.63; 0.41-0.96	0.034

HR, hazard ratio; 95%CI, 95% confidence interval; Cox p-val, p-value of Cox regression model

Results. Crude univariate analyses regarding the effect of androgen randomization on 5-year relapse incidence, LFS, EFS and OS are listed in Table 1. Of note, the effect of norethandrolone on relapse incidence, DFS, EFS, and OS, was not verifying the proportional hazards assumption (analysis of scaled Schoenfeld residuals). Since the 2 hazards rate functions crossed each other, which would result in ineffective comparisons if using log-rank tests, time-dependent models were considered. For all these endpoints, as estimates for the 2 arms were not significantly different during the first year, but diverged during the following years, favoring the AndroG arm (Table 2), a step-function at 1 year from diagnosis was considered. Thus, for patients alive and in complete remission at 1 year from

diagnosis, relapse incidence was lower, while LFS, EFS and OS were higher in the AndroG arm, even after adjusting for other significant covariates (p=0.029 for Relapse; p=0.038 for LFS; p=0.028 for EFS; p=0.029 for OS). *Conclusions*. the addition of low-dose norethandrolone to maintenance chemotherapy is associated with an improved outcome in elderly patients with AML; especially in patients who achieve and remain in remission for at least a year.

0838

THE COMBINATION OF FLOW-CYTOMETRY MINIMAL RESIDUAL DISEASE ASSESSMENT AND CYTOGENETIC/GENETIC PARAMETERS ALLOWS A PROPER RISK STRATIFICATION OF ADULT AML PATIENTS: IMPLICATION FOR TRANSPLANT CHOICE AND POST CONSOLIDATION THERAPY

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Background. prognostic stratification of patients with acute myeloid leukemia (AML) mostly relies on the cytogenetic/genetic risk profile assessed at diagnosis. We have shown by flow cytometry, that minimal residual disease (MRD) negativity (bone marrow residual leukemic cells $\leq 3.5 \times 10^{-4}$) at the end of consolidation therapy was independently associated with a significantly longer relapse free survival (RFS) and overall survival (OS). The aim of the study was to develop a comprehensive risk stratification of AML, integrating information derived from the initial cytogenetic/genetic assessment with those inherent the status of MRD. *Methods*. 158 patients affected with non-M3 AML were entered into the EORTC/GIMEMA protocols AML10/AML12 (age <61yrs) or AML13/AML15 (age >61 yrs); cytogenetics categories were defined according to MRC classification. *Results*. at the end of consolidation, 48 and 110 patients were MRD negative and MRD positive, respectively. Those MRD negative had a significantly longer OS and RFS (p<0.001 for both); multivariate analysis confirmed that post-consolidation MRD status was an independent factor affecting OS and RFS (p<0.001 for both). The combined analysis of MRD, FLT3-ITD and karyotype suggested that post-consolidation MRD status allows good and intermediate-risk karyotype categories to be further stratified in subgroups with remarkable differences in terms of OS (p=0.014 and 0.018, respectively) and RFS (p=0.02 and <0.001, respectively). Accordingly, we generated an integrated approach of AML risk assessment which allowed two populations of patients to be recognized: 1) high-risk: with FLT3-ITD mutations, unfavorable-risk karyotype or favorable/intermediate karyotype MRD positive at the post consolidation time-point. For this subgroup the clinical outcome is very poor with long-term OS and RFS below 20%. 2) low-risk: MRD negative at the post consolidation time-point, with no FLT3-ITD mutations and/or unfavorable karyotype. This category has a favorable outcome, with a long term RFS higher than 70%. *Conclusions*. Based on these observations, we believe that allogeneic transplant is recommended, not only for poor-risk karyotype or FLT3 positive AML, but also for good, intermediate and FLT3 unmutated categories not gaining MRD negativity, being this option able to provide a superior chance of prolonged RFS. On the other hand, patients who can experience a long term survival approaching 70-80%, such as those belonging to MRD negative good, intermediate and FLT3 unmutated categories, may have their life expectancy hampered by the choice of a therapeutic strategy with a disadvantageous risk/benefit ratio. Thus, the combined evaluation of baseline prognosticators (gene mutation, cytogenetics) and parameters inherent the quality of response (MRD), is useful to refine risk assessment of AML and to facilitate the decision making process in the direction of tailored options which take into account the actual clinical risk of the patients.

0839

PROGNOSTIC AND PREDICTIVE FACTORS FOR SURVIVAL AFTER COMPLETE REMISSION IN INDUCTION THERAPY OF ACUTE MYELOID LEUKAEMIA

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Background. In the AML 96 study of the German Study Initiative Leukaemia (DSIL), 622 patients with *de novo* or secondary acute myeloid leukaemia (AML) achieved complete remission (CR) after two cycles of induction therapy consisting of mitoxantrone, cytarabine, and etoposide in the first course and cytarabine and amsacrine in the second. *Aims*. Apart from the 36 patients with karyotype t(8;21), 586 patients were classified as patients with intermediate or poor cytogenetic risk for treatment success. For these 586 non-favourable-risk patients with CR, we sought to identify parameters evaluated at diagnosis which reveal prognostic information with respect to overall survival. Furthermore, the predictive value of these parameters for survival with specific post-remission therapies comprising allogeneic haematopoietic stem cell transplantation, chemotherapy with cytarabine and mitoxantrone, or autologous stem cell transplantation (autologous SCT) was investigated. *Methods*. Since survival probabilities depend on the kind of post-remission therapy, the prognostic influence of the candidate variables was evaluated by a stratified Cox regression model. Stratification by therapy allowed elimination of the correlation between treatment and survival. In order to estimate the predictive value for a particular therapy, within the sample of patients who were treated accordingly, ordinary Cox regression was applied. Candidate variables were diagnosis (*de novo*, post-myelodysplastic syndrome, or treatment-induced AML), age at diagnosis, sex, cytogenetic risk (intermediate or poor), white blood cell count, platelet count, FLT3-ITD mutant-to-wild-type ratio, NPM1 mutation status, and the percentages of CD34 expression, POX-positive blasts, and blasts in bone marrow at day 15 after start of induction therapy. *Results*. It proved to be appropriate to define an unfavourable-risk group by either poor cytogenetic risk or treatment-induced AML. All other patients formed the intermediate-risk group. The final multiple model comprised the variables age, risk group, platelet count, FLT3-ITD mutant-to-wild-type ratio, and the percentages of CD34 expression and of POX-positive blasts (all p<0.005). Of 449 patients with data to the six variables in the final model, 229 (51%) had died. As post-remission therapy, chemotherapy was allocated to 160 patients (36% of 449, 102 died). Here, age lost its significance (with α=0.05) whereas the five other variables remained to define the best predictive model. In the 155 patients (35%, 68 died) with autologous SCT, all six variables constituted the best model, again. When allocated to transplantation with a related donor (106 patients, 24%, 44 died), platelet count had to be removed to leave the remaining variables with significant information on survival. Patients transplanted with an unrelated donor (n=28, 6%, 15 died) were too few to estimate an own model. Conclusions: Stratified for post-remission therapy, six variables were identified as independent prognostic factors for survival after CR in induction therapy. Apart from age for chemotherapy-treated patients and platelet count for matched-related donor transplantation, all variables remained significant in the predictive models whereas none of the parameters not part of the common prognostic model reached significance in any predictive model. Further data will be collected and treatment-depended risk groups will be defined. The final results should boost risk-adapted therapy in AML.

0840

AMONAFIDE (AS1413) IS UNAFFECTED BY MULTIDRUG RESISTANCE AND IS ASSOCIATED WITH A HIGH RESPONSE RATE IN PATIENTS WITH SECONDARY AML

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Background. Secondary AML (sAML) is commonly associated with the multi-drug resistant (MDR) phenotype through overexpression of P-glycoprotein (Pgp) and other efflux transporters. A review of 80 studies including over 4500 patients with AML has identified a 1.7-fold reduction in complete remission (CR) rate associated with MDR+, as compared to MDR-, AML blast cells (Ajami *et al.*, in preparation). Amonafide is a DNA intercalator that, unlike anthracyclines such as daunorubicin, is unaffected by MDR. We evaluated amonafide plus cytarabine in a phase II trial of patients with secondary AML (sAML), and sought to assess whether the response to amonafide could be related to the ability of the drug to evade MDR. Leukemic blasts were obtained from 15 patients in this trial and analyzed for Pgp function and for efflux of daunorubicin and amonafide. *Methods*. Patients received amonafide 600 mg/m^2/day over 4 hours days 1-5 with cytarabine 200 mg/m^2/day IV continuous infusion days 1-7. Fourteen patients received a second course on day 14 for persistent leukemia. Post-remission therapy with either transplant or intermediate-/high- dose cytarabine was given, depending on age. Central bone marrow review was employed. The primary end-

point was CR + CRi. Secondary endpoints included duration of CR/CRi, survival and safety. AML blasts from 15 patients in this trial were retrospectively assessed for Pgp expression and function as well as amonafide and daunorubicin uptake and retention in the presence and absence of the Pgp inhibitor cyclosporine A. Pgp-mediated efflux was assessed by comparing uptake of the Pgp substrate DiOC2(3) in the presence and absence of the Pgp inhibitor PSC-833. Pgp-mediated transport (efflux) was calculated as differential uptake and retention of the drug (either amonafide or daunorubicin) in the absence or presence of a Pgp inhibitor, normalized to apparent influx, reported as the mean±s.e.m. *Results*. 88 patients were treated with amonafide + cytarabine: 47% were male; the median age was 62.5 years (range 23-87); the median performance status was 1; 48% had unfavorable cytogenetics. The overall CR+CRi rate was 42% (CRi: 3%); among the 42 patients with unfavorable cytogenetics it was 23.8%. AML blasts from the group of 15 sampled patients showed significantly less efflux of amonafide (5.32±3.2%) than of daunorubicin (16±2.1%; p=0.0083). Blasts from patients belonging to poor-risk subsets of sAML also showed significantly less efflux of amonafide than of daunorubicin (e.g. unfavorable cytogenetics, n=12; 0.13 ±3.7% vs 16±2.1%; p=0.015). *Conclusions*. The lack of Pgp-mediated efflux of amonafide, as compared to daunorubicin, from sAML blasts may provide a rationale for the efficacy observed with amonafide in the phase II trial described here. Further prospective assessment of these MDR parameters is underway in a randomized phase III clinical trial (ACCEDE), which compares amonafide + cytarabine with daunorubicin + cytarabine (7+3) for remission induction in patients with sAML.

0841

PRE- AND POST-TRANSPLANT FACTORS PREDICT LONG TERM SURVIVAL AFTER REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKAEMIA

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Background. Allogeneic stem cell transplantation utilising a reduced intensity conditioning (RIC) regimen represents an important new therapeutic modality in older patients with acute myeloid leukaemia (AML). Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after RIC allografts and T cell depletion is increasingly used. The factors determining outcome after a T cell depleted RIC allograft in AML have not been determined. *Aims*. We wished to identify pre and post transplant factors determining survival in patients with AML transplanted using a T cell depleted reduced intensity regimen. *Methods*. The outcome of 168 patients with AML transplanted using an alemtuzumab based RIC regimen was analyzed. The median age was 54 years (range 18-71 yrs). 76 patients were transplanted using an HLA identical sibling and 92 using a volunteer unrelated donor. 150 patients were in remission at the time of transplantation. 40 patients had adverse risk cytogenetics. The median duration of follow-up was 37 months. All patients received intravenous cyclosporine A (CsA) at a dose of 5 mg/kg on day-1. Patients were switched to oral CsA prior to discharge. Trough CsA levels were measured thrice weekly for the first three weeks post-transplant and the dose of CsA adjusted to achieve levels in the region of 200-300 microg/l during this period. Trough levels obtained during the first 21 days post-transplant were used to calculate CsA exposure for individual patients during this period using a previously determined regression equation. *Results*. The 100 day transplant related mortality was 10%. 7% of patients developed Grade III-IV acute GVHD and 21% chronic GVHD. The 3 year overall survival was 44%. 29% of patients relapsed. Multivariate analysis demonstrated that survival was influenced by status at transplant (p=0.008) and presentation cytogenetics (p=0.01). Patient age was not of prognostic significance and there was no difference in outcome between recipients of sibling or unrelated grafts. Increased post-transplant exposure to cyclosporin A (CsA) was associated with an increased relapse risk (p<0.0001) and decreased overall survival (p<0.0001) in univariate and multivariate analysis. *Conclusions*. Disease stage and presentation cytogenetics are important determinants of outcome after a T depleted RIC allograft for AML. A potent GVL effect is demonstrable even in this setting and post-transplant exposure to CsA represents an important, and manipulable, determinant of relapse and survival.

0842

MLN8237, A NOVEL ORALLY ACTIVE AURORA A KINASE INHIBITOR, IS HIGHLY ACTIVE ALONE AND IN COMBINATION WITH CYTARABINE IN PRECLINICAL MODELS OF ACUTE MYELOID LEUKEMIA

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Background. Acute Myeloid Leukemia (AML) most frequently affects the elderly, many of whom are unable to tolerate intensive chemotherapy. Therefore, improvements in clinical outcomes for patients with AML depend upon the development of novel targeted therapies. Aurora A is a serine/ threonine kinase that plays a key role in mitosis by regulating the G2-M transition, centrosome separation, spindle assembly and chromosome segregation. It is over-expressed in AML and has been implicated in genetic instability, disease progression and drug resistance. MLN8237 is a novel orally available Aurora A inhibitor that has entered Phase I and II trials in solid tumors and lymphoma. Based on the critical role of Aurora A in cell cycle regulation and its intrinsic overexpression in AML cells, we hypothesized that MLN8237 possesses significant antileukemic activity. *Aims*. To investigate the efficacy and mechanism of action of MLN8237 and to determine whether MLN8237 increases the efficacy of the standard of care agent, cytarabine in preclinical models of AML. *Methods*. We used MOLM13, MV411, HL60 and Kasumi1 AML cell lines and primary AML patient specimens to evaluate the preclinical activity of MLN8237. Induction of apoptosis and cell viability were assessed using PI/FACS and MTS assays. The prolonged *in vitro* effects of MLN8237 were assessed by MethoCult colony formation assays. Effects of MLN8237 on Aurora A activity and caspase activation were measured by western blot analysis. An *in vivo* model of AML was generated by injecting Molm13 cells into the flanks of immunodeficient mice. *Results*. Nanomolar concentrations of MLN8237 potently inhibited the *in vitro* growth and survival of all AML cell lines. MLN8237 reduced Aurora A kinase activity as evidenced by reduced phosphorylation of Aurora A at Thr288. MLN8237 treatment disrupted cell cycle kinetics and induced apoptosis in a dose- and time-dependant manner characterized by the accumulation of G2/M and aneuploid cells prior to the onset of apoptosis. MLN8237 effectively inhibited survival of primary human AML cells including those from patients with resistant disease at concentrations consistent with those achieved in humans following oral administration of the drug. We next investigated the ability of MLN8237 to increase the efficacy of cytarabine in AML. Treatment with the combination of MLN8237 and cytarabine resulted in significantly greater apoptosis and more effective inhibition of clonogenic survival than treatment with either agent alone. MLN8237 and cytarabine cooperated to enhance mitochondrial mediated apoptosis as evidenced by increased processing of caspases-9 and -3 to active forms. Daily oral administration of MLN8237 to immunodeficient mice bearing AML xenografts was well tolerated, effectively reduced tumor growth and significantly enhanced the activity of cytarabine (Figure 1).

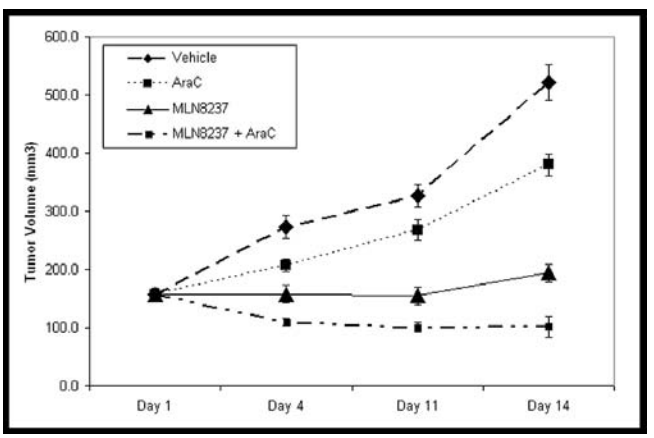

Figure 1. MLN8237 is active *in vivo* in AML.

Conclusions. Our data demonstrates that MLN8237 is highly active against AML cell lines, animal models and primary patient cells and has a multifaceted mechanism of action comprised of pro-apoptotic and growth inhibitory effects. Moreover, MLN8237 significantly potentiat-

ed the efficacy of cytarabine leading to tumor regression in animal models of AML. The combination of MLN8237 and cytarabine represents a novel and very promising therapeutic strategy for AML and clinical evaluation of this combination is warranted.

0843

IMPACT OF MYELODYSPLASIA-RELATED CHANGES AS DEFINED BY THE 2008 WHO CLASSIFICATION SYSTEM IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML) TREATED INTENSIVELY

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Background. Increased incidence of AML with myelodysplasia features with age is one factor often invoked to explain the poor outcome of elderly patients with AML. In two recent large studies, the presence of multi-lineage dysplasia at diagnosis has, however, not been found to be associated by itself with a worse outcome when unfavorable chromosome abnormalities commonly found in this age group were taken into account. In 2008, the revised WHO (WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues 4th edition, SH Swerdlow *et al*. IARC, Lyon 2008) has expanded this AML category into "AML with myelodysplasia-related changes" (AML-MRC) now including: 1) AML with multi-lineage dysplasia; 2) AML arising from prior myelodysplastic syndrome (MDS); or 3) AML with so-called MDS-related cytogenetic changes that include, but not exclusively, the most frequent standard unfavorable abnormalities. An individual case may fall into this category by meeting any of these criteria. *Methods*. We studied the prognostic value of this new AML-MRC category on complete remission (CR) rate, cumulative incidence of relapse (CIR), and overall survival (OS), but not considering the first multi-lineage dysplasia criterion. We compared it to prior categorization relying on standard cytogenetic subsets and presence of prior MDS in 391 elderly patients (median age, 71 years) prospectively included and intensively treated in the Acute Leukemia French Association (ALFA)-9803 trial (Gardin *et al.* Blood 2007). According to the British MRC classification, cytogenetics was favorable, intermediate, unfavorable, and failure in 13, 262, 61, and 55 of these patients, respectively. *Results*. Overall, 124 patients (32%) had AML-MRC, including 61 patients with post-MDS AML and 81 patients with WHO MDS-related chromosome abnormalities (18 patients had both criteria; p=0.08). Among these 81 patients, 59 and 22 had MRC unfavorable and intermediate anomalies, respectively (p<0.001). Patients with AML-MRC had more resistant disease (CR rate, 48 vs 61%, p=0.01; 2y CIR, 89 vs 67%, p=0.001) and shorter OS (2y OS, 21 vs 32%, p<0.001). Interestingly, the incidence of smoldering MDS relapses was significantly higher in the AML-MRC subgroup (2y cumulative incidence of MDS relapse, 32 vs 8%, p<0.001). Multivariate analyses revealed, however, that standard MRC unfavorable cytogenetics was a stronger predictive factor than AML-MRC for the three CR, CIR, and OS endpoints. Actually, when excluding patients with standard unfavorable karyotypes (-5/del(5q), -7, 3q abn, complex) from comparisons, AML-MRC was no longer predictive of lower CR rate (56 vs 62%, p=0.48), higher 2y CIR (85 vs 67%, p=0.13), or lower 2y OS (31 vs 32%, p=0.24). *Conclusions*. The newly defined WHO category of AML-MRC exhibits a worse clinical outcome when compared to non AML-MRC in this elderly population. The higher incidence of smoldering MDS relapses in patients with AML-MRC reinforced the interest of this new categorization. However, the predominant role of standard unfavorable cytogenetics pushes to still recommend to base treatment decision-making on this well-known simpler criterion. This also confirms that prior MDS by itself has no significant prognostic impact in this selected population of elderly AML patients included into an intensive chemotherapy program.

0844

FLUDARABINE-BASED INDUCTION THERAPY DOES NOT OVERCOME THE NEGATIVE EFFECT OF ABCG2 (BCRP) OVER-EXPRESSION IN ADULT ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Breast cancer resistance protein (BCRP), a recently identified multidrug resistance (MDR) protein of the ATP-binding cassette family, is associated with a worse prognosis in AML patients. On the other hand, fludarabine-based induction therapy displayed interesting results in AML, with high rates of complete remission (CR), and has been shown to overcome the negative effect of P-glycoprotein (PGP), another MDR-related protein, over-expression. No specific data are available about fludarabine effect on the outcome of AML patients with BCRP over-expression. *Aims*. We measured the levels of BCRP in 138 cases of AML, treated with a fludarabine-based induction therapy, to evaluate the possible effect of this regimen on CR rates and long-term survival according to BCRP expression. *Methods*. One hundred and thirty-eight patients with a diagnosis of *de novo* AML were included in our study. Cases with PML/RARα rearrangement were excluded. Median age was 56 years (range: 16-84), 21 patients had an unfavourable cytogenetics and 47 (37%) displayed a mutation of FLT-3 gene (33 ITD and 14 TKD). All patients were treated with an induction regimen containing fludarabine, cytarabine and idarubicin, and at least one consolidation course with high-dose cytarabine and idarubicin. BCRP expression was measured at diagnosis with flow cytometric analysis. *Results*. BCRP protein was over-expressed in 67/138 (48%) patients. A strong correlation was found between BCRP positivity and PGP (p<0.01) and MRP (p<0.001), and with immature (i.e. M0-M1) morphology, while no statistically significant differences were seen according to age, WBC count, karyotype, CD34 expression and FLT3 status between BCRP-positive and BCRP-negative cases. Ninety patients (65%) attained a CR after induction therapy. Advanced age, unfavourable cytogenetics and FLT3-ITD negatively affected remission rate. As expected, neither BCRP or other MDR-related protein over-expression was associated with CR obtainment. Relapse occurred in 30/90 patients (33%), with a higher relapse rate in BCRP+ cases (20/45) than in BCRP- patients (10/45, p=0.04). Moreover, relapse occurred earlier in the BCRP+ group (12 vs 24 months, p=0.01). As for disease-free survival, also overall survival was affected by BCRP status (RR 2.2, p=0.005), and a particular negative prognosis was found in the subset of patients with BCRP and PGP co-overexpression. *Conclusions*. BCRP over-expression did not influence the achievement of CR in fludarabine-treated AML patients, but significantly affected relapse rate, CR duration and overall survival. The inclusion of fludarabine in induction chemotherapy confirms its capacity to overcome the negative effect of MDR over-expression, but BCRP-positive patients have an unfavourable long-term prognosis, and could take advantage of an intensive post-remission therapy.

0845

SIX-YEAR OUTCOMES UPDATE FROM A RANDOMIZED PHASE 3 TRIAL IN AML: DURABLE EFFECT OF REMISSION MAINTENANCE IMMUNOTHERAPY WITH HISTAMINE DIHYDROCHLORIDE AND LOW-DOSE IL-2

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Background. A randomized multinational Phase 3 trial in adult AML patients (median age=56.5 years) in complete remission (CR; n=320) treated with histamine dihydrochloride (HDC) in conjunction with low-dose interleukin-2 (IL-2) met its primary endpoint, leukemia-free survival (LFS) at 3 years. A significant benefit on LFS over standard-of-care (controls; no treatment) (p=0.008 and 0.011) was observed in the overall and first remission (CR1) populations, respectively (Brune *et al.* Blood 2006; 108:88-96). Follow-up to determine long-term patient outcomes has been ongoing (Brune *et al*. Blood 2007;110:1846a) since the trial ended in 2004. *Aims*. To assess the durability of the treatment effect on the primary endpoint of LFS and on the secondary endpoint of overall survival (OS) in AML patients after treatment with HDC/IL-2 for up to 10 - 3-

week cycles over 18 months compared to controls. *Methods*. The randomized study of immunotherapy with HDC/IL-2, aimed at prolonging LFS, included 261 AML patients in CR1 and 59 in subsequent remission (CR>1). Study arms were balanced for all prognostic factors known at the time the study was conducted. HDC and IL-2 doses, both given sc BID, were 0.5 mg and 16,400 U/kg, respectively. In June 2008, 92 months after randomization of the last study patient, follow-up data captured whether patients were alive and still in CR, dates of relapse or death, and causes of death. Between-group differences were evaluated using log-rank tests of Kaplan-Meier estimates stratified by country and CR status. *Results*. Outcomes data were retrieved for 88% of 124 patients (median follow-up 7.4 years) who were alive at the original database lock. At 6 years, 26% of HDC/IL-2-treated patients remain leukemia-free vs. 21% of controls in all patients enrolled. For CR1 patients, 30% of HDC/IL-2-treated vs. 22% of controls remain leukemia-free. This enduring treatment benefit was statistically significant for both the overall and CR1 populations (hazard ratio [HR]=1.41; 95% CI: 1.08 to 1.82; p=0.011 and HR=1.43; 95% CI: 1.07 to 1.91; p=0.015, respectively) (Figure). The trial was not powered to detect a significant benefit in the secondary OS endpoint. However, a trend in OS favoring treatment was noted for both the overall and CR1 populations (HR=1.15; 95% CI: 0.87 to 1.52; p=0.33 and HR=1.18; 95% CI: 0.86 to 1.60; p=0.31, respectively). *Conclusions*. After a minimum of 6 years of follow-up, immunotherapy with HDC/IL-2 in AML patients provides enduring protection from leukemia relapse by maintaining a statistically significant benefit in LFS over no treatment.

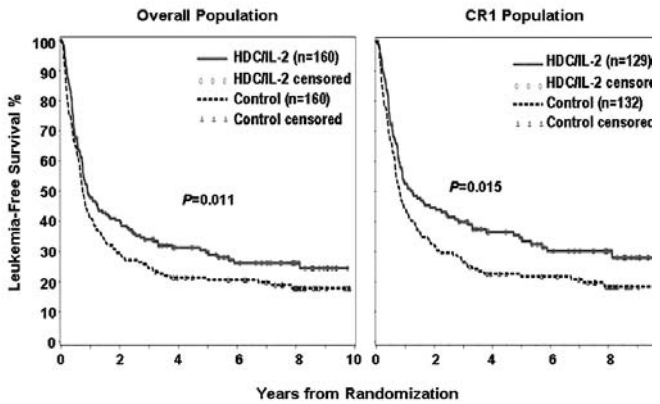

Figure. Long-term effect of HDC/IL-2 on LFS

0846

CXCR4 AS A PREDICTOR OF RESPONSE IN ACUTE MYELOID LEUKAEMIA

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Background. The expression of CXCR4 (CD184) has been associated with poor prognosis in Acute Myeloid Leukemia (AML) and it has also been suggested that the CXCL12(SDF-1a)/CXCR4 interaction contributes to the resistance of leukemia cells to chemotherapy-induced apoptosis. Inhibition of CXCR4 was found to enhance chemotherapy-induced apoptosis in a subset of leukemic myeloblasts that carry Flt3 mutations and to overcome chemoresistance associated with stromal activity. NPM variants with a cytoplasmic localization represent the most common mutation detected in myeloid malignancies and are associated with a favourable clinical outcome. A recent study provides biological evidence of a novel role for NPM as a negative regulator of CXCR4 signalling induced by CXCL12. Suppression of NPM expression enhanced chemotactic responses to CXCL12, and conversely, overexpression of a cytosolic NPM mutant reduced chemotaxis induced by CXCL12. *Aims*. We investigated whether CD184 expression could be a negative predictive factor for response to chemotherapy and if there is clinical evidence that NPM mutations could overcome chemoresistance to induction therapy in this subset of patients. *Patients and Methods*. The expression of CD184 was analyzed by flow cytometric methods in a group of 81 cases (42 males, 39 females; median age 56 years, range 15-75) of adult AML at onset of disease, diagnosed at our Institution since January 2006. The diagnosis was performed according to FAB/WHO criteria; all patients received intensive chemotherapy according to institutional protocols. AML cells were gated based upon their CD45 expression and samples were considered positive if CD184 was expressed by more than 20% of blasts. *Results*. CD184 was positive in 56 (69%) and negative in 25 (31%) cases. There was no significant difference between the two groups in terms of sex, age, Hb level, WBC and Plt counts, percentage of blasts, and occurrence of the NPM mutation. The CR rate was 44% in CD184$^+$ and 70% in CD184$^-$ (p=0.03); among CD184$^+$ cases, the CR rate was significantly lower in NPM unmutated cases, (p=0.002). *Summary*. Our results show that CD184 expression is associated with a lower rate of CR after induction therapy and this association is stronger in NPM unmutated cases, suggesting that CD184 expression is a negative predictive factor for response to chemotherapy. Further data are needed to verify if the biological role of the cytosolic NPM mutant as a negative regulator of CXCR4 signalling induced by CXCL12 could have a clinical role contributing to overcome the resistance of leukemic cells to induction chemotherapy.

0847

RASGRP1/APTX RATIO STRONGLY CORRELATES WITH CLINICAL RESPONSE AND SURVIVAL IN AML PATIENTS TREATED WITH TIPIFARNIB-BORTEZOMIB COMBINATION

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Background. Outcome of elderly acute myeloid leukemia (AML) patients is dismal. Targeted-therapies might improve current results by overcoming drug-resistance and reducing toxicity. In particular, the farnesyl-transferase inhibitor Tipifarnib (Zarnestra®), and the proteasome inhibitor Bortezomib (Velcade®), appeared synergistic in AML cells *ex vivo*, and their association was shown to be safe *in vivo* in a phase I trial by our group. *Aims*. We conduced a phase II study aiming to assess efficacy and toxicity of Tipifarnib-Bortezomib association in AML patients >18 years, unfit for conventional therapy, or >60 years, in relapse. Furthermore, we aimed to identify biological features potentially predictive of clinical response. In particular, we focused on the RASGRP1/APTX ratio, which was previously found to be effective in predicting treatment response in patients treated with Tipifarnib alone. *Methods*. Bortezomib (1.0 mg/m^2) was administered as weekly infusion for three consecutive weeks (days 1, 8, 15). Tipifarnib was administered at dose of 300-600 mg BID for 21 consecutive days. Response was assessed at the end of each cycle (28 days). Patients' withdrawn was planned in case of progression or stable disease after six cycles. Real-time quantitative-PCR (q-PCR) was used for RASGRP1/APTX quantification. *Results*. Seventy-two patients were enrolled. Median age was 70 years (42-84); 49% were secondary-AML. WBC at diagnosis was 3.05×10^9/L (0.3-40.3). Sixty-two patients actually initiated the treatment, 50 completed at least the first cycle while 12 early dropped out for non-leukemia related adverse event. Six patients achieved complete remission (CR) and 2 partial response (PR). Three patients obtained a hematological improvement (HI), and 3 patients died during marrow aplasia. Thirteen had progressive disease (PD) and the remaining showed stable disease (SD). The median time to response was 88 days, corresponding to 3 cycles. Marrow response (CR+PR) was significantly associated with overall survival (OS) (p<0.0001). RASGRP1/APTX was evaluated before treatment initiation on bone marrow (BM) and/or peripheral blood (PB). The median RASGRP/APTX value on BM was 15.3 (15-19.8) in responder patients and 2.2 (0.5-25.9) in non responders, respectively (p=0.00006). Its median value on PB was 31.6 (19.3-35.5) in responders and 6.4 (0.5-27.1) in non responders, respectively (p=0.00001). Interestingly, no marrow responses were recorded in patients with marrow RASGRP1/APTX ratio <15, while the response rate was 73% in patients with RASGRP1/APTX >15 (p<0.0001). Finally, RASGRP1/APTX levels significantly correlated with OS (p=0.005). Conversely, there was no correlation between WBC, age, FLT3 and NMP status, and response or overall survival. Toxicity was overall mild, the most common adverse event being febrile neutropenia. *Conclusions*. We conclude that the clinical efficacy of the combination

Tipifarnib-Bortezomib was similar to what reported for Tipifarnib alone. However, noteworthy, we could confirm that the RASGPR1/APTX BM or PB level is an effective predictor of response. Though higher RASGRP1/APTX is relatively rare (~10% of cases), Tipifarnib (±Bortezomib) may represent an important option in a subset of high risk/frail AML patients.

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0848

IMPACT OF MOLECULAR MARKERS AND CYTOGENETIC ABNORMALITIES ON LONG-TERM OVERALL SURVIVAL AND DISEASE FREE SURVIVAL IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS RECEIVING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATIONS

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We performed a retrospective analysis from our registry on first allogeneic hematopoietic stem cell transplantation (HSCT) for AML patients (pts). Principal objective: to analyze the impact of molecular markers on the long-term OS and DFS after first allo-HSCT. We found 364 pts, only 63 pts had available conserved cells at diagnosis. Among these 63 pts, there were 27 (43%) males and 36 (57%) females, median age of 41 years [18-64]. Concerning the karyotype, 6 pts were good prognostic, 24 intermediate and 32 poor. Four (6%) pts had Flt3mut, 19 (30%) FLT3 ITD+, 3 (5%) MLLmut, 10 (16%) Hoxa9 over-expression, 7 (11%) Evi1 over-expression, 15 (24%) NPMmut, 25 (40%) WT1 over-expression and 1 CEBPαmut(evaluated in 12 pts) (Table1).

Table 1. OS and DFS for different karyotype subgroups and molecular markers.

	OS	DFS
FLT3 ov-ex	50%(25-75)	50%(25-75)
FLT3 nomal	40%(33-47)	34%(32-46)
FLT3 ITD+	27%(16-38)	25%(15-35)
FLT3 ITD -	46%(38-54)	47%(41-55)
Hoxa9 ov-ex	50%(35-65)	50%(35-65)
Hoxa9 nomal	38%(31-45)	38%(31-45)
EVI1 ov-ex	28%(11-45)	28%(11-45)
EVI1 normal	43%(36-50)	42%(35-49)
NPM1mut+	32%(20-44)	33%(21-45)
NPM1mut-	43%(35-55)	42%(35-49)
WT1 ov-ex	37%(22-47)	34%(25-43)
WT1 normal	43%(34-52)	44%(35-53)
Fav. Prognosis	83%(67-98)	83%(67-98)
Int. Prognosis	50%(39-61)	52%(42-62)
Poor. Prognosis	24%(16-32)	24%(16-32)

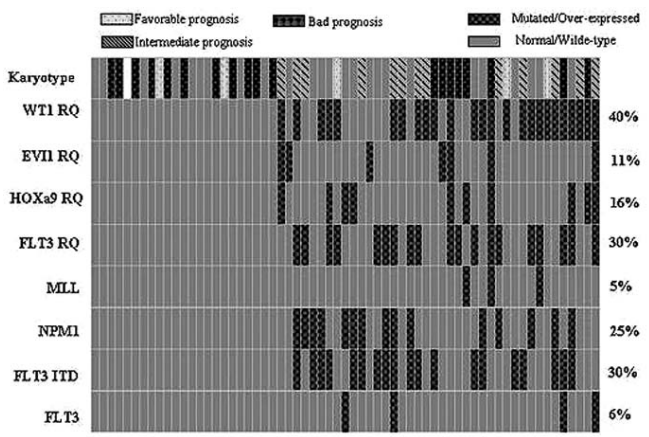

Figure 1. Frequencies and distribution of different markers, mutations, and karyotype subgroups.

Twenty three(36%)pts had no abnormal molecular markers and 40(54%) pts with at least one abnormal marker: 10(16%) 1 marker, 10(16%) 2 markers, 12(19%) 3 markers, 4(6%) 4 markers and 4(6%) 5 markers. Concerning the karyotype, among the 23 negative molecular pts, there were, 9 (41%) normal, 11(55%) poor and 2(4%) good; and among the 40 positive pts, 16(40%) were normal, 8(20%) poor, 13(32.5%) intermediate and 3(7.5%) good. The median interval diagnosis-transplantation was 6 months (2.6-68.5). Before conditioning, 41 pts were in CR (26 CR1, 14 CR2 and 1 CR3), 8 in PR and 14 in relapse. Twenty five (40%) pts received a non-myelo-ablative conditioning and 38 (60%) a myelo-ablative one. There were 34 sex-mismatched (21 M'F and 13 F'M), 21 ABO incompatibility (6 minor and 15 major), 55 were HLA matched and 8 mismatched. Twenty three (36.5%) pts received PBSC, 37 (59%) bone marrow and 4 (6.5%) cord blood cells from 47(75%) HLA siblings and 16(25%) unrelated donors. Fifty nine(94%) pts engrafted, 42 developed AGVHD (21gr1, 13 gr2 and 8 gr4), and among 51 evaluable pts, 13 developed cGVHD (7 limited and 6 extensive) and 20 pts relapsed. At the last follow-up 29 pts are alive (28 CR and 1PR) and 34 died [18(53%) from TRM and 16(47%) from relapse].At the median follow-up of 48 months, the OS and DFS for the whole population were 40% (33-47) and 40%(34-46) respectively, and for the different subgroups according to karyotype and molecular markers are shown in Table 1. The univariate analysis only showed a significant impact of FLT3 ITD and over-expression of FLT3RQ on long-term DFS, (p=0.03 and p=0.02) respectively, and a trend on long-term OS (p=0.08).Concerning the karyotype and some other markers (MLL, EVI1, NPM1 and Hoxa9), we did not observe any significant difference because of small number of pts in each subgroup. The known benefic impact of NPM1 muted, was erased because the majority of this group presented an FLT3 ITD+. We are performing a multivariate analysis that will be presented. In conclusion, the allo-HSCT in this high risk population of pts, allowed a good probability of long term OS and DFS, despite the presence of high number of bad molecular markers and cytogenetic abnormalities. Finally, AML pts with FLT3 ITDmut seem not benefit from allogeneic HSCT even if FLT3 ITDmut was associated to NPM1mut

0849

PHASE 1B/2 PHARMACOKINETIC/PHARMACODYNAMIC (PK/PD) STUDY OF COMBINATION VORELOXIN AND CYTARABINE IN RELAPSED OR REFRACTORY AML PATIENTS

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Background. Voreloxin is a naphthyridine analog that intercalates DNA and inhibits topoisomerase II, inducing apoptosis. Clinical activity is observed in ovarian cancer and AML. Voreloxin combined with cytarabine shows supra-additive activity preclinically. Interim results from a Phase 1b/2 study in relapsed or refractory AML are reported. *Aims*. Safety and MTD of escalating doses of voreloxin in combination with cytarabine; voreloxin pharmacokinetics; clinical activity by IWG; pharmacodynamic markers of patient response; *ex vivo* voreloxin sensitivity in bone marrow aspirates (BMA). *Methods*. Phase 1b dose escalation in relapsed/refractory AML patients with ≤3 prior induction regimens; Phase 2 expansion in first relapse patients (CR1 ≥3 months) at MTD. Voreloxin given D1 and D4, combined with one of 2 cytarabine schedules: A, continuous infusion 400 mg/m^2/d x 5d cytarabine (CIV), or B, bolus 1 g/m^2/d IV x 5d cytarabine. Voreloxin starting dose: A, 10 mg/m^2/dose; B, 70 mg/m^2/dose. Treatment: induction, reinduction if needed, and up to 2 courses for consolidation. DLT for dose escalation, PK and PD were assessed in cycle 1. Patients' PBMC were evaluated for induction of DNA damage response markers. *Ex vivo* sensitivity of patient BMA to voreloxin and cytarabine was evaluated by CellTiter-Glo® proliferation assay. *Results*. 70 patients treated to date (A: 39 patients, dose-escalation; 15 patients Phase 2; B: 16 patients dose-escalation). A: MTD is 80 mg/m^2/dose voreloxin. Infections are the most common G3 or higher toxicity. Voreloxin PK were dose proportional to 50 mg/m^2, then plateaued. Evaluation of PBMC pre- and post-treatment suggests modulation of pDNA-PKcs and pChk2 may reflect response. *Ex vivo* BMA assay results suggest that voreloxin is the primary contributor to the majority of CRs observed and that the combination of voreloxin and cytarabine can overcome resistance. Phase 1b: A: 9 CR + CRp were observed in multiply relapsed or 1° refractory patients. B: 70 mg/m^2: No DLT or remission; 80 mg/m^2: 1 CR, 2 in heme recovery, 3 PD and 1 DLT. Currently, patients are enrolling at 90 mg/m^2. Phase 2: A: 8 patients currently evaluable for response: 4 CR (50%), 3 PD and one

death. *Conclusions*. Phase 1b: voreloxin in combination with CIV cytarabine is generally well-tolerated, with CRs observed in relapsed/refractory patients. Voreloxin in combination with bolus cytarabine has shown early evidence of activity. Dose escalation continues. Phase 2 voreloxin activity with CIV cytarabine in first relapse patients appears robust thus far, with remissions observed in poor prognosis patients with CR1 < 1 year. *Ex vivo* activity assay results suggest that voreloxin is the primary contributor to the majority of CR and that the combination of voreloxin and cytarabine can overcome resistance. Induction of pDNA-PKcs and pChk2 in PBMCs from treated patients may reflect response.

0850

THE SAFETY AND EFFICACY OF AZACITIDINE IN PATIENTS WITH NEWLY-DIAGNOSED AND REFRACTORY/RELAPSED AML NOT ELIGIBLE FOR OR RESISTANT TO CHEMOTHERAPY: A MULTI-CENTER PHASE I/II-STUDY OF THE EAST GERMAN HAEMATOLOGY AND ONCOLOGY STUDY GROUP (OSHO)

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5-azacitidine is an approved treatment for MDS. In this multi-center phase I/II-study the safety and efficacy of a 5 days-schedule of azacitidine in patients with newly-diagnosed and refractory/relapsed AML not eligible for intensive chemotherapy were studied. *Patients and Methods*. From April-October, 2008, 40 pts with AML [19m/21f, median age 72(range 32-84)years] were included. Median WBC was 3.6(range 0.7-187)×10^9/L. Median marrow blasts were 42%. High-risk cytogenetics were found in 12/38 (32%), FLT3mut in 9/34 (26%) and NPM1mut in 7/34 (21%) pts. Median WT1-level was 1209copies/10000ABL. All pts [newly-diagnosed AML (groupA, n=20) and refractory/relapsed AML (groupB, n=20) received azacitidine 75 mg/m^2/day sc for 5 days every 4 weeks. Hematological response was assessed according to the International Working Group Criteria for AML. For non-hematologic toxicity, the NCI CTC and for hematologic toxicity the NCI CTC hematology criteria for leukemia studies or bone marrow infiltrative/myelophthisic processes were used. *Results*. GroupA pts were older (median age 78years) compared to groupB (median age 67years) (p=0.001). To date, a total of 164 treatment-cycles with a median of 3 cycles/patient (groupA, n=6, groupB, n=2) were applied. 29 (73%) patients received at least 2 treatment-cycles. Overall response was 68% [CR, PR, hematologic improvement (HI) n=11 (28%) and stable disease (SD) n=16 (40%)]. Response occurred after a median of 2 months. The probability of response in groupA was 69% compared to 15% for groupB (p=0.07). Similarly, median survival time in groupA (8 months) tended to be higher compared to groupB (3 months) (p=0.09). Interestingly, median survival time for pts with SD was similar to pts with CR,PR, or HI. High-risk cytogenetics had no negative impact on response or survival. Marrow blasts on day 15 of the first azacitidine course correlated with response (p=0.01) but not with survival. Patients achieving CR, PR or HI after 2 courses had a median of 13% marrow blasts day 15 compared to 52% for pts achieving SD only. Lower WT1-expression at screening and on day 15 of the first course correlated with a higher response as well as survival (p=0.03). Pts with WT1-levels >1000copies/10000ABL at screening had a median survival time of only 3 months compared to pts with WT1-levels <1000copies/10000ABL with a plateau at 6 months and a median survival time which is not yet reached. FLT3mut tended to be associated with a shorter survival (p=0.007) but had no impact on response. Surprisingly, NPM1mut also tended to correlate with shorter survival time (p=0.03). Non-hematologic events >grade III were reported 39 times (infections n=21, hepatotoxicity n=5, bleeding n=5, nephrotoxicity n=3, myocardial infarction n=1, and hyperkalemia.n=1, tumorlysis n=1, diarrhea n=1, hypoglycaemia n=1). Neutropenia and thrombocytopenia >grade III occurred in 26 (65%) and 29 (73%) patients respectively. *Conclusions*. Azacitidine applied in a 5 days schedule every 4 weeks is well tolerated in patients with AML and can often be given in an outpatient setting. It induces remarkable hematologic responses especially in patients with newly-diagnosed AML. Patients achieving only SD have a similar survival time to patients with CR, PR or HI.

Chronic myeloid leukemia - Biology and Clinical

0851

NILOTINIB SIGNIFICANTLY INDUCES APOPTOSIS IN IMATINIB RESISTANT K562 CELLS, AS EFFECTIVELY AS IN PARENTAL SENSITIVE COUNTERPARTS

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Background. Chronic myeloid leukemia (CML) is a hematological malignancy characterized by increased numbers of immature white blood cells. BCR/ABL fusion protein, resulting from a translocation between long arms of chromosomes 9 and 22, is the main cause of CML. BCR/ABL is a strong oncogenic protein which has significant roles in the regulation of cell growth, proliferation, apoptosis, migration, differentiation and adhesion. Thus, inhibition of BCR/ABL activity will prevent leukomogenesis. 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 imatinib era, 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 apoptotic effects of novel BCR/ABL inhibitor, nilotinib, on parental and 3 μM imatinib resistant human K562 chronic myeloid leukemia cells (K562/IMA-3) in addition to investigating the development of possible cross resistance of these cells to nilotinib. *Methods*. The Ph^+ human K562 cells were exposed to step-wise increasing concentrations of imatinib for a two-year time period starting from 50 nM. Subpopulations of cells that were able to grow in the presence of 3 μM imatinib, were then selected, and referred to as K562/IMA-3. The IC50 values of imatinib (drug concentration that kills 50% of the cell population) were determined from cell survival plots obtained 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. *Results*. We calculated IC50 value of imatinib as 0.24-, and 14.5 μM in parental and K562/IMA-3 cells, respectively. These results showed that K562/IMA-3 cells are 60 times more resistant to imatinib as compared to parental sensitive counterparts. To examine cytotoxic effects of nilotinib on parental and resistant cells, both cells were exposed to low concentrations of nilotinib for 72 hours and the changes in caspase-3 enzyme activity and MMP were determined. The results revealed that there were 1.29 and 1.25-fold increases in caspase-3 enzyme activity in 1 nM nilotinib applied K562 and K562/IMA-3 cells, respectively, as compared to untreated controls. On the other hand, steady state levels of caspase-3 enzyme activity were 2% decreased in resistant cells comparing to parental sensitive cells. When we compare changes in caspase-3 enzyme activity in 1 nM nilotinib applied K562/IMA-3 cells with untreated counterparts, there were 1.27-fold increase in the activity. In order to examine the changes in MMP in response to nilotinib, K562 and K562/IMA-3 cells were exposed to 10 nM nilotinib for 72 hours. There were 1.55-, and 1.23-fold increases in cytoplasmic/monomeric JC-1 in K562 and K562/IMA-3 cells as compared to untreated controls. Steady state levels of cytoplasmic/monomeric JC-1 were decreased 13% in K562/IMA-3 cells compared to parental sensitive cells. When we compare changes in cytoplasmic/monomeric JC-1 in 10 nM nilotinib applied K562/IMA-3 cells with untreated counterparts, there were 1.41-fold increase in cytoplasmic/monomeric JC-1 ratio. *Conclusions.* In this study, we have shown that nilotinib application induces apoptosis in K562/IMA-3 cells as effectively as the parental sensitive K562 cells. Nilotinib induces apoptosis through the increase in caspase-3 enzyme activity and decrease in MMP almost at the same levels both in parental sensitive and K562/IMA-3 cells. Taking all these data together supports the usage of nilotinib as an anti-cancer drug in imatinib-resistant CML patients. The effectiveness of nilotinib at the lower concentrations might provide a more target-oriented therapy while reducing the risk of the undesired cytotoxic effects to the remaining healthy cells in the body.

0852

LACK OF KRAS MUTATIONS IN BCR-ABL MUTATION NEGATIVE CML PATIENTS RESISTANT TO FIRST AND SECOND GENERATION TYROSINE KINASE INHIBITORS

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Introduction. BCR-ABL kinase domain mutations are the dominating cause of resistance to tyrosine kinase inhibitors (TKI) in CML. BCR-ABL independent mechanisms are heterogeneous and include clonal evolution and activating of pathways bypassing BCR-ABL signal transduction. Recently, downstream activation of signal transduction by an activating KRAS mutation in codon 58 was demonstrated in an imatinib-resistant CML patient. The mutation was associated with resistance to imatinib and dasatinib in experimental models (Agarwal A, Leukemia 2008). *Aims*. We therefore established and compared strategies for the detection of KRAS point mutations: (i) Light CyclerTM based High Resolution Melting (HRM) and (ii) conventional direct sequencing of cDNA. The aim of this study was to determine the frequency of KRAS mutations in a group of BCR-ABL mutation negative CML patients resistant to imatinib, nilotinib, and dasatinib with unmutated BCR-ABL. *Methods*. The assays were established on serial dilutions of the colorectal cancer cell line Colo678 bearing a codon 12 KRAS mutation in the HCC 827 cell line with wildtype KRAS. BCR-ABL mutations were excluded by D-HPLC and conventional sequencing. A cohort of 157 TKI resistant CML patients with unmutated BCR-ABL (chronic phase, n=96; advanced phase, n=61) were investigated. Eighty-two patients (43 male, 39 female; median age 59, range 23-85 years) were resistant to imatinib, 39 resistant to second line nilotinib (13 male, 16 female, median age 65, range 22-82 years) or dasatinib (18 male, 18 female, median age 61, range 32-76 years), respectively. In addition, a group of 20 patients with chronic myelomonocytic leukemia (CMML) known to harbor KRAS mutations at a higher frequency were screened for assay validation and as positive controls. *Results*. HRM analysis was optimized for the detection of 1-5% Colo678 cells in a background of wildtype cells. The detection limit for G12D KRAS point mutations by conventional sequencing was 10-15%. The median transcript ratio BCR-ABL/ABL ratio at the time of resistance was 46% (range 11-152%). There was not a single KRAS mutation found in this series of 157 resistant CML patients by HRM analysis as well as by direct sequencing. In comparison, in the CMML control group 20% of cases (4/20) showed KRAS mutations G12R, G13D, G14S, and G59H. *Conclusions*. (i) HRM analysis offers a more sensitive KRAS mutation screening compared with direct sequencing. (ii) KRAS mutations causing activation of the signal transduction downstream of BCR-ABL are very rare events in BCR-ABL mutation negative CML patients with resistance to first and second generation TKI.

0853

ACQUIRED IMATINIB RESISTANCE IN CML IS ASSOCIATED WITH TKD MUTATIONS OF BCR-ABL AND/OR ADDITIONAL CHROMOSOMAL ABERRATIONS: CERTAIN TKD MUTATIONS ARE ASSOCIATED WITH CHROMOSOME INSTABILITY

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Imatinib resistance in CML was correlated to secondary mutations in the BCR-ABL tyrosine kinase domain (TKD) in 50% of all cases. Besides,additional chromosomal abnormalities (ACA) secondary to the Philadelphia translocation have been implicated in resistance. In this study 88 CML pts with acquired imatinib resistance were analyzed in parallel for ACAs and TKD mutations. In 40 of the 88 pts (45.5%) at least one TKD mutation was detected. Of the 40 mutated cases 6 (15%) revealed two different TKD mutations whereas in 48 pts (54.5%) no mutation was detected with a sensitivity of 10-20%. In the group not affected by TKD mutations 15 of 48 (31.3%) cases showed ACAs. In the group with resistance mutations a nearly equal number of 15/40 (37.5%) cases revealed ACAs and the spectrum of aberrations was very similar. Thus the total amount of pts with ACAs, the spectrum of ACA as well as the number of ACAs per patient is nearly equal in the mutated and the unmutated cohort. In the 40 TKD mutated cases 16 different mutations were detected. Most of the recurrent mutations are distributed equally in the groups with or without ACAs. Solely in 12 cases with M244V, G250E or Y253H we never observed ACAs indicating that mutations within this region are strong enough to cause high resistance. In comparison 5 of 11 cases with T315I and 3 of 4 with H396R mutations are associated with multiple or complex chromosomal aberrations (p=0.014). Five pts were analyzed at 2-4 times under dose escalation of imatinib and show that ACAs can preceed TDK mutations and vice versa. TP53 was sequenced in 16 TKD mutated cases (8 with and 8 w/o ACAs) but no TP53 mutation was detected. In conclusion, 1) ACAs are equally distributed between TKD mutated and unmutated cases. 2) AA exchanges in the region 244-253 are not observed together with ACA whereas T315I and F359C are associated with multiple aberrations. 3) TP53 mutations probably do not underly the genetic changes associated with imatinib resistance.

0854

ROLE OF ABC TRANSPORTERS IN THE DEVELOPMENT OF IMATINIB RESISTANCE

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Background. Imatinib mesylate (IM), the front-line treatment of Chronic Myeloid Leukemia (CML) induces complete cytogenetic remission in more than 80% of newly diagnosed patients. Drug transporters are among the mechanisms underlying primary resistance observed in the remaining 20% and in most patients in advanced phase. It is now well-established that different (ATP)-binding cassette (ABC) efflux transporters such as P-glycoprotein (P-gp or ABCB1), multidrug resistance-associated proteins (MRPs, e.g. ABCC1) or the breast cancer resistance protein (BCRP or ABCG2) actively regulate the traffic of small molecules across the cell membrane being therefore key determinants of intracellular drug concentrations, including IM. *Aims.* To assess expression levels of the genes ABCB1, ABCG2 and ABCC1 in sub-lines of cells resistant to different concentrations of IM in order to evaluate if alterations in the efflux activity of these transporters are one of the molecular mechanisms underlying resistance. *Methods*. The K562 cell line (CML cell line expressing BCR-ABL) was incubated with increasing concentrations of IM to establish 0.25, 0.5, 1.0, 2.0 and 5.0 μM IM-resistant sub-lines, enabling us to study different resistance levels. The final concentration was chosen to reach approximately a 5x concentration of that normally encountered in patients' plasma. After RNA extraction and cDNA synthesis, TaqMan quantitative real-time PCR was used to evaluate expression level of ABCB1, ABCG2 and ABCC1 transporter genes and also the expression level of the BCR-ABL transcript. The threshold cycle (Ct) values of all the samples were first normalized to the Ct value of an endogenous housekeeping gene (GAPDH for transporters and GusB for BCR-ABL) in the same sample and then this normalized value was compared to the normalized value of the reference sample. *Results*. All drug transporters were found to be overexpressed in all (ABCB1) or some (ABCG2 and ABCC1) resistant sub-lines (Figure 1).

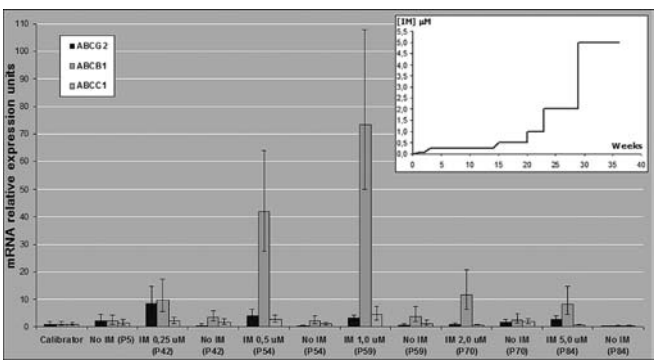

Figure 1.

The most significant overexpression was observed for the ABCB1 gene in the 1.0 μM IM-resistant sub-line. Albeit lower, also ABCC1 presented the same trend as ABCB1: both increased their expression up to the 1.0 μM IM-resistant sub-line and then decreased in the following sub-lines. ABCG2 attained its higher expression in the 0.25 μM IM-resistant sub-line. In 2.0 and 5.0 μM IM-resistant sub-lines the role of the drug transporters is reduced. The overall levels of BCR-ABL transcripts in the different resistant sub-lines as compared with each corresponding control and with the starting population did not present any significant variation. *Summary and Conclusions*. The overexpression of the drug transporters (mainly ABCB1) is probably a major cause for the acquisition of the initial resistant phenotype. BCR-ABL amplification

with its consequent overexpression is unlikely to contribute significantly to resistance in the IM-resistant K562 sub-lines studied. The involvement of other mechanisms like mutations, elevated expression of anti-apoptotic proteins, unfaithful DNA repair and increased biotransformation in the development of resistance in sub-lines of cells resistant to higher doses of IM is currently being evaluated. In agreement with other studies, we show that resistance is the result of multiple mechanisms.

0855

EIGHTEEN YEARS EXPERIENCE WITH AUTOGRAFTING FOLLOWED BY IFN-A AND IMATINIB IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA (CP-CML). THE GENOA EXPERIENCE

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Background. Our team was able to demonstrate many years ago that diploid stem cells could be collected after conventional chemotherapy and G-CSF in the peripheral blood of patients with CP-CML (Carella *et al.* JCO 1997;15:1575-82). *Methods*. In the period 1991-2000, 50 patients with "early" CP-CML were autografted with myeloablative therapy and Ph-negative cells rescue. High-dose therapy consisted of Cy/TBI or high-dose busulfan. After autograft, one patient died of fulminant hepatitis and another patient progressed in blastic phase. The remaining 48 patients were given IFN-a after stem cell engraftment. *Results*. At a median of 95 months (range, 65-128 months) 8 patients evoluted in blastic crisis and died. Forty patients are alive: 12 patients under IFN-a, of whom 9 in CCyR/MMR and 3 CCyR/CMR and 28 patients, relapsed cytogenetically under IFN-a, received Imatinib. Nowdays, 5 patients have been relapsed under Imatinib and have been treated with other TK inhibitors (2 patients) or allografted (3 patients); 4 patients are in CCyR, 6 in CCyR/CMR, 13 in CCyR/MMR. *Conclusions*. These data confirm the previous results published many years ago. A possible explanation for these very good results could be due to the leukemic stem cell debulky with myeloablative therapy in the first months of diagnosis; this intensive approach might have been capable of eliminating a major fraction of the progeny of the Ph-positive stem cells. Subsequently IFN-a, with its specific ability to induce myeloblastin-specific T-cell response, was able to control the disease in some patients. For IFN-a relapsed patients, after 2000 Imatinib was available and it was able to reduce or eliminate in many patients the target cells, though not the stem cell itself, for disease progression.

0856

COMPARISON OF HIGH PREFORMANCE LIQUID CHROMATOGRAPHY-ULTRAVIOLET DETECTION (HPLC-UV) VS LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS) TO DETERMINE IMATINIB PLASMA LEVELS IN CML-CP PATIENTS

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Background. Sensitive, efficient, and readily available methods for determining imatinib blood levels are needed for therapeutic drug monitoring, pharmacokinetic and metabolic studies. Liquid chromatography followed by tandem mass spectrometry (LC/MS/MS) is the standard method for determining imatinib blood levels. This approach is highly sensitive but is expensive and not readily available. HPLC-UV is more readily available and represents a potential alternative to LC/MS/MS. *Aims*. To compare LC/MS/MS and HPLC-UV for their applicability and for the quantitation of imatinib in human plasma. *Methods*. Fifty patients with chronic myeloid leukemia (CML) in chronic phase (CP) receiving 400 mg/day imatinib for a minimum of 1 month were enrolled. Blood samples were taken 1 hour before the next dose of imatinib and plasma was divided in 2 equal aliquots. One aliquot (500 µL) was tested by the HPLC-UV method while the second (200 µL) was tested by LC/MS/MS. Both analytical methods were validated adhering to international guidelines under GLP environment. Analysis of variance (ANOVA) was performed to test if drug concentrations assayed by the 2 methods were statistically different. *Results*. The HPLC-UV method exhibited an acceptable linear range over the imatinib concentration range 100-4000 ng/mL with an approximate correlation coefficient of 0.99. Intra-day accuracy of the HPLC-UV method ranged from 93.68% - 96.00%, while intra-day precision ranged from 1.26-3.79% at the concentrations of 300, 2000 and 3500 ng/mL. Inter-day precision of the method ranged from 3.74% -7.23% for the same drug concentrations. The lower limit of quantitation (LLOQ) for HPLC-UV was 100 ng/mL using 500 µL of plasma. LC/MS/MS led to short retention times of 0.5 min for the drug and internal standard, respectively (total runtime 2.5 min) and high selectivity and sensitivity. Correlation coefficients >0.999 confirmed that the calibration curves for LC/MS/MS were linear over the imatinib concentration range 10 - 4000 ng/mL. The LLOQ was 10 ng/mL using 200 µL of plasma, at which the calculated accuracy and precision were 98.32% and 5.75%, respectively. The intra- and inter-day precisions of LC/MS/MS ranged from 1.94-7.99% and from 2.11-12.01%. The accuracy ranged from 89.72-106.29%. These values were within the acceptable range and demonstrated that the method was accurate and precise. *Summary and Conclusions*. In this study the HPLC-UV assay performed well and demonstrated high concordance with the LC/MS/MS method, suggesting that HPLC-UV could be substituted as an imatinib quantitation method. Disadvantages of the HPLC-UV system include relatively longer run times, less sensitivity leading to the need for larger sample, and less selectivity for drug of interest over endogenous compounds and comedications. However, the large number of patients tested here demonstrates that the UV method has sufficient selectivity and accuracy for the analysis of patients' samples. It was proved there were no significant differences between the two methods at the 0.05 level after logarithmic transformation of the concentrations. The correlation coefficient between both methods was 0.95.

0857

NO INHIBITION OF WARFARIN PHARMACOKINETICS AND PHARMACODYNAMICS BY NILOTINIB IN HUMAN SUBJECTS

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Background. Nilotinib (Tasigna®), a highly selective and potent BCR-ABL tyrosine kinase inhibitor, is approved for the treatment of patients with Philadelphia chromosome-positive (Ph+) chronic myeloid leukemia (CML) in chronic phase (CP) and accelerated phase (AP) who are resistant or intolerant to prior therapy including imatinib. Nilotinib has shown competitive inhibition of CYP2C9 *in vitro*, but its effect on CYP2C9 activity in humans is unknown. Aims. This study was to evaluate the effects of nilotinib on the pharmacokinetics and pharmacodynamics of warfarin, a sensitive CYP2C9 substrate, in healthy subjects.

Table 1.

	Warfarin N=20	Warfarin+nilotinib N=22
S-warfarin PK parameters		
C_{max} (ng/mL)	1092 (19.9)	1094 (19.6)
$AUC_{0\text{-}inf}$ (h.µg/mL)	49.1 (32.2)	48.6 (24.0)
T_{max} (h)	4.94 (2.97 - 7.97)	5.06 (1.97 - 12.0)
$T_{1/2}$ (h)	34.1 (21.3)	32.5 (17.0)
R-warfarin PK parameters		
C_{max} (ng/mL)	1129 (18.8)	1149 (19.9)
$AUC_{0\text{-}inf}$ (h.µg/mL)	70.7 (23.5)	72.3 (22.9)
T_{max} (h)	5.50 (3.97 - 10.0)	7.00 (3.93 - 12.0)
$T_{1/2}$ (h)	44.8 (18.9)	42.7 (18.5)
Warfarin PD parameters		
PT_{max} (s)	31.7 (25.8)	30.7 (25.0)
PT_{auc} (h.s)	2539 (20.7)	2475 (19.9)
INR_{max}	1.72 (21.6)	1.68 (22.3)
INR_{auc} (h)	182 (16.0)	179 (15.4)

Values are median (range) for T_{max}, and geometric mean (CV%) for all other parameters.

Methods. Twenty-four subjects (6F, 18M, age 21-65 years) were enrolled to receive a single oral 25 mg warfarin with either 800 mg nilotinib or matching placebo (all administered 30 minutes after consumption of a high-fat meal) in a cross-over design. Serial blood samples were collected post-dose for determination of nilotinib, S- and R-warfarin concentrations. Prothrombin Time (PT) and International Normalized Ratio (INR) were determined as pharmacodynamic measures for warfarin. CYP2C9 genotyping was performed in all subjects using TaqMan assay. *Results*. Sixteen subjects were identified as CYP2C9 extensive metabolizers (EM)

and 8 as intermediate metabolizers (IM), but none was poor metabolizer. Pharmacokinetic parameters of S- and R-warfarin were found to be similar between two treatments (warfarin+nilotinib versus warfarin alone) for both EM and IM groups. The geometric mean ratios (90% CIs) for the Cmax and AUC0-° of S-warfarin in all subjects were 0.98 (0.95-1.02) and 1.03 (0.99-1.07) respectively, and for R-warfarin were 1.00 (0.96-1.04) and 1.02 (0.99-1.06) respectively. Mean ratios for the maximum value and AUC of PT were 1.00 (0.96-1.04) and 1.00 (0.98-1.02) respectively, and for INR were 1.00 (0.97-1.01) and 1.00 (0.99-1.01) respectively. Nilotinib Cmax was 1872±560 ng/mL, which is comparable to the mean steady-state Cmax in patients with CML or gastrointestinal stromal tumors (GIST) who receive nilotinib 400 mg twice daily doses. Adverse effects observed following either treatment were generally consistent with the known safety profiles of both drugs, and there were no new safety issues observed. *Summary and Conclusions*. The study results demonstrate that nilotinib has no effect on the pharmacokinetics and pharmacodynamics of warfarin. This implies that nilotinib does not inhibit CYP2C9 activity in human subjects. These findings suggest that warfarin and nilotinib can be used concurrently without the risk of increased anticoagulant effect and irrespective of CYP2C9 metabolizer status.

0858

EFFICACY AND SAFETY OF IMATINIB IN THE FIRST LINE CHRONIC MYELOID LEUKEMIA (CML) TREATMENT. AN ANALYSIS OF A COMPREHENSIVE POPULATION-BASED DATABASE

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Background. Imatinib has revolutionized the treatment of CML and is particularly effective in newly diagnosed patients in chronic phase (CP). Most of the data about the efficacy and safety of imatinib in first-line setting have originated from the IRIS trial and recently from published single centre experience. However, confirmatory data from real life are still needed. Aims. To describe and evaluate the results of imatinib in the first line treatment of a consecutive unselected population of CML patients in CP. *Methods*. Data about 1st CP CML patients from the defined region were collected into the database called INFINITY (tyrosine kinase Inhibitors iN the FIrst aNd followIng CML Treatment) One of the main goals was to include all consecutive patients treated with imatinib between June 2003 and December 2008. Rates of hematologic, cytogenetic and molecular responses in defined time-points were evaluated. We assessed also adverse events, time to progression according to IRIS trial including events of failure according to European LeukemiaNet recommendations, time to alternative treatment and survival. *Results*. A total of 147 patients (median age 54 years, 22-77; 67 males and 80 females) underwent the analysis. The median follow-up on imatinib treatment was 22 months (1-56). Imatinib was commenced at a median of 1.7 months (0-35) from the time of diagnosis and the median of actually administered dose was 400 mg. The response rates at 3, 6, 12 and 18 months were as follows: complete hematologic response (CHR): 84%, 87%, 90% and 94%, major cytogenetic response (MCgR): 82%, 82%, 85% and 81%, complete cytogenetic response (CCgR): 35%, 49%, 62% and 74%. Major molecular responses (MMolR) at 12, 18 and 24 months were 41%, 49% and 52%, respectively, with undetectable transcript levels in 5%, 10% and 20% of patients, respectively. Non-hematological toxicity of any grade decreased from 67% (month 3) to 31% (month 24) with gr.3/4 in only 13 cases. Hematological toxicity decreased from 51% to 12% at the same time points. In total, 28/147 patients (19%) permanently discontinued imatinib after a median of 15.5 months (1-51) from various reasons: allogeneic stem-cells transplantation (alloSCT) (n=4), non-hematological (n=5) and hematological intolerance (n=1; the patient subsequently died from septic shock), progression to the accelerated phase or blast crisis (n=2), failure to achieve CHR (1), failure to achieve MCgR or CCgR (n=5) and loss of MCgR or CCgR (n=8). In total, 25 patients (17%) were given on alternative treatment: aloSCT (n=4), dasatinib (n=18), nilotinib (n=1) and hydroxyurea (n=2) in a median of 15 months (1-51). There were 5 deaths in total: 1 case is described above, 2 patients died while on imatinib from the not-CML related reasons and 2 patients died after the discontinuation of imatinib because of disease progression. *Summary*. We confirmed very good efficacy and tolerability of imatinib with improving tendency over time also in unselected patient population managed outside of clinical trials. Early identification of small subgroup of patients with no or little benefit from imatinib could further improved the results.

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0859

CHRONIC MYELOID LEUKAEMIA PATIENTS WITH THE E13A2 BCR-ABL TRANSCRIPT HAVE INFERIOR RESPONSE TO IMATINIB THAN E14A2 PATIENTS

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Background. The reciprocal t(9;22) translocation that characterises CML creates the functional fusion gene BCR-ABL. The breakpoints in chromosome 22 almost always occur between exon e13 and e14 or between e14 and e15, and fuse with ABL exon 2. This leads to the creation of e13a2 and e14a2 BCR-ABL transcript types (differing in length by 75bp; 25 amino acids). The clinical significance of the BCR-ABL transcript type in newly diagnosed chronic phase (CP) patients treated with imatinib 400mg from initial diagnosis remains unknown. *Aims*. Here, we investigate the effect of BCR-ABL transcript type on clinical outcome in 71 newly diagnosed imatinib treated CP patients. *Methods*. All patients aged 16 or over in our geographical area with newly diagnosed CML between January 1st 2003 and October 31st 2007 with either e13a2 or e14a2 transcripts are included in this study if they received imatinib 400mg daily from original diagnosis (preceded only by up to 6 weeks of hydroxycarbamide). Measurement of BCR-ABL transcripts was conducted by real-time quantitative PCR, using a LightCycler. *Results*. Thirty-two patients presented with e13a2 transcripts and 39 with e14a2 transcripts. During the first 12 months of treatment, three e14a2 cases failed imatinib treatment (2 disease progression and 1 intolerance), while eight e13a2 cases failed (7 disease progression and 1 intolerance). After 12 months of treatment, 53.8% of e14a2 patients achieved a complete cytogenetic response (CCR), compared to 25% of e13a2 patients (p=0.01). Similar trends were seen after 18 and 24 months of treatment. Kaplan-Meier analysis of the time to achieve CCR revealed that e14a2 patients demonstrated more rapid response rates, compared to e13a2 patients, which continues throughout treatment (p=0.006). e14a2 patients demonstrated a higher event-free survival in the first 12 months of treatment, though overall survival did not differ significantly different between the transcript types. During the period of study, a total of five additional cases presented in blast crisis of CML; all expressed the e13a2 transcript type. hOCT1 mRNA levels did not differ between the transcript types. The pre-treatment pCrKL/CrKL ratio (a surrogate marker of BCR-ABL tyrosine kinase activity) was higher in e13a2 than e14a2 patients (p=0.017) Furthermore, *in vitro* treatment of samples from e14a2 patients with 5μM imatinib produced a greater suppression of pCrKL/CrKL ratio than in e13a2 samples. *Conclusions*. In conclusion, e14a2 patients have a higher and more rapid CCR rate than e13a2 patients. Knowledge of the transcript type may yield additional prognostic information, though this requires testing on larger datasets.

0860

OVERVIEW OF CHRONIC MYELOID LEUKEMIA AND CURRENT DIAGNOSIS AND TREATMENT PATTERNS FROM 15 HOSPITALS IN CHINA

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Background. The prevalence data including CML patients characteristics are not clear in China due to lack of a mature nationwide leukemia registration system. Limited available data show that the estimated inci-

dence of CML in China is from 0.22 to 0.36 per 100,000 per year. It seems lower than the incidence in western countries.Imatinib mesylate (Glivec) is becoming the first line choice for CML patients who are not appropriate for HSCT. However, it has not been covered by insurance since launch. The price strongly limits CML patients to access it.CML treatment pattern in China was changed much but might be some differ with the pattern in western countries. *Aims*. To explore demographic characteristics, current diagnosis and treatment patterns of chronic myeloid leukemia (CML) patients in China. *Methods*. Data of hospitalized CML patients in 2005 and outpatient information (July 1 through September 30 in 2006) from haemotology departments of 15 hospitals throughout Chinese were reviewed and analyzed. *Results*. A total of 1,824 CML cases were analyzed, including 722 inpatients and 1,102 outpatients. M/F ratio was 1.78:1. The median age at diagnosis was 40.02 (2.45~83.29) years old, 90.41% of the patients were diagnosed at the chronic phase. Proportion of accelerated phase or blast crisis patients increased to 21.66% during study period. 93.20% patients received routine blood testing and bone marrow morphologic examination at diagnosis and monitoring; 70.29% patients were diagnosed and monitored by routine blood testing, bone marrow morphologic examination, and cytogenetic analysis. Only 51.54% patients received hematological test, cytogenetic examination and molecular measurement. The most common drug for CML treatment was hydroxycarbamide. Proportion of patients treated with imatinib and interferon were 37.45% and 25.55%, respectively. 164 cases (22.72%) out of 722 inpatients received hemopoietic stem cell transplantation (HSCT). Proportion of chronic phase, accelerated phase and blast crisis patients treated with imatinib were 35.94%,48.28% and 48.42%, respectively ($p<0.05$). The mean imatinib dosage in three phases did not differ significantly. Imatinib resistance rates were 6.87% and 16.28%, for outpatient and inpatient, respectively. In the outpatient group, resistant to imatinib occurred primarily as secondary resistance (68.75%) while primary resistance was the majority of the inpatient group (65.71%). The intolerance rates of imatinib for outpatient and inpatient were 3.21%, 11.63%, respectively. The majority of patients treated with imatinb were not monitored in time: 63.38% patients evaluated hematologic response after 3 months treatment proportions of patients received cytogenetic examination after 6 months and 12 months treatment were 41.41% and 27.35%, respectively. Mean cost for HSCT was 213092±125890 RMB. *Conclusions*. CML in China tends to afflict a younger population than in Western countries. Most patients were diagnosed in the chronic phase. Due to restriction of funds, only one third CML patients in China were treated with imatinib; the majority of the treated ones were not monitored in time. Lastly, clinicians should pay attention to resistance and intolerance to imatinib treatment.

0861

CHRONIC MYELOID LEUKEMIA AS SECOND MALIGNANCY; A RETROSPECTIVE MULTICENTRIC STUDY

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Background. Neither studies on the effects of alkylating agents and topoisomerase inhibitors nor epidemiological studies on secondary leukaemias following cytotoxic therapy have so far indicated a relationship between chemical exposure and increased risk of BCR-ABL-positive CML. Although cases of treatment-related CML are increasingly reported in the literature, few are the studies focusing on the survival of patients with CML occurring after a prior malignancy. Moreover, it is not clear if the previous exposure to an antiblastic treatment can modify the clinical course of a "secondary-CML" and its response to tyrosine-kinase inhibitors. *Aims*. To examine the incidence, the presenting clinical and biological features and the outcome of CML diagnosed after a prior malignancy. *Methods*. All incident cases of CML diagnosed during the last 10 years by 12 hematological centres of Southern Italy were reviewed and those occurring after a prior malignancy were included in the present analysis. *Results*. Among the 569 patients with CML, 47 (8.2%) were diagnosed following a previous malignancy. Of them, nineteen (40.5%) were male with a median age at the time of diagnosis of the primary malignancy and of CML of 52 yrs (range 22-84) and 60 yrs (range 29-86) respectively. The most common preceding malignancy was breast cancer (23.4% of all preceding cancers), followed by colon/rectum (21.3%), prostate (8.5%), lymphoma (8.5%) and hypophysis (6.4%). The median time from primary malignancy to CML was 54 months (range 2-328) with the following distribution according to Sokal's score: 36% low, 45% intermediate, and 18% high. Only two patients showed additional cytogenetic abnormalities besides Ph chromosome. Thirty-one patients received treatment with Imatinib and all attained at least an haematological response at 3 months (48.4% Major or Complete Cytogenetic response and 6.4% molecular response); at 12 months 54% achieved a molecular response (complete 9.1%) and 31.8% a Cytogenetic Response (Complete 27.3%) and at 18 months the percentage of complete molecular responses raised up to 31.8% with only two patients (9%) losing response or progressing. With a median observation time of 28 months (range 1- 166) only one out of 37 patients treated with Imatinib and 3 out of 10 who received alternative therapies died of CML (overall survival at 5 yrs 94% vs 60% $p=0.017$). Looking at the series according to the treatment received for the primary neoplasm, 19 patients had received chemotherapy and/or radiotherapy while 28 surgery with or without hormonal therapy. The median age at primary tumor diagnosis was similar in the two groups (52 vs 57 yrs) while in the former group the median age at CML diagnosis was significantly lower (57 vs 65 $p=0.04$) and a trend toward a shorter interval between first neoplasm and CML diagnosis was detected (62 vs 103 months; $p=0.06$). *Conclusions*. CML secondary to other neoplasms emerges from this study as a well-defined entity with biological, clinical and prognostic features paralleling those of primary CML and in which the role of a prior chemotherapeutic treatment seems to be related to the progression of the leukaemic clone more than to its rise.

0862

LONG-TERM FOLLOW-UP AND SAFETY OF DASATINIB IN CML/ALL PH1+ IMATINIB RESISTANT PATIENTS IN A COMPASSIONATE USE PROGRAM

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Dasatinib is a potent second-generation ABL kinase inhibitor (TKi). Its activity against nearly all imatinib-resistant BCR-ABL mutations has been confirmed clinically. From May 2006 to June 2007 93 pts with CML imatinib resistant or intolerant and 33 ALL Ph1+ with same characteristic have been treated with dasatinib in a compassionate use. Forty-five patients were affected from CML-Chronic phase (CP), 19 accelerate-phase, 29 blast crisis and 33 were ALL Ph1+. Dasatinib was administered 70 mg BID, and dose escalation to 100mg BID and reduction to 50 mg or 40 mg BID were allowed for inadequate response or adverse events (AEs), respectively. Imatinib resistance was primary (39 pts) and acquired (68 pts), median age was 55 years (range 17-76). The median time from CML/ALL Ph+ diagnosis to enrolment in the compassionate use was 48 months. All pts received prior imatinib at median dose 439mg/d for CML-CP, 449 mg for CML-advanced phases (AdP) and 600 mg for ALL Ph+, for a median time of 1191 days for CP, 1020 for AdP and 277 for ALL Ph+. The best previous response to imatinib was CCyR in 27% of CML-CP, 42% in ALL Ph1+, and 31% in CML-AdP. Prior treatment for CML included IFN-α in 2 ALL Ph1+ pts (1,5%) and in 46 CML pts (36%), chemotherapy in 44 ALL Ph1+ pts (35%) and stem cell transplantation in 7 ALL Ph1+ pts (5,5%) and in 9 CML pts (7%). Twenty-seven patients

(21,4%) received other TKi before dasatinib administration. With a median follow-up of 24 months, the CHR rate was 84% for CML-CP, 71% for CML-AP, 68% for ALLPh1+, respectively. The CCyR rate was 40% in CML-CP, 16% in AdP and 42% in ALLPh1+. The median dose of dasatinib has been 111 mg for CML-CP, 108 mg for CML-AP and 140 mg for ALLPh1+. Thirty-five pts (27,7%) required a dose interruption; 4,7% were due to hematologic toxicity and 11% due to non-hematologic toxicity. Fifty pts remain on treatment with a median of 485 days (33 CML-CP, 11 AdP, 6 ALL Ph1+); 43 had progression of disease. Seven patients had allogeneic bone marrow transplantation. AEs (all grades) considered drug related included headache 4,2%, diarrhea 10,4%, nausea 16%, fatigue 10,4%, rash 8,3%, edema 2,1%, dyspnea 6,3%, pleural effusion 9%, myalgia 7%, arthralgia 4,2%, pneumonia 6,3%, and gastrointestinal hemorrhage 1%. The PFS at 24 mo was 86% in CML-CP, 39% in CML-AP and 18% in ALLPh1+. OS at 24 mo was 90% for CML-CP, 35% for CML-AP and 21% for ALLPh1+. An update with molecular response data and a durable stable disease condition will be considered.

0863

EFFECT OF THE PROTON PUMP INHIBITOR ESOMEPRAZOLE ON THE ORAL ABSORPTION AND PHARMACOKINETICS OF NILOTINIB

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Background. Nilotinib (Tasigna®), a highly selective and potent BCR-ABL tyrosine kinase inhibitor (TKI), is approved for the treatment of patients with Philadelphia chromosome-positive (Ph+) chronic myeloid leukemia (CML) in chronic phase (CP) and accelerated phase (AP) who are resistant or intolerant to prior therapy including imatinib. The solubility of nilotinib is pH dependent, with lower solubility at higher pH. *Aims*. This study was to evaluate whether esomeprazole, a potent proton pump inhibitor that can significantly increase gastric pH, could affect the oral absorption and pharmacokinetics of nilotinib in healthy subjects. *Methods*. Twenty-two subjects (6 F, 16M, age range 18-64 years) were enrolled to receive nilotinib alone or in the presence of steady-state esomprazole during two treatment periods. Nilotinib was given as a single oral 400 mg dose on Days 1 and 13, and esomeprazole 40 mg once-daily on Day 8 through Day 13. Serial blood samples were collected up to 72 hours after nilotinib dosing for determination of nilotinib serum concentrations by a validated liquid chromatography-tandem mass spectrometry assay. Gastric pH was monitored in all subjects at baseline (prior to nilotinib and esomeprazole dosing), before and during the first 4 hours of esomeprazole steady state dosing (5th dose on Day 12).

Table 1.

Nilotinib PK Parameter	Nilotinib alone (n=22)	Nilotinib + esomeprazole (n=15)
C_{max} (ng/mL)	387 (23.2)	275 (45.4)
AUC_{last} (h.µg/mL)	9.75 (34.3)	6.08 (54.4)
$AUC_{(0\text{-}inf)}$ (h.µg/mL)	10.4 (45.6)	6.28 (58.5)
T_{max} (h)	4.00 (2.00 - 10.0)	5.98 (2.00 - 10.0)
$T_{1/2}$ (h)	15.2 (52.4)	14.7 (43.4)
Cl/F (L/h)	38.4 (45.6)	63.7 (58.5)
V_z/F (L)	843 (29.1)	1350 (56.0)

Values are median (range) for T_{max}, and geometric mean (CV%) for all other parameters.

Results. When co-administered with esomeprazole, nilotinib Cmax was decreased by 27% and AUC0-° decreased by 34% respectively. The median time to reach nilotinib Cmax was slightly prolonged from 4.0 hours to 6.0 hours, but its elimination half-life was not altered. Median gastric pH was increased from 0.8 at baseline to 2.0 at pre-dose, and to 3.9, 5.8, 5.5 and 5.7 at 1, 2, 3 and 4 hours after esomeprazole dosing. Administration of nilotinib alone or in combination with esomeprazole was generally well tolerated in the study subjects. *Summary and Conclusions*. The study results suggest a modest reduction in the rate and extent of nilotinib absorption when co-administered with esomeprazole. Such an effect is unlikely to have clinically significant consequences for nilotinib therapy. Thus nilotinib is a TKI that can be used concurrently with esomeprazole or other proton pump inhibitors as needed.

0864

IMATINIB TREATMENT IN ELDERLY PATIENTS (>65 YEARS) WITH CHRONIC MYELOGENOUS LEUKEMIA (CML) IN EARLY CHRONIC PHASE

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Background. Data concerning Imatinib treatment in elderly patients with CML in early chronic phase are still very scarce. Aims and Methods. The impact of age was evaluated in a cohort of 117 consecutive newly diagnosed CML patients observed at our Institution from 9/02 to 3/08 and treated with Imatinib as first-line treatment. *Results*. Of them, 40 were aged > 65 years and 77 <65 years. In the elderly group, 34 patients (85%) had at least 1 concomitant disease compared to 39 patients (50.6%) in the younger group (p<0.001). Thirty-four elderly patients (85%) assumed at least 1 concomitant drug compared to 33 younger patients (42.9%) (p<0.001); in addition, 12 elderly patients (30%) needed ≥4 different concomitant drugs compared to 4 younger patients (5.2%) (p<0.001). Complete cytogenetic response (CCyR) was achieved by 33/37 evaluable elderly patients (89.1%) while 4/37 (10.9%) were resistant; among younger patients, 70/75 evaluable patients (93.3%) achieved CCyR and 5/75 (6.7%) were resistant. No statistical differences in the CCyR and resistance rates between the 2 groups were observed. Severe haematological toxicity (grade 3-4 WHO) was observed in 10 elderly patients (25%) as compared to 7 younger patients (9.1%) (p=0.02). Severe extra-haematological toxicity (grade 3-4) was recorded in 11 elderly patients (27.5%) compared to 8 younger patients (10.4%) (p=0.017). As to the follow-up of Imatinib treatment, the rates of both permanent discontinuation (5/40 or 12.5% vs 1/77 or 1.3%, p=0.009) as well as dose reduction to 300 mg or less (12/40 or 30% vs 7/77 or 9.1%, p=0.001) were significantly higher in elderly patients. Conclusions. Imatinib treatment in elderly with newly diagnosed CML seems to produce same cytogenetic results as in younger patients, but higher haematological and extra-haematological toxicities, leading to increased rate of Imatinib permanent discontinuation or dose reduction. To overcome this problem, future trials addressing the best dosage in this subset of patients could be useful.

0865

EXPANDING NILOTINIB ACCESS IN CLINICAL TRIALS (ENACT) STUDY IN ADULT PATIENTS WITH IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA (CML): FINAL SAFETY ANALYSIS OF 117 CHINESE CML PATIENTS

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Background. Nilotinib, 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. *Aims*. The ENACT study, which is a Phase IIIb, open label, multicenter study, was initiated to obtain additional safety information in pts with imatinib-resistant or -intolerant CML in chronic, accelerated, or blast (BC) phase in a clinical practice setting outside of a registration study. *Methods*. Pts received nilotinib 400 mg twice daily (BID). Dose escalation was not permitted. 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), lack of response, or persistent disease at the investigator's discretion. Efficacy was provided by investigator assessment. *Results.* A total of 117 Chinese pts enrolled in the ENACT study between 01/2006 and 10/2008, including 82 CP pts (70%), 14 AP pts and

21 BC pts. The median age of all pts was 39 years and 75% were imatinib-resistant. The majority of patients had received prior interferon (72.6%). At study completion, 55 (47%) pts were continuing on nilotinib. Median (range) duration of nilotinib exposure was 302 (19-520) days for CP pts, 178 (18-534) days for AP pts, and 58 (1-428) days for BC pts; median average dose intensity was 760, 798 and 700 mg/day, respectively. The main reasons for treatment discontinuation were inadequate responses (26%) and AEs (13%). The majority of study drug related grade 3/4 AEs were hematologic and the most common of these toxicities were thrombocytopenia (32.5%) and anemia (17.9%). Non-hematologic AEs were mostly grade 1/2 and included rash, headache and fatigue. Study drug related grade 3/4 non-hematologic AE's were infrequent. Death on study was reported for 4 pts, equally divided among AP (n=2; 14%) and BC (n=2; 10%) pts. No incidence of grade 3/4 QT prolongation (QTcF > 500 msec) was observed. Grade 3/4 lipase elevation was observed in 3 patients (2.6%) but none were associated with treatment discontinuation. None of the patients experienced pancreatitis or discontinued treatment due to hyperglycemia. No patient experienced pleural effusion on study. Dose reductions were 49.6% and interruptions were 58.1% and are similar to that observed in the overall study safety cohort. Overall major cytogenetic response (MCyR) rates were 44% in CP, 29% in AP and 19% in BC pts. Likewise, complete hematologic response (CHR) rates were 59% in CP, 21% in AP and 14% in BC pts. *Conclusions*. This final safety analysis of the Chinese subset of a large expanded access study further demonstrates that nilotinib is generally well tolerated in heavily pretreated pts in all phases of CML with safety profile similar to that observed in Caucasian patients. This data supports the use of nilotinib at 400 mg bid as the recommended dose in Chinese patients.

0866

PROANGIOGENIC CYTOKINES IN CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB MESYLATE

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Background. although there are many publications that address elimination of Ph^+ clone in patients with chronic myelogenous leukemia (CML) treated with tyrosine kinase inhibitors (TKIs), other biological features of these drugs are not yet clear. *Aims*. of this study was to investigate antiangiogenic and proapoptotic features of imatinib in order to search for additional criteria for treatment efficiency assessment and leukemia monitoring. *Materials and Methods*. 75 patients with Ph^+ chronic phase CML: 11 with first diagnosis, 30 pretreated with Interferon alfa (IFN) and 34 pretreated with cytostatics were studied. Methods of conventional cytogenetics and fluorescent *in situ* hybridization (FISH) were used. Levels of proangiogenic cytokine - vascular endothelial growth factor (VEGF), proapoptotic cytokine - tumor necrosis factor alfa (TNF-alfa) and its soluble receptors of type I and II (TNF-RI, TNF-RII) were determined by immunoenzyme method using standard kits: "Human VEGF ELISA Kit", "Human TNF-alfa ELISA Kit", "sTNF-RI (p55) ELISA" "sTNF-RII (p75)" (Biosource USA); blood chemistry and serum proteins were tested as well. *Results*. Investigation of clinical efficiency of particular CML treatment options showed that the highest levels of hematological and cytogenetic remissions were achieved with imatinib mesylate (IM), the result was obtained quicker and it was the best in first diagnosed patients. Therefore conclusion was made that optimum first line treatment of CML was IM. Patients with prior ineffective treatment with IFN treated subsequently with IM showed response rate and time to its achievement similar to those in the first line IM patients. Patients with and without major cytogenetic response (MCyR) (97% vs 63%), but all of them with hematological remission, had significantly different disease free survival (DFS). Time to progression in patients with complete hematological response (CHR) but without MCyR was 25 months. In all the patients significantly increased serum levels of VEGF were revealed prior to IM treatment, simultaneously levels of TNF-α and its soluble receptors (type I and II) were also several times higher than normal. Comparance of the levels of these cytokines with the results of clinical, hematological and cytogenetic tests showed that increased level of VEGF reflects adequately the activity of leukemic process, particularly regulation of angiogenesis. Similar results were obtained for TNF-alfa which belongs to proapoptotic and proangiogenic cytokines. Level of serum VEGF got several times lower already after 1,5 months of IM initiation when no reduction of Ph^+ clone was yet detected. At the same time in those patients who later did not achieve MCyR (after 12-24 months of treatment), VEGF level stayed high after 3, 6 and 12 months of IM initiation. During the course of IM treatment serum levels of TNF-alfa and its soluble receptors decreased to normal ranges irrespective of cytogenetic results. *Conclusions*. The obtained results apparently reflect process of myelopoiesis suppression. Importantly this effect can be detected at the early stages of treatment before the cytogenetic signs of leukemic clone reduction. Therefore determination of serum (plasma) levels of VEGF and TNF-alfa in CML patients treated with IM could be recommended as independent prognostic factor for treatment monitoring.

0867

IMATINIB DOSE ESCALATION FOR CML RESISTANT PATIENTS IS USEFUL IN GENERAL PRACTICE: IMPACT ON CYTOGENETIC REMISSION

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Imatinib has become a golden standard in general practice for CML treatment. Few data about efficacy of dose escalation for resistant CML patient are available. This topic is of special value for general practice. The aim of the study was the evaluation of efficacy of Imatinib and its dose escalation, The starting dose of Imatinib was 400 mg. Patients were regularly monitored by cytogenetics and the data were reported. Physicians were free to escalate the dose either in case of suboptimal response or 'failure'(ELN criteria). Physicians had access to unlimited dose escalation in case of any kind of resistanse in CML chronic phase patients.The data of all patients were reported, retrospectively resistant patients were revealed. The data base was analyzed with SPSS. The whole group comprised 134 chronic phase CML patients (m-53, f-82, median age 49). Median time to Imatinib treatment was 14 mons. 35 patients appeared to be primary or secondary resistant and needed Imatinib dose escalation(m-20, f-15, median age 43). Median time to Imatnib therapy was 22 mons in this group of patients. In the whole group, probability of major cytogenetic response(MCyR) was 82% by 18 mons, the median time to MCyR was 6.8 mons.The probability of MCyR loss was 33% by 5 years, the rate was 11.5%. In resistant patients, probabilty of MCyR after dose escalation was 18% by 6 mons, 52% by 12 mons, 71% by 18 mons. The rate of MCyR was 49% with median time to MCyR 10mons. The rate of MCyR in patients dose escalated for ' suboptimal response' was significantly higher than in 'failure' patients (75% vs 51%). Probability of MCyR loss was 11% by 3 years after dose escalation, whereas in patients without resistance the probablity of MCyR loss was 17% (p=0.7). *Conclusions*. Dose escalation is effective tool for achievement of CyR in resistant patients. Early dose escalation revealed better results. CyR after dose escalation is stable.

Myeloproliferative disorders - Biology

0868

IDENTIFICATION OF A NOVEL MODE OF KINASE INHIBITOR RESISTANCE: AN F604S EXCHANGE IN FIP1L1-PDGFRA MODULATES FIP1L1-PDGFRA PROTEIN STABILITY IN A SRC-DEPENDENT MANNER

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FIP1L1-PDGFR α is a constitutively activated protein kinase which was reported in chronic eosinophilic leukemia (CEL) and in cases of hypereosinophilic syncrome and mastocytosis with eosinophilia. Imatinib is clinically active against FIP1L1-PDGFRA positive disease. However, clinical resistance to imatinib has been observed in FIP1L1-PDGFRA positive leukemia and was shown to occur due to a secondary mutation (T674I) in the PDGFR α kinase domain. Using a screening strategy to identify imatinib resistant mutations, we generated numerous imatinib resistant cell clones. Analysis of the PDGFRA kinase domain in these cell clones revealed a broad spectrum of resistance mutations including the clinically reported exchange T674I. Interestingly, one of the abundant mutations was a Phe to Ser exchange at position 604 (F604S), which occurred alone or in combination with other exchanges. Surprisingly, FIP1L1-PDGFRA/F604S in contrast to D842H and F604+D842H did not increase the biochemical or cellular IC50 value to imatinib when compared to wild-type (wt). However, F604S and F604S+D842H transformed Ba/F3, NIH3T3 and mouse bone marrow more efficiently compared to wt and D842H, respectively. Also, F604S and F604S+D842H showed strong activation of Stat5, ERK and Akt compared to wt and D842H. Immunoprecipitation and immunoblotting indicated increased amounts of FIP1L1-PDGFRA protein in F604S versus wt cells. Moreover, SRC coimmunoprecipitated with FIP1L1-PDGFRA in wt, but not F604S cells. We hypothesized that F604S might interfere with FIP1L1-PDGFRA protein stability, and that SRC might be involved in this process. GST pull down experiments using SRC-SH2 domain showed lesser binding of FIP1L1-PDGFRA/F604S compared to wt. Similarly, using a GST-PDGFRA fragment, more SRC was precipitated with wt compared to F604S. Importantly both, the SRC inhibitor PD166326 and SRC siRNA mimicked the F604S phenotype and resulted in stabilization of the wt protein. Also, co-expression of SRC in 293T cells augmented degradation of wt, but not F604S FIP1L1-PDGFRA, indicating that SRC is a negative regulator of FIP1L1-PDGFRA protein stability. Similar results were obtained with an exchange in near proximity to F604. However, kinase-defective SRC had no effect on FIP1L1-PDGFRA stability, indicating that kinase activity of SRC is necessary for its effect on FIP1L1-PDGFRA stability. Moreover, kinase defective FIP1L1-PDGFRA (G610R) was not degraded indicating that kinase activity of FIP1l1-PDGFRA is necessary for its own degradation. Taken together, imatinib resistance screening in FIP1L1-PDGFRA identified a novel class of resistance mutations, that do not act by impeding drug binding to the target, but rather increase target protein levels by interfering with its SRC mediated degradation.

0869

C-ROS IS AN ORPHAN TYROSINE KINASE RESPONSIBLE FOR ABNORMAL PROLIFERATION AND ADHESION IN CHRONIC MYELOMONOCYTIC LEUKEMIA

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Background. Chronic myeloproliferative disorders are usually characterized by an abnormal activation of tyrosine kinases although in the majority of the cases these are completely unknown. Perturbation of RTK signalling by genetic alterations results into an abnormal proliferation advantage and finally into a malignant transformation. c-Ros is an orphan RTK displaying transforming activity whose role has been established in the development of neuronal neoplasia. *Aims*. of this study were to evaluate the involvement of c-Ros in the pathogenesis of chronic myelomonocytic leukemia (CMML) and to establish the effects of c-Ros activation. *Methods*. c-Ros expression was evaluated by RQ-PCR in 133 samples collected from 96 CMML patients at diagnosis (96 BM and 37 PB) and 60 healthy donors (30 PB and 30 BM). The protein amount and localization was analyzed by westen blot and immunofluorescence assay. In order to establish the effects of c-Ros activation we generated a chimeric receptor containing the extracellular domain derived from epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domains from c-ros (ER). The chimeric receptor was then transfected in NIH3T3 and HEK293T cells. Transfected and control cells were then stimulated with 100 nM EGF ligand and proliferation and apoptosis evaluated by incorporation of 3H timidine and MTT assay and by FACS for the detection of annexin V, respectively. *Results*. The study demonstrates that Ros it is overexpressed in both BM and PB cells collected from CMML (p<0,0001) with a median value of 2-ΔΔCt of 380 in BM (range 10-63303) and 212 in PB (range 6-30012). By contrast, it is undetectable in healthy subjects. WB confirmed the presence of c-Ros protein in CMML cells but not in normal controls. Immunofluorescence staining localized the protein within the cytoplasm. We found that ROS is highly expressed in $CD34^+$ cells and monocytes from CMML patients but not in their normal counterparts. Sequence analysis revealed the absence of mutations of c-Ros promoter. SNPs analysis exclude the presence of duplications or deletions of the gene. Moreover we found that the EGF induced activation of c-Ros affects proliferation by increasing of 3.5 folds the proliferation rate as compared to cells transfected with the empty vector and stimulated with EGF under the same conditions. Furthermore cell adhesion was 4 folds decreased as compared to control. By contrast apoptosis is not affected by c-ROS activity (p=0,2). *Conclusions*. This study demonstrates the aberrant expression of c-Ros in CMML. The ectopic expression of c-Ros results in loss of adhesion and increased proliferation. Therefore c-ROS is likely to be involved in the pathogenesis of CMML and could represent a target for future molecular therapies.

0870

MIR-28 EXPRESSION IN PLATELETS OF MYELOPROLIFERATIVE PATIENTS VIA STAT5 CONSTITUTIVE ACTIVITY

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Polycythemia vera (PV), Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF) are three distinct myeloproliferative neoplasms (MPNs) characterized by a key molecular determinant: JAK2 V617F. Along with the erythropoietin and G-CSF receptors, the thrombopoietin receptor (MPL, TpoR) is a major JAK2 coupled receptor postulated to play a key role in JAK2 V617F signaling. Furthermore, platelet TpoR levels exhibit an inverse correlation with JAK2 V617F allele burden in these diseases. In order to explore the mechanisms of MPL/TpoR regulation, we assessed whether known microRNAs could target the MPL/TpoR 3'UTR sequence. We found that miR-28 and two other related miR interact with the MPL 3'UTR sequence and inhibit the luciferase activity of a reporter vector, when the MPL 3'UTR is cloned downstream of the luciferase coding region. Interestingly, miR-28 was overexpressed in approximately one third of PV, ET and PMF patients' platelets. In such patients TpoR protein levels were down-modulated, but TpoR down-modulation was not restricted only to the miR-28 positive patients. miR-28 was also overexpressed in MPN patients negative for JAK2 V617F. In the JAK2 V617F-positive group, platelet miR-28 overexpression correlated with a JAK2 V617F allele burden >50%, suggesting that high JAK2 V617F signaling may induce miR-28 expression. Indeed, JAK2 V617F, TpoR W515A, TpoR Δ5, Bcr/Abl and the constitutively active STAT5-1*6 were all able to induce miR-28 in retroviraly transduced cell lines, indicating that miR-28 expression is induced by high STAT5 constitutive activation. Moreover, miR-28 is expressed from an upstream alternative promoter in the presence of JAK2 V617F, as shown by 5'RACE and ChIP using antibodies against STAT5 and RNA polII. Finally, in addition to MPL, we identified other miR-28 targets involved cell cycle regulation. We propose that induction of miR-28 in approximately 30% of MPN patients reflects STAT5 activation and that it might play, via down-modulating its targets, a significant role in the evolution of an important fraction of MPNs.

0871

JAK2V617F-INDEPENDENT OVEREXPRESSION OF AUTOCRINE/PARACRINE HEPATOCYTE GROWTH FACTOR (HGF) AND/OR INTERLEUKIN-11 (IL-11) IN POLYCYTHEMIA VERA AND INHIBITION OF CLONAL EYTHROID PROGENITOR GROWTH BY BLOCKING THE HGF/IL-11 PATHWAYS

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Background. Polycythemia vera (PV) is a clonal myeloproliferative disorder arising from a multipotent progenitor and resulting in excessive erythropoiesis. Activating mutations of JAK2, a key signalling molecule for hematopoietic cytokines, are found in almost all PV. However, other abnormalities may affect clonal proliferation and disease phenotype. The known dependence of PV erythroid progenitors on cytokines prompted us to investigate the possibility of abnormal production in PV of pro-erythroid cytokines. *Aims*. To identify JAK2-activating cytokines with altered production in PV, on which clonal erythroid progenitors might depend for their growth. *Methods*. We used cytokine antibody arrays (Raybiotech Inc.) to establish the cytokine profile of serum from 20 PV patients and 27 patients with second erythrocytosis (SE) and of bone marrow (BM) plasma (21 PV, 21 SE). Molecules of interest were then measured by ELISA in serum (32 PV, 33 SE) and in BM plasma (27 PV, 26 SE). Quantitative RT-PCRs were used to analyse cytokine expression in cells purified from PV and SE patients: BM mesenchymal stromal cells (BMMSC), $CD3^+$ lymphocytes, $CD34^+$ progenitors, GPA+ erythroblasts. Correlations between cytokine levels, blood cell counts and %JAK2V617F were analysed using Pearson's or Spearman's rank correlation tests. *Results*. Analysis of serum, BM plasma and purified cells of PV patients revealed overproduction of hepatocyte growth factor (HGF) and interleukin 11 (IL-11). The main producers of the two cytokines, known to promote erythroid cell proliferation, were BMMSC and erythroblasts. Exposure of PV BMSC to HGF induced a dose- and time-dependent increase in IL-11 production. Over-expression of HGF and IL-11 in PV erythroblasts is unlikely to be a consequence of JAK2V617F as: i) HGF and IL-11 mRNA levels did not correlate with those of JAK2V617F; ii) hematocrit of patients correlated with IL-11 levels in serum, not with JAK2V617F; iii) expression of JAK2V617F in BaF-3/EpoR cells had no effect on HGF and IL-11 mRNA expression. The relevance of HGH and IL-11 deregulation in PV was then investigated using neutralizing antibodies specific for c-MET, the receptor for HGF, or for IL-11 in *in vitro* cultures: both types of antibodies inhibited the growth of JAK2V617F-mutated erythroblasts from PV patients, as well as JAK2V617F+/+ HEL cells. *Conclusions*. We found JAK2V617F-independent deregulation of autocrine/paracrine HGF and IL-11 in PV, and provide evidence that targeting the c-MET/HGF/IL-11 pathways may be of interest in PV.

0872

CIRCULATING ENDOTHELIAL COLONY-FORMING CELLS ARE INCREASED IN PATIENTS WITH PRIMARY MYELOFIBROSIS, DO NOT BEAR THE JAK-2V617F MUTATION, AND COEXIST WITH JAK-2V617F-POSITIVE MATURE ENDOTHELIAL CELLS

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Background. Primary myelofibrosis (PMF) is characterizaed by increased angiogenesis both in the bone marrow and in the spleen. Recently, a role for circulating endothelial progenitor cells (cEPCs) in new vessel formation has been hypothesized. *Aims*. To evaluate the frequency of cEPCs, grown as endothelial colony-forming cells (ECFCs) *in vitro*, in patients with PMF and other Ph-negative chronic myeloproliferative disorders (CMPDs), and to assess the presence of the JAK-2V617F mutation both in ECFCs and in mature endothelial cells derived from the spleen of PMF patients. *Methods*. cEPCs were studied in 121 PMF patients, 32 patients with PV or ET, and 12 healthy subjects. Spleen sections were obtained from 5 patients undergoing therapeutical splenectomy. ECFCs were grown *in vitro* according to Yoder *et al.*, whereas the JAK-2V617F mutation was evaluated by semi-nested PCR both in ECFC-derived colonies and in endothelial cells obtained from spleen sections by laser microvascular dissection. In two patients carrying major chromosomal abnormalities in their granulocytes, ECFCs were also studied by array-comparative genomic hybridization (array-CGH). *Results*. The median frequency of ECFC per 10 mL of peripheral blood (PB) was statistically higher ($p<0.05$) in PMF patients (0.48, range 0-6) than in PV patients (0.13, range 0-0.57), ET patients (0.25, range 0-1), and normal subjects (0.14, range 0-0.5). Frequency was higher ($p<0.05$) in patients with prefibrotic PMF (0.8 ECFC/10 mL PB; n=33) than in patients with fibrotic MFI (0.45/10 ml PB; n=88). In patients with PMF, a history of previous splancnic vein thrombosis was associated to statistically higher ($p<0.01$) ECFC frequency (1.45 ECFC/10 mL; n=17) compared to patients without thrombosis (0.46/10 mL; n=104), as well as to healthy subjects, either with or without history of thrombosis (0.5 and 0.14, respectively, $p<0.05$ for both). ECFC frequency of all PMF patients also inversely correlated with patients' age (r=-0.22, $p<0.02$), whereas no correlation was found with the JAK-2 mutational status. However, at a multivariate regression analysis, the only parameter that independently correlated with ECFC frequency was a history of previous vein thrombosis ($p<0.001$). The presence of the JAK-2V617F mutation was assessed in 49 ECFC-derived colonies obtained from 28 PMF patients with the mutation in their granulocytes, and in 15 ECFC-derived colonies from 18 JAK-2V617F-positive PV or ET patients. All colonies harbored only the wild type allele. Array-CGH showed the absence of chromosomal abnormalities in all ECFC-derived colonies (n=7) from 2 PMF patients whose granulocytes were characterized by trisomy 8 and by del(4)(21.21-q25), del(7)(pter-p13), and del(7)(q21.11-qter), respectively. Surprisingly, in 2 out of 5 splenectomized PMF patients, homozygous for the JAK-2V617F mutation in their granulocytes, mature endothelial cells obtained by laser microdissection of splenic vessel walls showed the presence of the mutation. *Summary*. cEPCs, assessed as ECFC, are increased in PMF patients compared to healthy subjects and do not belong to the JAK-2V617F mutated clone. The presence of the JAK-2V617F mutation in mature endothelial cells derived from splenic vessels suggests that contribution of the malignant clone to neoangiogenetic processes is possibly accomplished by other cells endowed with angiogenic potential but derived from the hematopoietic lineage

0873

CIAP-1 AND CIAP-2 ANTI-APOPTOTIC MOLECULES ARE OVEREXPRESSED IN ESSENTIAL THROMBOCYTHEMIA AND MYELOFIBROSIS

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Background. Chronic Myeloproliferative disorders (MPD) are clonal hematopoietic stem cell malignancies characterized primarily by hypercellular bone marrow, proliferation of cells from one or several myeloid lineages and hematopoietic progenitor independence of or hypersensitivity to cytokines. The apoptotic machinery deregulation may be associated to MPD physiopathology. *Aims*. To quantify ciap-1 and ciap-2 gene expression in bone marrow $CD34^+$ hematopoietic stem cells (HSC) and peripheral leukocytes in Essential Thrombocythemia (ET) and Myelofibrosis (MF). We also attempted to correlate the gene expression results with JAK2V617F allele burden. *Subjects and Methods*. 24 TE, 12 MF patients and 53 healthy subjects. Patients were 13 males and 23 females with a mean age of 61.1y and controls were 23 males and 30 females with a mean age of 45.9y. 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 Trizol® method, High Capacity® Kit was used to synthesize cDNA and real time PCR to quantify ciap-1 and ciap-2 expression. The gene expression results were given as $2^{(-\Delta\Delta Ct)}$. The JAK2 V617F mutation was detected by real time allelic discrimination PCR assay. Statistical analyses were performed by Mann-Whitney and Spearman tests. *Results*. In $CD34^+$ HSC, ciap-1 mRNA levels were increased in ET (median=5.71) and MF (22.84) patients in comparison to controls (0.75) ($p=0.004$; $p=0.009$, respectively). ciap-2 are also overexpressed in ET (11.32) and MF (31.37) patients compared to healthy subjects (0.73) ($p=0.005$;

p=0.002). In leukocytes, we found no statistically difference in ciap-1 or ciap-2 expression between patients and control group. Interestingly, we observed that CD34+ HSC from ET patients who are negative for the JAK2V617F mutation showed higher ciap-2 expression (41.57) than that positive (15.22) (p=0.04) and we found a negative correlation between JAK2V617F allele burden and ciap-2 expression in ET patients CD34+ HSC (r=-0.3882; p=0.04). *Conclusions*. The deregulation in ciap-1 and ciap-2 expression in MPD patients might reduce the degree of CD34+ HSC apoptosis, consequently increasing the accumulation of myeloid cells in MPD patients. Moreover, a negative correlation between ciap-2 and JAK2V617F allele burden suggests that ciap-2 overexpression is not linked to JAK2V617F mutation.

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GENE EXPRESSION PROFILING OF CD34+ CELLS IN MYELOPROLIFERATIVE DISORDERS BY CDNA MICROARRAY TECHNOLOGY

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Myeloproliferative disorders (MPD) are clonal hematopoietic diseases that include essential thrombocytosis (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) as well as BCR-ABL+ chronic myelogenous leukemia (CML). In the past several years, studies with cDNA microarrays have defined patterns of gene expression corresponding to specific molecular abnormalities, oncologic phenotypes, and clinical outcomes in hematologic malignancies. This study was aimed at the description of a gene expression signature in MPD which would eventually present a new pathogenetic approach and also diagnostic as well as prognostic information. Using cDNA microarray analysis, involving 25,100 unique genes, we studied the gene expression profile of the pluripotent hematopoietic CD34+ stem cells and mature granulocytes obtained from peripheral blood of ET, PV, PMF and CML patients compared with healthy individuals. The average number of CD34+ cells (cells/μL) in peripheral blood was approximately 6 in PV and ET, 111 in PMF and 2880 in CML as measured by flow cytometry. A somatic point mutation JAK2V617F was detected in 93% of PV, 73% of PMF and 55% of ET patients within genetically homogenous populations. The JAK2V617F mutant allele burden was the highest in PV (60%), less prominent in PMF (42%) and low in ET (11%) patients. The JAK2V617F mutation negative patients were also negative for exon 12 mutations. By microarray analysis, approximately 420, 680 and 1130 genes were uniquely over expressed in CD34+ cells derived from ET, PV and PMF patients, respectively. When we analyzed EPO and JAK-STAT signaling pathways-related genes in MPD, we found that FOS, RAF1 and JAK2 genes, related to EPO signaling pathway, were elevated in ET, PV, PMF and reduced in CML compared to healthy controls. Interestingly, JAK2V617F mutation homozygous and heterozygous patients generally displayed more significant differences compared to patients with no mutation for FOS, RAF1 and JAK2 genes. STAT5 gene expression was decreased in all MPD patients. CSF3R, STAT1 and STAT3 gene expression, related to JAK-STAT signaling pathway, was elevated in ET, PV, PMF and reduced in CML compared to healthy controls. The nuclear transcription factor cAMP-response elements-binding protein (CREBB) gene expression was reduced in CD34+ cells of ET, PV and PMF patients, but during maturation its expression was enhanced in granulocytes. Compared to previous microarray reports, related to JAK-STAT signaling pathway in MPD, we found similarities in SOCS2 and PIM1 increased genes expression in favor of JAK2V617F-positive patients. In conclusion, molecular profiling of CD34+ cells and granulocytes from MPD patients revealed gene expression changes that is beyond their recognized function in disease pathogenesis and can be related to patients' clinical characteristics and may have imminent prognostic relevance.

0875

THREE NOVEL MUTATIONS IN THE PROLYL HYDROXYLASE DOMAIN PROTEIN 2 GENE OF THE OXYGEN SENSING PATHWAY IN PATIENTS WITH ISOLATED ERYTHROCYTOSIS

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Background. Cellular and physiological responses to changes in oxygen tensions in mammalians are mediated via the post-translational oxidation of hypoxia-inducible transcription factor (HIF). Hydroxylation of conserved prolyl residues in the HIF-α subunit is catalyzed by HIF prolyl-hydroxylases (PHDs), belonging to the Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase family. Such hydroxylation promotes interaction with the von Hippel-Lindau E3 ubiquitin ligase (VHL) and, consequently, proteolytic destruction by the ubiquitin-proteasome pathway. PHD2 isoform employs molecular oxygen as obligatory substrate and its inhibition mimics the hypoxic response, including an increased erythropoietin (Epo) production. Heterozygous PHD2 mutations in humans are associated with erythrocytosis. *Aims.* To identify the genetic lesion in a cohort of subjects with isolated erythrocitosis (defined by the presence of absolute polyglobulia, normal platelet and white blood cell count, absence of splenomegaly, decreased level of serum erythropoietin), either sporadic or familial (almost one first-degree relative with the same diagnosis). *Methods*. Peripheral blood samples at diagnosis from 67 consecutive patients were collected. 12 subjects with familial erythrocitosis, and 55 cases of isolated erythrocytosis were included in the study. PCR of the PHD2 coding region (exon 1-5) and sequencing analyses were performed under standard conditions. To confirm the identity of the newly identified mutations, allele-specific PCR were carried out for each variant. The three VHL exons, HIF2A-exon 12, JAK2 exon 12 and JAK2V617F mutations were also analyzed. *Results*. We identified three novel PHD2 heterozygous mutation (Figure 1 A, B). N203K occurred in a subject with erythrocytosis resulted positive for the JAK2 (547insL+I540F547dup8) mutation in the exon 12, with low serum Epo level.

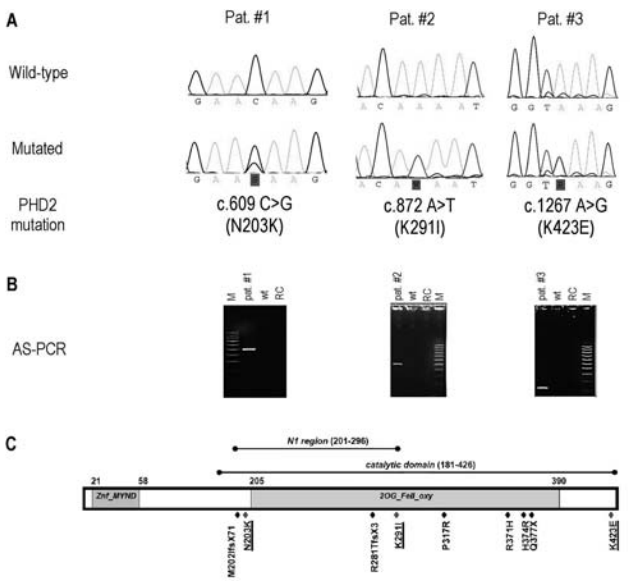

Figure 1. Mutational analysis results and localization of the PHD2 mutations (**A**) Sequencing results of wild-type and mutated alleles in 3 patients. Nucleotide positions (GenBank accession, NM 022051), nucleotide changes and corresponding amino acid changes are indicated below (**B**) Confirmation of the new mutations by allele-specific PCR (AS-PCR) and agarose gel electrophoresis. AS-PCR fragments lenghts agree with the respective primers positions. Lane M: molecular weight marker, lane pat #: patient's DNA amplified with mutation specific primer, lane wt: control DNA amplified with mutation specific primer, lane RC: reaction control (no template) (**C**) Schematic diagram of the human *PHD2* gene. *Znf_MYND*: Zinc finger MYND-like domain, *2OG_FeII_oxy*: 2-oxoglutarate and Fe(II)-dependent oxygenase-type domain. Diamonds indicate the location of the mutations, underscored are the novel genetic variations described in this study. Numbers indicate amino acid residue positions.

Figure 1.

One of his niece and her respective son were erythrocytosic as well, but they did not carry the N203K mutation and they were both JAK2 wild-types. The second mutation, K291I, was found in a 29-year-old male, that referred a familial history of erythrocytosis. The last mutation, K423E, was found in a case of sporadic idiopathic erythrocytosis.

The patients with a PHD2 mutation did not carry VHL, HIF2A-exon 12 genetic lesions or JAK2V617F mutation. *Conclusions*. The three erythrocytosis-associated PHD2 mutations described above are likely to be all of the "partial-loss type", as the six previously reported, and involve the catalytic domain of the protein (Figure 1 C). N203 and K291 map in the region responsible for the differential target preference of PHD2 (N1 region, Figure 1 C). K423E causes the loss of a positive charged side chain with the acquisition of a complementary negative charged glutamate. K423 is an evolutionally conserved residue in orthologues forms of PHD2 but it was absent in the two closely related human isozymes, PHD1 and PHD3. It is possible that removal of the C-terminus of the enzyme actually promotes uncouples turnover of 2OG. Identification of the disease-causing genes will enable better classification of familial and acquired erythrocytosis as, to date, in many cases of this disorder the molecular basis remain unknown. The co-presence of PHD2 and JAK2 exon 12 mutation in a patient and the contrasting Epo levels highlight the difficulties in clearly classify primary or secondary forms rather than acquired or hereditary cases. It will be of interest to asses the substrate binding affinity and the differential effect of these new mutations on HIF-α hydroxylation.

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REACTIVE OXYGEN SPECIES ARE DIFFERENTIALLY REGULATED IN CHRONIC MYELOID LEUKEMIA VERSUS PHILADELPHIA NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

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Background. Reactive Oxygen Species (ROS), generated by cellular metabolism, are deleterious for the cells when produced in excess. Their production is increased by tyrosine kinase activation and can be responsible for additional mutations favouring the evolution of myeloproliferative neoplasm from chronic phase to more aggressive acute leukemias. The role of ROS in generating genetic aberrations has been mainly studied in cell lines. *Aims*. In this work, we compared the basal and stimulated ROS production in primary cells of CML or Philadelphia-negative myeloproliferative neoplasm (Ph-MPN) as an approach to ROS metabolism in these diseases and a potential predictor of adverse evolution. *Methods*. Cells were obtained from patients diagnosed with CML [22 from bone marrow (BM), 18 from peripheral blood (PB)], or with Ph-MPN (PB: 6 Polycythemia Vera, 9 Essential Thrombocythemia, 4 Primary Myelofibroses, 2 atypical CML) and from healthy donors. They were incubated with DCFDA (a fluorogenic probe revealing ROS production), labelled with an anti-CD45 antibody or an anti-CD34 antibody, and then stimulated with either the oxidant hydrogen peroxide (H_2O_2) or the PKC activator Phorbol Myristate Acetate (PMA). ROS production was analysed by flow cytometry in granulocytes, monocytes, lymphocytes and $CD34^+$ cells. In some experiments, $CD34^+$ cells were sorted using magnetic beads. *Results*. In our study, the basal level of ROS was not higher in myeloproliferative neoplasms cells as compared to healthy donors, including in $CD34^+$ cells. This is in contrast to some reports based on cell lines or smaller populations of patients. ROS levels was even significantly lower in CML lymphocytes, either from the BM (2.8 Arbitrary Units vs 8.3 AU, $p<10\text{-}6$) or PB (2.9 AU vs. 7.4 AU, $p<10\text{-}6$) and in CML granulocytes from PB (14 AU vs 45 AU, $p<10\text{-}8$), but not from bone marrow. The ROS levels of Ph-MPN cells were similar to control cells, except for granulocytes from peripheral blood (29 AU vs 45 AU, $p=0.01$). Upon H_2O_2 stimulation however, ROS production increased significantly more in CML cells as compared to normal cells (5 fold increase in lymphocytes and 10 fold increase in granulocytes) whatever their origin (PB or BM). Interestingly, H2O2-dependent ROS increase in $CD34^+$cells correlated with that observed in PB granulocytes, indicating that studying granulocytes is a possible alternative to CD34 cells for this aspect of ROS regulation. In contrast, for Ph-MPN cells, ROS production was close to that of normal cells (only two fold higher than control BM granulocytes and lymphocytes). After PMA stimulation, which yielded a more modest ROS production than H_2O_2, CML cells behaved similarly to normal cells whereas ROS production was four fold higher in Ph-MPN cells, whatever their type and origin. *Conclusions*. ROS levels at the basal stage are not higher in primary cells of myeloproliferative neoplasms, but CML cells are more sensitive to oxidants whereas Ph-MPN cells respond more to the "cytokine mimicking PMA". These results suggest that the mechanisms of ROS generation and thus of genetic instability are differently regulated in CML and Ph-MPN cells. Some of these elements could be easily appreciated in peripheral blood cells.

0877

TYROSINE PHOSPHORYLATED SOCS3 IS UNABLE TO REGULATE THE MYELOPROLIFERATIVE NEOPLASM ASSOCIATED- JAK2 V617F KINASE

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Backgound. JAK2 V617F, identified in the majority of patients with myeloproliferative neoplasms (MPN), tyrosine phosphorylates SOCS3 and escapes SOCS3 inhibition. We have demonstrated that the JAK2 exon 12 mutants described in a subset of V617F-negative MPD cases, also stabilise tyrosine phosphorylated SOCS3. We have also observed SOCS3 tyrosine phosphorylation in peripheral blood mononuclear cells and/or granulocytes isolated from patients with JAK2 V617F, H538QK539L or JAK2 F537-K539delinsL mutations. *Aims*. We hypothesise that inappropriate SOCS3 tyrosine phosphorylation by JAK2 V617F prevents the SOCS3-Elongin C interaction rendering SOCS3 unable to function as an E3 ligase to degrade the mutant JAK2 kinases. *Methods*. We use co-expression studies in 293T cells to investigate which tyrosine residues within SOCS3 V617F phosphorylates. Protein pull down assays are also employed to determine how tyrosine phosphorylation of SOCS3 affects its interaction with ElonginC. We also use cycloheximide treatment to track the turnover of wild-type JAK2 or V617F in the presence of wild-type SOCS3 and a SOCS3 tyrosine mutant. *Results*. In this study we demonstrate that JAK2 V617F phosphorylates SOCS3 at Y204 and Y221. We also establish that phosphorylation of both Y204 and Y221 prevents the SOCS3-ElonginC interaction. Consequently, wild-type SOCS3 is unable to form a function E3 ligase in the presence of V617F and therefore unable to ubiquitinate JAK2 V617F. However a SOCS3 mutant lacking Y204 and Y221 can. This SOCS3 tyrosine mutant can also regulate the phosphorylation status of JAK2 V617F. *Summary*. In this study we address the relevance of SOCS3 phosphorylation as a potential mechanism by which JAK2 V617F can overcome the suppressive function of SOCS3.

0878

A ROLE FOR TIF1GAMMA IN CHRONIC MYELOMONOCYTIC LEUKAEMIA

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Background. Transcriptional Intermediary Factor 1 gamma (TIF1γ) belongs to the TIF1 family of transcriptional co-regulators. In mice, Tif1γ deletion is embryonic lethal (R. Losson, unpublished data), demonstrating a crucial role of this gene during development. In zebrafish, tif1γ gene mutation disrupts embryonic and adult haematopoiesis with severe red blood cell aplasia. *Aims*. Our goal is to investigate the role of TIF1γ in haematopoiesis. *Methods*. Mice expressing the Tif1γ floxed allele were crossed with mice in which the Cre recombinase is expressed under the human cFES promoter. *Results*. Mice transplanted with Tif1γΔ/Δ bone marrow (BM) survived from lethal irradiation, and cells in which Tif1gamma gene was deleted were detected in the peripheral blood (PB) of these mice confirming that the deletion occurred in HSCs. Tif1γΔ/Δ mice older than 6 months exhibited a monocytic hyperplasia associated with an increase in the number of megakaryocytes harbouring morphologic abnormalities, together with dysmegakaryopoiesis, dyserythropoiesis, and dysgranulopoiesis. Flow cytometry analyses confirmed the increased population of monocytes in the BM and in the spleen. The Tif1γΔ/Δ mice with monocytosis also displayed a hepatosplenomegaly. Altogether, the dysplastic and myeloproliferative features observed recapitulate the human chronic myelomonocytic leukaemia (CMML). We showed that the number of $Lin^-Sca1^+cKit^+$ (LSK) cells was increased in Tif1γΔ/Δ before 6 months of age, then returned to normal values as mice develop a CMML-like phenotype. We also identified an abnormal differentiation of produced mature monocytes. The CMML phenotype associated with Tif1γ deletion in mice suggests that Tif1gamma deficiency may contribute to transformation by a loss of proliferative control at the stem/progenitor stage or in a committed myeloid lineage. Since the phenotype observed in the Tif1γΔ/Δ mice recapitulated the human CMML, we studied the expression level of TIF1gamma in monocytes obtained from 22 CMML patients compared to 12 healthy donors, after that informed consent was obtained. RQ-PCR analysis demonstrated that TIF1gamma gene expression was almost undetectable in 8 out of the 22 patients. In addition, a three days exposure of PB monocytes

from CMML patients to decitabine, a demethylating agent that was recently shown as a potentially efficient therapeutic in about 30% of these patients, increased the expression of TIF1γ. Several reports have described TIF1γ as a member of TGF-β/BMP signalling pathways even if its mechanism of action is still unclear. Among the TGF-beta family members, BMP4 has demonstrated a crucial role in haematopoietic stem cells maintenance and megakaryopoiesis. We showed that Bmp4 transcript is strongly decreased in Tif1γΔ/Δ BM cells. By IHC, we also started to study the expression and cell localisation of Smad4. *Conclusions*. In conclusion, our animal model not only closely mimics human CMML, but also allows genomic analysis of a large series of primary patient samples leading to the identification of a subset of patients expressing low levels of TIF1gamma. Furthermore, this animal model will be useful as a powerful system to identify other hits in CMML pathogenesis, but it will also permit to assess the therapeutic efficacy of molecular agents like DNA hypomethylators.

0879

A NEW PROLYL HYDROXYLASE DOMAIN PROTEIN 2 MUTATION IN A JAK2(V617F) POSITIVE PATIENT WITH A FAMILIAL MYELOPROLIFERATIVE DISEASE

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Background. Polycythemia vera (PV) is the most common cause of primary erythrocytosis, almost always due to the somatic V617F allele in the JAK2 gene, within erythroid progenitor cells, that results in constitutive intracellular signalling and clonal proliferation. Less often, JAK2(V617F) and MPL(W515L/K) mutations are associated with essential thrombocythemia (ET) or primary myelofibrosis (PMF), while JAK2 mutations located in exon 12 have been found in patients with isolated erythrocytosis (IE) or in JAK2(V617F) negative PV cases. There is evidence to support the possibility that diseases alleles other than those described above are involved in the pathogenesis of Philadelphia-negative chronic myeloproliferative disorders (MPDs). Recent insights of the genetic causes of erythrocytosis came from the fuctional and biochemical investigation of the oxygen-sensing (OS) pathway. Aim: To investigate the possibility that diseases alleles other than JAK2 could be involved in the pathogenesis of familial MPDs. *Methods*. Peripheral blood samples at diagnosis from 23 familial PV or ET patients were collected. The von Hippel-Lindau tumor suppressor (VHL), prolyl hydroxylase domain protein 2 (PHD2) and the exon 12 of the 2α-subunit of the hypoxia-inducible factor (HIF2A) genes were analyzed for their role as key proteins of the OS pathway. PCR of the PHD2 (exon 1-5) and VHL (exon 1-3) coding regions and sequencing analyses were performed under standard conditions. To confirm the identity of the newly identified mutation, allele-specific PCR was carried out. JAK2 and MPL mutational status were previously assessed in all the patients. *Results*. We identified a novel PHD2 heterozygous mutation (Figure 1 A, B). Molecular biology studies revealed a G>C missense mutation at coding nucleotide position c.471 resulting in a Q157H replacement in the amino acid sequence. It was found in a PV patient with normal serum Epo level, raised hematocrit (55.2%) and high platelets count (703×10^9/L) previously resulted positive for the homozygous JAK2(V617F) variation. His sister was affected by PV as well. At the time of the study she was already deceased (Figure 1 C).

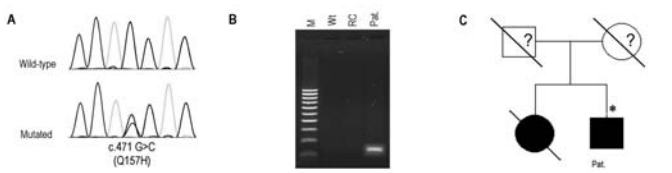

Figure 1. Mutational analysis results. (**A**) Sequencing results of wild-type DNA and mutated alleles of the patient. Nucleotide positions (GenBank accession, NM 022051), nucleotide changes and corresponding amino acid changes are indicated below (**B**) Confirmation of the new mutation by allele-specific PCR (AS-PCR) and agarose gel electrophoresis. Lane M: molecular weight marker, lane Pat.: patient's DNA amplified with mutation specific primer, lane wt: control DNA amplified with mutation specific primer, lane RC: reaction control (no template) (**C**) Pedigree of the family with MPD. Squares represent males, circe females, affected individuals are indicated in black and slashes indicate deceased members. Genetically tested individual is indicated by an asterisk.

Figure 1.

The patient with the new PHD2 mutation did not carries VHL, HIF2A-exon 12, MPL or JAK2-exon 12 mutations. Conclusions: Q157H is the most N-terminal mutation reported to date in the PHD2 protein, lying between the N-terminal MYND zinc finger-like domain (amino acids 21-58) and the highly conserved C-terminal catalytic domain (amino acids 181-426). This amino acidic variation do not directly involves the catalytic domain, it introduces a positively charged histidine, with a bulky and reactive side chain, instead of an indifferent glutamine. A truncated version of PHD2, lacking residues 76-177 from its N-terminal unique region behaved as full-length PHD2, but further studies will be required to estabilish the function of this PHD2 unique region. Noteworthy, for the first time, a mutation affecting PHD2 has been identified in a PV patient, while other partial-loss PHD2 mutations are all erythrocytosis-associated. The co-presence of PHD2 and JAK2(V617F) genetic lesions support the hypothesis that JAK2 mutation is a secondary, not a disease-initiating, genetic event and that the JAK2(V617F) mutation might occur as a secondary event on the background of an inherited genetic predisposition.

0880

SOCS1, SOCS3 AND PTPN6 METHYLATION IN PATIENTS WITH PHILADELPHIA-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASES (CMPD)

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Background. The Jak2 V617F mutation can be detected in the majority of CMPD patients and leads to constitutively activated Jak2 proteins. SOCS1, SOCS3 and PTPN6 are members of the Suppressors Of Cytokine Signalling family or Protein Tyrosine Phosphatase family, respectively, thus directly interacting and inhibiting Jak2 kinase activity. Epigenetic gene silencing of these proteins by DNA methylation activates the Jak-STAT pathway similarly as the Jak2 V617F mutation and might be an alternative mechanism in patients without any activating mutation. *Aims*. Elucidation of the methylation status of CpG islands of SOCS1, SOCS3 and PTPN6 in blood of 48 CMPD patients (36 Jak2 V617F mutated, 12 wildtype) as compared to 22 healthy controls. *Methods.* DNA was prepared from blood of 21 patients with polycythemia vera (PV), 19 patients with essential thrombocythemia (ET), 8 patients with myelofibrosis (MF) and 22 healthy controls. Methylation-specific PCR (MSP) for SOCS1 exon2, SOCS3 exon2, and PTPN6 promoter was performed according to Capello D. (Br J Haematol 141, 2007), for SOCS3 intron corresponding to Fernandez-Mercado M. (Leuk Res 32, 2008). *Results*. SOCS1 exon2 was methylated in 46%, 62%, 32% and 13% of healthy controls, PV, ET and MF, respectively. SOCS3 exon2 was methylated in all patients and controls, whereas SOCS3 intron and PTPN6 methylation was only detected by increasing the number of PCR cycles to 40. Accordingly, SOCS3 intron methylation was detected in 36% of healthy controls and in 19% PV, 26% ET and 38% MF, while PTPN6 promoter methylation in 9%, 19%, 0% and 13%, respectively. Between Jak2 V617F positive and negative CMPDs no significant differences in the frequency of methylation of these four genomic regions were observed so far. *Conclusions*. Methylation of CpG islands in the examined Jak2 pathway suppressing genes were found in the peripheral blood from CMPD patients as well as from healthy controls. The methylation status observed in this study joins the conflicting results from previous publications. However, whether differences between PV, ET and MF patients observed is of any clinical relevance should be analysed further on.

0881

AME AFFECTS MEGAKARYOCYTIC DIFFERENTIATION AND INCREASES PRO-PLATELATS FORMATION *IN VITRO*

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Background. The t(3;21)(q26;q22) resulting in the AML1/MDS1/EVI1 (AME) fusion gene is associated with several hematological disorders often characterized by severe dysmegakaryopoiesis, suggesting that AME alters the megakaryocytic maturation program. *Methods*. We expressed the AME fusion protein in bone marrow lineage negative cells by retroviral infection. The cells were cultured in presence of TPO to induce megakaryocytic differentiation and on day 2 the morphology was analyzed after Wright Giemsa staining. We also expressed AME in the human erythromyeloblastoid cell line K562. PMA was used to induce megakaryocytic differentiation. The cells were collected after 48 and 96 hours and then compared with the control differentiated K562. The DNA content was measured after propidium iodinate (PI) staining by FACS analysis. *Results*. In the AME positive cultures we counted a 5 fold increase (35%) of MKs percentage compared with the control cells

(7%). AME positive MKs were smaller in size in comparison to the control. In AME cultures we frequently observed micro-megakaryocytes and other features of dismegakaryopoiesis. During 12 days in liquid culture in presence of TPO, while the control MKs persisted for the entire experiment, the AME positive MKs quickly decreased after 7 days. AME positive MKs produced platelets until day 6. The platelets appeared immature, bigger in size with several features of pro-platelets. The AME platelets were found 10 fold increased as compared to controls and their formation was prolonged for more than 7 days while the control MKs produced platelets for only 2 days. AME-K562 showed a block in megakaryocytic differentiation. As previously demonstrated in murine cells, also AME-differentiated-K562 were smaller in size in comparison to the controls. The DNA content measured after 96 hours, pointed out a significant lower (4n) level of polyploidy in the AME positive cells compared with the controls (8n). We also checked in the K562 cell line by semi-quantitative PCR the expression of several regulatory genes implicated in megakaryopoiesis. Before PMA incubation, AME positive cells showed a slight up-regulation of GATA1, cMPL, PF4 and BCL2, while NFE2 and BCL-XL were slightly down-regulated. Interestingly, after PMA stimulation, except GATA1 and BCL-XL that were up-regulated, all the others were strongly down-regulated. *Conclusions*. It seems that AME interferes with the megakaryocytic program, causing impairment in MKs differentiation with arrest in polyploidization, increased apoptosis and overproduction of immature platelets. The molecular mechanism appears complex, affecting concurrently transcription factors essential for MKs differentiation, apoptosis regulatory genes and genes implicated in platelets production.

0882

CONTRIBUTION OF BONE MARROW DERIVED CELL ANGIOGENESIS IN BCR/ABL NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASE

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Background and Aims. Circulating cells derived from bone marrow (BM) have been reported to induce and modulate angiogenesis and vasculogenesis in condition of ischemia and/or cancer. Endothelial progenitor cells (EPCs) are non hematopoietic bone marrow-derived stem cells that can home into the site of active neo-vascularization and are found increased in setting of enhanced angiogenesis. Recent studies underline the role of other subsets of hematopoietic BM-derived cells in triggering vessel growth. They contribute by releasing pro-angiogenic factors and by directly incorporating into nascent blood vessels and are characterized by the expression of the angiopoietin receptor Tie2/Tek or by the presence of VEGFR-1. Increased angiogenesis is thought to have a central role in bcr/abl negative chronic myeloproliferative disorders (CMPDs). To assess the role of BM-derived cells in this setting, we evaluated the level of EPCs, Tie2 and VEGFR-1 expressing circulating cells in a cohort of bcr/abl negative CMPDs. We additionally evaluate microvessel density (MVD) on BM biopsies and a set of cytokines related to angiogenesis. *Methods*.We analyzed 88 patients composed as follow: ET (25), PV (18), CIMF (45) and 25 controls. EPCs (defined as $CD34^+/CD133^+/CD45^-$), $TIE2^+/CD45^+$ and $VEGR1^+/CD45^+$ cells were evaluated by flow cytometry. Serum concentration of VEGF, Ang-1, Ang-2 and FGFb were assessed by commercial ELISA kits. Forty-one BM biopsies were available; MVD was evaluated using the 'hot spots' method. The data were statistically analysed using the Mann-Whitney U test for between-group comparisons and Spearman's correlation coefficient to evaluate the associations between variables. $p<0.05$ was taken as a cut-off point for statistical significance. *Results*. Bcr/abl negative CMPDs were characterized by elevated EPCs (median: 0.17/mcl [range: 0.00-2.40] vs 0.04 [0.00-0.27] $p<0.0001$), VEGF (1940 pg/mL [200-18351] vs 574 [92-1241] $p=0.0005$) and FGFb (33 pg/mL [0.5-269.6] vs 22.4 [7.6-46.1] $p=0.02$) and BM-MVD (24.3 vessels/hpf [5.3-86.2] vs 6.0 [4.6-16.3]). No differences were found regarding the other cytokines evaluated. Absolute and relative number of $VEGFR1^+/CD45^+$ were increased in CMPDs than controls (2086.7/mcl [333.0-9954.6] and 32.9% of total WBC [6.1-90.4] vs 953.2 [436.5-2597.2] and 16.1% [4.9-34.8], $p=0.01$). $TIE2^+/CD45^+$ cell number was not different than controls, they were positively correlated with serum VEGF ($p=0.03$), Ang-1 ($p=0.04$) and bFGF ($p=0.02$). Most of the $TIE2^+/CD45^+$ cells expressed CD14 (73% [21,2-93,1]), and $TIE2^+/CD45^+/CD14^+$ still correlate with the same cytokines while the $CD14^-$ part does not; moreover, they were negatively correlated with BM-MVD ($p=0.007$) *Conclusions*. CMPDs patients have an abnormally enhanced angiogenesis as shown by high EPCs, VEGF, bFGF and BM-MVD. In this group of patients $VEGFR1^+/CD45^+$ cells are found increased while the $TIE2^+/CD45^+$ are correlated with serum markers of angiogenesis and BM-MVD. These preliminary data put forward the concept that different subset of cells can contribute to the pro-angiogenic phenotype of bcr/abl negative CMPDs. It may be therefore interesting to further explore the role of these cells in a setting of pathological maturation and proliferation of the hematopoietic BM compartment as in CMPDs.

0883

STUDY OF MEGAKARYOPOIESIS AND PROPLATELET FORMATION IN PRIMARY MYELOFIBROSIS

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Background. Primary myelofibrosis (PMF) is an uncommon chronic myeloproliferative disorder. Several abnormalities that could explain the myeloproliferation have been reported, and most important is a striking involvement of the megakaryocyte (Mk) lineage, with hyperplasia and dysplasia resulting in an excessive production of several cytokines and chemokines. Whereas molecular defect(s) associated with the development of PMF have been described, its pathogenesis is not thoroughly elucidated. *Aims*. The general objective of this research is to study the alterations of megakaryopoiesis in PMF and to correlate it to histological and clinical features of the disease. *Patients and Methods*. We analyzed *in vitro* Mk differentiation and maturation, as well as proplatelet formation (PPF) in 8 patients diagnosed with PMF. Mks were differentiated from peripheral blood $CD34^+$ cells for 12 days in the presence of TPO. Mature Mks were grown in suspension or plated onto glass coverslips coated with collagen I or fibrinogen (FNG). Mk differentiation-maturation and PPF were evaluated by phase contrast and fluorescence microscopy upon cell staining with anti α-tubulin and CD41 antibodies. Measurement of Mk diameters was performed on acquired images, at least one hundred Mks were analyzed for each sample. Mk ploidy was evaluated by flow cytometry after staining with propidium iodide. Controls were analyzed in parallel with each patient sample. *Results*. Immunomorphological evaluation of cultured progenitors at day 12 revealed that the percentage of $CD41^+$ cells with Mk morphology was slightly increased, but not significantly different in patients and relative controls. Moreover, classification of Mk morphology, according to standard criteria, revealed no significant differences in the differentiation profiles between patients and controls. However differences were observed in the maturation pattern as, at the end of the culture, patients presented a significant decreased number of very mature, large, polyploid Mks with respect to controls. When reseeded in fresh medium, mature Mks derived from peripheral blood of both patient and control extended proplatelets. However, a time course analysis revealed a reduction of PPF by Mks derived from the patient: in fact, while 10±1.9% of control Mks were forming proplatelets after 16-24 hours of culture, the percentage of Mks with PPF from the patient's peripheral blood was 2.5±1.9%. In morphologic analysis proplatelets derived from patient peripheral blood presented a significant defect in branching, which resulted in a lower number of proplatelet tips formed by each megakaryocyte. Interestingly, some patients presented thrombocytopenia that was positively correlated to proplatelet decreased numbers and branching defects. *Summary and Conclusions*. Mks from patients affected by PMF presented both quantitative and qualitative abnormalities of *in vitro* PPF. The decreased capacity of completing maturation was positively correlated with decreased Mk ploidy and diameter. Altogether these data indicate that, although patients' peripheral blood progenitors easily differentiate through the megakaryocytic lineage, PMF affects the final steps of Mk maturation, proplatelet formation and platelet release.

0884

MURINE MYELOPROLIFERATIVE DISORDER WITH LIVER LESION: CHARACTERISTICS OF LEUKEMIA STEM CELLS AND PHENOTYPE OF CELLS INVADED LIVER

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Background. Tumors are morphologically and functionally heterogeneous, whether due to their hierarchical structure or due to stochastic processes. The stochastic model predicts that all tumor cells are biologically homogeneous, equally subjected to unpredictable reversible influences resulting in morphological and functional heterogeneity. The hierarchy model suggests the existence of leukemic stem cells (LSC) capable of self-maintenance that can mature to generate heterogeneous progeny that lack stem cell properties. If stochastic model is correct LSC activity may be observed in any tumor fraction and most of leukemic cells have potential for tumor initiation. *Aims*. The aim of this study was the investigation of LSC in different cell populations with phenotype of stem and committed cells in hematopoietic stem cells hierarchy. *Methods*. The model of transplantable MPD-like myeloid leukemia with histiocytic sarcoma was used. Bone marrow (BM) and liver cells of affected mice were fully transplantable, recipients became moribund within 17-32 days since cells injection. All the ill animals had enlarged liver (M 4,1±0,1 g versus normal 1,44±0,03 g) and spleen (M 304,9±15,5 mg versus normal 93,1±1,8 mg). BM and liver cells from the moribund mice were stained with antibodies against different surface markers and analysed with FACS, simultaneously BM cells were sorted by Miltenyi Biotec® MACS® magnetic separation columns and injected into syngeneic recipients. Limiting dilutions of cells were used in some sorted populations to evaluate the concentration of cells initiating leukemia. *Results*. Phenotype of leukemic and normal BM cells didn't differ. Each injected population of the cells led to the development of fatal leukemia: c-kit-CD45- in 23.9, c-kit+ in 22.2, c-kit-CD45+ in 15.4, Ter119+ in 18.2 and Ter119- in 17.7 days. The data suggest that: neoplastic transformation may take place in very early progenitor cells (c-kit-CD45-); myelopoiesis is involved; the ability to give rise to leukemia is present even in cells committed to erythroid differentiation. The analysis of leukemia initiating cells' concentration by limiting dilutions of c-kit+ BM cells and unsorted liver cells of the ill mice was made with Poisson statistics. It turned out to be 1 cell per 37000 c-kit+ cells and 1 cell per 45 unsorted cells from the liver. Taking into consideration the differences in time of disease development, the frequencies of leukemia initiating cells in other cell populations were calculated as 1 LSC per 2500000 c-kit-CD45- cells, 200 c-kit-CD45+ cells, 4500 Ter119+ cells, 2600 Ter119- cells. So, the LSC properties in the described disease preserved in cells committed to erythroid and myeloid lineages. The concentration of cells capable to transfer leukemia increased with differentiation from c-kit-CD45- to c-kit-CD45+ cells population. The frequency of leukemia initiating cells reaches maximum in liver invading population. *Conclusions*. Leukemic stem cells retain hierarchical organization in the studied model and are able to differentiate at least among myeloid and erythroid lineages without the loss of self-renewing ability. However LSC activity was observed in all tested fractions that support the stochastic model of tumor heterogeneity. So, this murine leukemia combines two models of tumor heterogeneity.

0885

EPIGENETIC CHANGES IN JAK2V617F POSITIVE MYELOPROLIFERATIVE DISORDERS

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Background. There is increasing evidence that, in addition to genetic alterations, epigenetic changes play an important role in the pathogenesis of hematopoietic malignancies. Aberrant methylation of CpG islands near gene promoter regions has been established as an important mechanism for gene silencing. Hypermethylation of genes involved in the JAK-STAT signalling pathway has been recently reported in Cronic Myeloproliferative Disorders (CMPDs) in alternative or together with JAK2 mutations. *Aims*. In order to further investigate the role of DNA methylation changes in the pathogenesis of CMPDs, we analyzed the methylation status of the promoter-associated CpG islands of 8 cancer-related genes by methylation-specific polymerase chain reaction after DNA bisulphite modification. *Methods*. Forty-seven patients with Chronic Myeloproliferative Disorders (10PV, 10ET, 5MF, 4CML, 14CMPD unclassifiable) who have been previously characterized for the presence of JAK2V617F mutation were analyzed. Four peripheral blood from controls were also evaluated. The target genes chosen are involved in many cellular pathways as cell cycle regulation (p16INK4a, PTEN), cell-cell adherence (E-cadherin), the canonical Wnt pathway (WIF1, DKK3) and JAK/STAT3 signalling pathway (SOCS1, SOCS3, SHP1). The correlation between methylation and JAK2 mutation status was assessed by Fisher exact test or Chi-square test. *Results*. Overall, 44 out of 47 patients (93%) presented at least one hypermethylated gene and 38% (18/47) had aberrant methylation in more than two genes. Hypermethylation was detected in 22% of patients for p16, 51% for DKK3, 23% for WIF1, 58% for E-cadherin, 14% for SHP1. In our casistics SOCS-1 and SOCS-3 resulted methylated in 83% and 100% of patients, respectively, while PTEN was methylated in only two patients with CML and unclassifiable CMPD. Only 5/47 cases (10.6%) resulted simultaneously methylated for p16, DKK3, E-Cadherin, SOCS1. Methylation frequencies did not differ between mutant and wildtype JAK2 carriers. However, analysis of the methylation patterns among JAK2V617F positive patients showed that WNT antagonist genes were more frequently methylated than p16 and SHP1 and the difference was statistically significant with a p value of 0.002. *Conclusions*. In summary, we found that either aberrant methylation of WNT antagonists or the absence of p16 or SHP1 epigenetic inactivation may be cooperating with JAK2V617F mutation in myeloproliferative disease.

0886

PRIMARY MYELOFIBROSIS CD34+ CELLS SHOW DIFFERENT CXCR4 CHEMOKINE RECEPTOR PROFILES AT SITES OF DISEASE ACTIVITY

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Background. In previous studies we found that the reduced expression of CXCR4 by peripheral blood (PB) CD34+ cells is a characteristic of Primary Myelofibrosis (PMF). We documented that the number of circulating CD34+CXCR4+ cells in PMF patients, as well as the membrane CXCR4 expression, was directly related to CXCR4 mRNA level and that reduced CXCR4 mRNA level was not due to SDF-1-induced down-regulation but to a hypermethylation of CXCR4 promoter CpG island 1. Therefore, abnormal methylation of the CXCR4 promoter has been suggested to be a mechanism contributing to constitutive migration of CD34+ cells in PMF. *Aims*. To investigate the pattern of CXCR4 receptor expression on CD34+ cells from PB, bone marrow (BM) and spleen of patients with PMF. *Methods*. We studied PB and BM paired samples in 16 patients, and PB and spleen paired samples in 8 patients with PMF. Patients had been splenectomized for refractory and symptomatic splenomegaly. After extensive rinsing, each spleen sample was minced to obtain a single cell suspension. PB, BM, and spleen cells were stained with FITC-CD34 and PE-CXCR4 monoclonal antibodies. The expression of CXCR4 was evaluated as percentage and mean fluorescence intensity (MFI) of CD34+ gated cells. *Results*. In patients with PMF the percentage of PB CD34+ cells expressing CXCR4 (34%, 9.1-68.7%) was significantly higher than that found in BM CD34+ cells (19.8%, 2.7-57.8%)(Wilcoxon paired test, p=0.019), Similarly, MFI of the CXCR4 antigen on CD34+ cells from PB (0.6, 0.1-2.4) was significantly higher than that of BM (0.3, 0.05-2.2) (Wilcoxon paired test, p=0.015). In spleen tissue, percentage of CD34+CXCR4+ cells (10.7%, 3.9-20.0%) and MFI (0.3, 0.2-0.7) were decreased with respect to the values found in PB. We had no paired samples of bone marrow and spleen tissue, but the percentage and the MFI of CD34+CXCR4+ cells from spleen tissues were not statistically different from those of BM. Interestingly, the percentage of CD34+CXCR4+ cells obtained in apheresis samples of 3 healthy subjects mobilized with G-CSF (11.3, 7.6-13.4) was comparable to that found in cells from spleen tissues. Summary These data suggest that CD34+ cells of patients with PMF display different CXCR4 profiles in different sites of disease activity. Micronvironmental factors might play a role in stem cell functional behaviour.

0887

IDIOPATHIC HYPEREOSINOPHILIC SYNDROME IN CHILDREN: MOLECULAR ANALYSIS AND CLONAL EVOLUTION

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Background. Idiopathic hypereosinophilic syndrome (HES) is a rare hematologic disorder characterized by unexplained sustained overproduction of eosinophils in the bone marrow, persisting eosinophilia (>1.5×10^9/L for>6 months), tissue infiltration and organ damage. In the majority of HES, diagnosis is based on exclusion criteria and frequently remains doubtful due to the lack of genetic markers. Early identification of HES eventually evolving towards a lympho-myeloproliferative disease is relevant in light of its obvious prognostic and therapeutic implications. Only few cases of pediatric HES are described in literature. *Aims*. To analyse molecular features and clinical outcome of 10 pediatric patients with idiopathic HES. *Methods*. BCR-ABL and FIP1L1/PDGFRA fusion genes were analysed by routine RT-PCR. WT1 gene copy number was assessed by RQ-PCR using Taqman technology. TCR clonality was investigated by DHPLC-sequencing of TCRb genomic regions. IgH clonality was performed amplifying CDR-I, CDR-II and CDR-III regions using oligo-degenerate-sequence primers mapping the frameworks FR2 and FR3 of variable and joining-Heavy chain of immunoglobulin genes. *Results*. All patients had normal karyotype on conventional cytogenetics. T-cell receptor analysis disclosed germline configuration in all cases. Five children showed a IgH clonality (FR2-CDRII region) at presentation; of these, 2 developed a B-NHL and a B-lineage ALL at 6 and 12 months, respectively, with tumor cells harbouring the same IgH monoclonal rearrangement documented at diagnosis; 2 had spontaneous reversion to polyclonal IgH profile in the follow-up, and the last one persisted with HES without B-clonal evolution at 19 mos. The PDGFRA/FIP1L1 fusion gene was identified in 1 patient who achieved hematologic and molecular remission after treatment with imatinib mesylate. Three of the 6 patients analysed for WT1 had over-expression and 3 had gene expression levels within normal range. No correlation was found between WT1 expression and HES clonality. *Conclusions*. IgH rearrangement is a frequent molecular feature of pediatric HES (50%) and may precede B-clonal expansion and evolution into B-cell malignancies, although spontaneous regression may occur. The analysis of more cases with detailed molecular characterization including IgH gene status and WT1 is warranted to identify features predictive of clonal evolution.

Myeloproliferative disorders - Biology and Clinical

0888

CONSISTENT UP-REGULATION OF STAT3 INDEPENDENTLY OF JAK2 MUTATIONS IN A NEW MURINE MODEL OF ESSENTIAL THROMBOCYTHEMIA

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Background. Janus-activated kinase 2 (JAK2) mutations are common in myeloproliferative disorders; however, although they are detected in virtually all polycythemia vera patients, they are found in approximately 50% of essential thrombocythemia (ET) patients, suggesting that converging pathways/abnormalities underlie the onset of ET. Recently, the chromosomal translocation 3;21, leading to the fusion gene AML1/MDS1/EVI1 (AME), was observed in an ET patient. *Methods and Results*. After we forced the expression of AME in the bone marrow (BM) of C57BL/6J mice, all the reconstituted mice died of a disease with symptoms similar to ET with a latency of 8 to 16 months. Peripheral blood smears consistently showed an elevated number of dysplastic platelets with anisocytosis, degranulation, and giant size. Although the AME-positive mice did not harbor Jak2 mutations, the BM of most of them had significantly higher levels of activated Stat3 than the controls. With combined biochemical and biological assays we found that AME binds to the Stat3 promoter leading to its up-regulation. *Conclusions*. Signal transducers and activators of transcription 3 (STAT3) analysis of a small group of ET patients shows that in about half of the patients, there is STAT3 hyperactivation independently of JAK2 mutations, suggesting that the hyperactivation of STAT3 by JAK2 mutations or promoter activation may be a critical step in development of ET.

0889

ELEVATED PRV-1 PLASMA LEVEL IS A RISK FACTOR FOR TRANSIENT ISCHEMIC ATTACK (TIA) IN ESSENTIAL THROMBOCYTHEMIA

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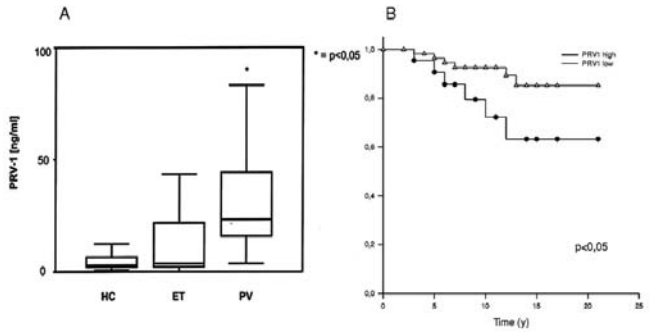
Background. PRV-1 mRNA overexpression has been described as a molecular marker in patients with myeloproliferative disorders (MPD). The PRV-1 gene is overexpressed in over 90% of Polycythemia vera (PV) patients and approximately 50% of patients with Essential thrombocythemia (ET) and Primary myelofibrosis (PMF). While PRV-1 mRNA overexpression is diagnostically sensitive for MPDs, there is a large overlap between PRV-1 protein surface expression on neutrophils in healthy controls and MPD patients. However, PRV-1, a member of the uPAR/CD59/Ly6 family of GPI-linked cell surface receptors, is shed into the plasma. Since there are very few predictive markers for the development of thromboembolic complications in patients with ET, we assessed the utility of PRV-1 plasma concentration as a biomarker for the prediction of thrombotic episodes. *Aims.We* therefore determined PRV-1 plasma levels in MPD patients and age matched healthy controls. Subsequently we correlated elevated PRV-1 plasma concentrations with clinically recorded thrombo-embolic episodes in a prospectively collected cohort of 89 ET patients. *Patients and Methods*. PRV-1 protein concentration was measured using a sandwich ELISA comprised of two monoclonal antibodies generated in our laboratory. A purified PRV-1 protein-standard curve was used for quantification. PRV-1 plasma levels were detected in a prospectively collected cohort of 89 ET patients, a cross sectional cohort of 55 PV patients as well as 100 age matched healthy controls. Kruskal-Wallis One Way ANOVA on Ranks and the Mann-Whitney Rank Sum test were used to compare PRV-1 plasma level between cohorts. The ET cohort [38 males (43%) and 51 females (57%)] was prospectively monitored for thromboembolic complications including the occurrence of stroke, transient ischemic attack (TIA), deep vein thrombosis (DVT) and occlusive artery disease (OAD) with a median follow up of 12 years (range 2-21 years). Median age at diagnosis was 55 years (range 20-85). Fisher's Exact Test as well as Kaplan Meier survival estimates were used to assess the relationship between PRV-1 plasma-level and thrombo-embolic events. *Results*. PRV-1 plasma levels were significantly elevated in PV patients compared to either healthy controls or ET patients (Figure 1A). However, among ET patients, a large range of PRV-1 concentrations were observed, prompting us to divide this pop-

ulation into two groups. ET patients with PRV-1 plasma levels less than one standard deviation above the median of the healthy controls (<21ng/mL) were considered PRV-1 low (n=64), while ET patients with PRV-1 plasma levels more than one standard deviation above the median (>21ng/mL/n=25) were considered PRV-1 high. Comparison of the thrombotic events in the two groups of ET revealed a significantly higher incidence of TIA in the PRV-1 high patients [p=0,014 with Fisher's exact (one-sided) and p<0,05 with Kaplan Meier survival estimate(Figure 1B)]. Likewise the combined endpoint of TIA and stroke occurred at a significantly higher incidence in PRV-1 high ET patients [p=0,037 with Fisher's exact (one-sided) and p<0,05 with Kaplan Meier survival estimate]. PRV-1 plasma levels were independent of the white blood cell (WBC) count, suggesting that it constitutes an independent parameter for the assessment of thrombotic risk in ET. *Conclusions*. We could identify two different groups of ET patients are heterogeneous with respect to their PRV-1 protein plasma levels. In our prospectively analyzed cohort, individuals with high PRV-1 plasma levels seem to have a higher probability for developing either a TIA or a stroke.



(A) The level of PRV-1 plasma protein was measured with a sandwich ELISA in 55 PV and 89 ET patients as well as 100 age matched healthy controls (HC). PRV-1 concentrations between groups were analyzed using Kruskal-Wallis One Way ANOVA on ranks and the Mann-Whitney Rank Sum test (* p<0,05 for PV vs. healthy control and vs. ET). (B) Kaplan Meier survival estimates for the probability of development of a TIA in ET patients with high and low PRV-1 plasma levels (p<0,05 for PRV-1 high ET vs. PRV-1 low ET).

Figure 1. PRV-1 Plasma levels in MPD.

0890

INCREASED EXPRESSION OF THE ATP9A GENE IN ESSENTIAL THROMBOCYTHEMIA

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Essential thrombocythemia (ET) is a chronic myeloproliferative disorder whose molecular pathogenesis has been associated predominantly with a somatic mutation in the JAK2 gene (JAK2V617F). However, JAK2V617F is not present in approximately half of ET patients and those that are positive for the mutation display hematological and clinical features that more closely resemble polycythemia vera. These data indicate that other genetic abnormalities may account for megakaryocyte hyperplasia in ET. We have previously found an ET patient with a 550-kilobase deletion at chromosome 20q13 that included the nuclear factor of activated T-cells cytoplasmic 2 (NFATC2), ATPase class II type 9A (ATP9A) and SAL-like 4 (SALL4) genes. We observed that diminished NFATC2 transcription factor activity as a result of drug-induced inhibition of NFATC2-calcineurin interaction leads to megakaryocyte proliferation and decreased cytokine expression in an ET-derived cell line (SET-2). We now report molecular analysis of the yet uncharacterized ATP9A gene in ET patients. Bioinformatic analyses predicted that ATP9A contains 7 plasma transmembrane regions and shares a 39% aminoacid similarity with plasma membrane Ca^{2+} atpase 4b (PMCA4b) protein including a putative calcineurin-binding domain. This indicates that ATP9A may regulate the activity of NFATC2 through interaction with calcineurin. Screening of the entire coding sequence (28 exons) of ATP9A in granulocyte DNA (6 samples) and mononuclear cell mRNA (16 samples) by polymerase chain reaction (PCR) and direct sequencing showed no presence of mutations in ET patients. We also used quantitative-PCR to determine ATP9A mRNA levels in granulocytes and mononuclear cells isolated from peripheral blood of ET patients and normal individuals. Quantification of ATP9A expression was normalized to ABL transcript numbers. We found that the ATP9A gene is expressed at significantly higher levels in ET patients compared to normal individuals in either granulocytes (t-test: p<0.001) or mononuclear cells (Mann-Whitney test: p<0.05). No significative differences were found between the level of ATP9A expression and JAK2V617F-positivity (14 patients) or JAK2V617F-negativity (10 patients) in granulocyte DNA (Mann-Whitney test: p=0.266). ATP9A mRNA expression levels in SET-2 cells were not affected using siRNA-mediated NFATC2 suppression or inhibition of NFATC2-calcineurin interaction, indicating that NFATC2 does not regulate transcription of ATP9A. In conclusion, our results indicate ATP9A as a novel candidate gene that operates in the calcineurin-NFATC2 pathway and reinforce the notion that this pathway plays a role in the etiology of ET.

0891

SOCS3 TYROSINE PHOSPHORYLATION AS A BIO-MARKER FOR THE MYELOPROLIFERATIVE DISORDER-ASSOCIATED MUTANT JAK2 KINASES

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Background. Polycythaemia vera (PV) is a myeloproliferative disorder (MPD) attributed to a somatic mutation in Janus kinase (JAK) 2, resulting in a valine-to phenylalanine substitution at position 617 (V617F). Recently, other mutations within JAK2 have been identified in patients with V617F-negative PV. Like JAK2 V617F, these exon 12 mutations render JAK2 constitutively active and confer cytokine-independent growth *in vitro*. Suppressor of cytokine signalling (SOCS) 3 is a key negative regulator of the erythropoietin receptor (EPOR) and the receptor-associated JAK2 kinase. We have shown before that JAK2 V617F tyrosine phosphorylates SOCS3 and escapes its inhibition. *Aims*. To establish if SOCS3 is stabilised and phosphorylated by the other MPD-associated mutant exon 12 JAK2 kinases. *Methods*. Transfection of EPOR, SOCS3 and JAK2 mutants into HEK293T cells followed by analysis of protein expression and phosphorylation; Analysis of proliferation of stable BaF3 cells lines expressing EPOR along with wt and mutant JAK2; Treatment of cells with JAK inhibitors; Analysis of SOCS3 phosphorylation in PBMCs and granulocytes isolated from a healthy control or MPD patients with JAK2 exon 12 mutations. *Results*. The JAK2 exon 12 mutants described in a subset of V617F-negative MPD cases are able to stabilise tyrosine phosphorylated SOCS3. SOCS3 tyrosine phosphorylation was also observed in peripheral blood mononuclear cells and granulocytes isolated from patients with JAK2 H538QK539L or JAK2 F537-K539delinsL mutations. JAK kinase inhibitors, which effectively inhibited the proliferation of cells expressing V617F or K539L, also caused a dose-dependent reduction in both mutant JAK2 and SOCS3 tyrosine phosphorylation. *Conclusions*. We propose therefore that SOCS3 tyrosine phosphorylation may be a novel bio-marker of MPDs resulting from a JAK2 mutation and a potential reporter of effective JAK2 inhibitor therapy currently in clinical development.

0892

CONCURRENT JAK2 V617F MUTATION AND BCR-ABL REARRANGEMENT IN A ESSENTIAL THROMBOCYTHEMIA PATIENT

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Background. Myeloproliferative disorders (MPDs) are clonal hematopoietic diseases, distinct from Philadelphia chromosome positive (Ph^+) chronic myeloid leukemia (CML) for the absence of BCR-ABL. Previously the coexistence of BCR-ABL fusion transcript and JAK2V617F mutation has been reported in a few cases of both Polycythemia Vera (PV) and Myelofibrosis, their coexistence appearing during the evolution of the disease. Only in one case of myelofibrosis and one case of PV these two abnormalities were observed at diagnosis (Cambier 2008). We described here a 46-year old woman referred at our hospital because of marked thrombocytosis (1406×10^3/μL PLTS) and leukocytosis (27.9×10^3/μL WBC); Hb levels were normal and differential leukocyte count showed: neutrophils 70%, eosinophils 1%, basophils 4%, lymphocytes 14%, monocytes 7%, metamyelocytes 1%, myelocytes 3%. She showed erythromelalgia at both feet and complained of intermittent left limbs paresthesia. Molecular investigation showed a BCR-ABL

fusion transcript b2a2b type and AS-PCR detected the JAK2V617F mutation. Conventional karyotype and FISH analysis confirmed the presence of t(9;22) in 100% of cells. *Aims*. To evaluate the concomitant BCR-ABL rearrangement and JAK2V617F mutation in the same clone, we performed clonogenic assays. Patient follow-up was monitored using real-time quantitative-PCR for both molecular abnormalities. *Methods*. Erythroid colonies cultures were prepared by isolating peripheral blood mononuclear cells on Ficoll-Hystopaque density gradient (Sigma-Aldritch); harvested cells were washed and risospended in RPMI medium solution (Sigma-Aldritch). Mononuclear cells were plated at final concentration of 2.5 -105 cells/mL in Methocult medium with 3 units/mL Epo (Methocult GF H4434) and without Epo (Methocult GF H4534), (StemCell Technologies). Cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2. Erytroid colonies were scored on day 14 and 19. Thirty-eight individual colonies were plucked and submitted to RNA extraction following RNAqueous-Micro Micro scale RNA isolation kit instruction manual (Ambion 1931). BCR/ABL rearrangement and JAK2V617F were tested using qualitative-PCR assay. Furthermore quantitative-PCR for bcr/abl and JAK2 was performed at diagnosis and in 3 follow-up samples collected monthly during Imatinib treatment. *Results*. As previously described for chronic phase CML patients the culture pattern was characterized by excessive scattering of CFU-GM and poorly hemoglobinized BFU-E with atypical morphology. None of the colonies showed the coexistence of both molecular abnormalities. During the follow-up, quantitative-PCR analysis showed a 2,37 logarithmic decreased of BCR/ABL copy number, whereas the JAK2 V617F allele burden showed a trend towards a progressive decrease from 46% at diagnosis to 32% in the last sample. *Conclusions*. Here we report the first case of ET with V617F mutation and concomitant Ph^+ CML in a patient at initial presentation. Clonogenic assays suggest the coexistence of two distinct myeloproliferative disorders involving different clones. Imatinib therapy induced normalization of the patient's blood white cells and platelets count within two months, with a decrease in both BCR/ABL fusion transcript and JAK2 V617F allele burden, contrary to previous observations about a JAK2 V617F clone expansion in patients who responded to CML therapy.

0893

NATURAL EVOLUTION OF JAK2V617F ALLELE BURDEN IN POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA PATIENTS NOT RECEIVING CYTOREDUCTIVE TREATMENT

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Background. The natural evolution of the mutant allele burden in JAK2V617F-positive polycythemia vera (PV) and essential thrombocythemia (ET) patients not receiving cytoreductive therapy has not been extensively studied. *Aims*. To analyze the evolution of the JAK2V617F allelic burden in untreated PV and ET patients. *Patients and Methods*. A cohort of 45 JAK2V617F-positive patients (21 PV and 24 ET) consecutively diagnosed in a single institution according to WHO criteria were included in a prospective study. No patient received cytoreductive treatment during the study period. PV patients were treated with phlebotomies to maintain an Hct below 0.45 L/L (M) and 0.42 L/L (F), plus low-dose of aspirin. ET patients were clinically observed and/or received aspirin. JAK2V617F allelic burden was determined at diagnosis and every 6-12 months during follow-up. JAK2V617F was quantified in purified granulocytes by allele-specific real-time PCR assay. Determination of the relative change of the allelic ratio of JAK2V617F was calculated as follows: (% last JAK2V617F minus % first JAK2V617F) divided by % first JAK2V617F x 100. *Results*. Median follow-up was 49 (9-72) and 45 (9-67) months for PV and ET, respectively (p=0.211). All ET patients and 16 out of 21 (76%) PV patients were heterozygous for the JAK2V617F mutation; the remaining five PV patients were homozygous. A mean number of 5 samples per patient were drawn during the study. Thirty-eight patients (84%) were evaluated at month 24. The mean percentage of JAK2V617F allele load at time of first sample was 45±20 and 25±7 for PV and ET, respectively (p<0.001). The mutant allele load at 24 months was 65±48 for PV patients and 26±7 for ET patients, with the difference being statistically significant (p=0.012). The mean JAK2V617F allele variation was 31±40 and 6±18 for PV and ET, respectively (p=0.02). *Conclusions*. The natural evolution of JAK2V617F allele burden showed a progressive increase in PV whereas it remained stable in ET at 2 years of follow-up.

0894

POLYCYTHEMIA VERA (PV) BUT NOT ESSENTIAL THROMBOCYTHEMIA (ET) REDUCES THE LIFE EXPECTANCY IN ELDERLY

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Background. Malignancies are more common in old people. Also myeloproliferative neoplasms interest mainly median-advanced age, with a mean age at diagnosis of 60-70 years. A Sweden study has demonstrated that the incidence of PV and ET increases progressively with age, achieving 12-16/100000 inhabitants/year and 5-7/100000/year respectively in patients over 70 years. Life expectancy is the main concern of patients with PV and ET at the time of the disease diagnosis, but the studies on this issue are relatively scarce. There is some evidence that life expectancy of PV patients is reduced when compared with that of the general population, whereas the survival of patients with ET is not significantly shortened. In this prospective observational study over 7 years we evaluated the survival of old patients with PV and ET compared with that of a control population. *Patients*. Our study included 205 patients (PV 102 cases, 45 males and 57 females; ET 103 cases, 30 males and 73 females) diagnosed in our Department in agreement with the criteria in use at the time of first observation. All the patients were older than 65 years at the time of diagnosis. As controls we used 3099 (1245 men and 1854 women) age- and sex-stratified subjects of the Progetto Veneto Anziani (Pro.V.A.) Study, an observational cohort study of the Italian population aged 65 years and older. Both patients and controls lived in the same geographical area of east-south Veneto, Italy. *Statistical analysis*. The Kaplan-Meier product-limit method was used to estimate univariate survival curves and the log-rank test was adopted to compare the survival curves. The chi-square test was used for categorical variables. All analyses were performed using SPSS 16.0 software. *Results*. During the 7 years follow-up period, 64 (62.7%) patients with PV, 42 (40.7%) with ET and 745 (24%) controls were died. The death rate of PV was higher than that of ET (p=0.0016). Therefore, the survival probability of PV patients resulted lower than that of ET (p=0.012). Comparing the survival of patients with that of general population we did not find a significant difference; however, while the survival curve of ET is superimposed to the curve of general population, the survival curve of PV appeared clearly diverging (Figure 1). *Discussion*. This study shows that, among old people, PV patients have a life-expectancy reduced as compared to ET patients, while the life-expectancy of ET does not seem different from that of the general elderly population. In spite of a higher death rate of PV than ET patients the differences of the survival curves is not statistically different. When compared to control population probably because of a low number of PV patients.

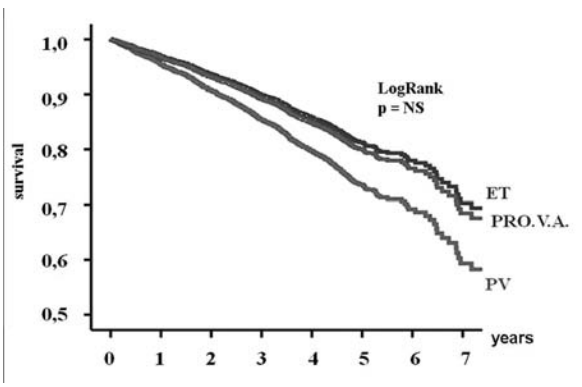

Figure 1.

0895

ANALYSIS OF BONE MARROW PATHOLOGY, ANGIOGENESIS AND CLINICAL PARAMETERS IN PATIENTS WITH PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE DISORDERS

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Background. The abnormal megakaryocytic proliferation, dysplastic changes in megakaryocytes (MKC) and fibrosis are phenomena associated with chronic myeloproliferative disorders (MPD) as idiopathic

myelofibrosis (IM), polycythaemia vera (PV) and essential thrombocythaemia (ET). Substantial data have been accumulated about the increased angiogenic activity in these disorders as well. It is not elucidated the relation between the established histo-pathological criteria and angiogenesis and clinical characteristics in these patients. *Aims*. The present study aimed to analyze patients with Ph-negative MPD according to the proposed clinico-pathological criteria, the level of bone marrow (BM) angiogenesis and MKC pathomorphology. *Materials and Methods*. Totally of 134 patients were analyzed (45 with IM, 38 with PV, 39 with ET and 12 controls) after the informed consents were obtained. We used standard methods for BM analysis (hematoxyllin-eosin and Gomori staining) as well as specific immunoperoxidase technique for immunohistochemistry (anti-von Willebrand Factor (vWF) as an endothelial and megakaryocytic marker and anti-Vascular Endothelial Growth Factor (VEGF) antibodies). SPSS 15.0 for windows was used for the statistical analysis. *Results*. We found most increased microvessel density (MVD) in patients with IM (77,4±23/mm^2), comparing to PV, ET and control groups (50±19/mm^2, 49.4±17.8/mm^2 and 13±5,2 mm^2 resp.). The analysis of the micro-megakaryocytic population showed statistically increased mean number in patients with IM (25,25±11.25/mm^2) comparing to PV and ET (11.97 and 8.10, resp.). The highest VEGF expression by MKC was found in IM patients (VEGF index 3.38±0.72) as compared with PV and ET (2.08±0.65 and 2.31±0.72 resp.). We subdivided the IM patients into the following clinicopathological stages: early-prefibrotic (N=9), manifest (N=6) and advanced stage (N=25). When we reanalyzed the MVD and the level of fibrosis with regard to the stages, the MVD was found to be significantly higher in the advanced stage of IM (p<0.007) and we confirmed a significant positive correlation between these two parameters as well (r=0.51, p<0.001). In IM patients we confirmed significant correlations between MVD and overall survival (OS), degree of fibrosis (DF), transfusion dependency and splenomegaly. In PV group significant positive correlations were shown between MVD and the patient's risk and DF. *Conclusions*. In cases with thrombocytosis and mild fibrosis, the analysis of the level of neo-vascularization, VEGF expression by MKC and micro-megakaryocytes could serve as differentiation markers among the earlier stages of MPDs. The analysis of VEGF and vWF expression by MKC could extend the morphological assessment of MKC dysplasia which is a characteristic feature for IM. MVD could serve as a diagnostic marker as well as a parameter for determining the risk in patients with IM and PV but not in ET. We propose a routine use of anti-vWF antibody as an easily approachable method that could be used in visualizing the micro-vessel density, vessel structure and micro-megakaryocytic populations. In the present study we confirm the predictive value of MVD with respect to the overall survival of IM patients.

0896

THY-1, C-KIT AND TFR EXPRESSIONS ON CD34+ MYELOBLASTS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES AND THEIR RELATIONS TO IPSS

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Background. Myelodysplastic syndromes (MDSs) are heterogeneous diseases of bone marrow cell precursors for which immunophenotypic characterization is still considered irrelevant despite the accuracy and sensitivity of flowcytometry (FCM) techniques. *Aims*. The aim of this study is to estimate the antigenic expression of Thy-1, c-Kit and TfR on the CD34$^+$ blasts in Bone marrow (BM) aspirates from *de novo* and therapy related MDSs (t-MDSs) patients to determine their prognostic values in relation to cytogenetic risk factors as defined by International Prognostic Scoring System (IPSS). *Methods*. this study was carried out on 20 patients with *de novo* MDSs and 12 patients with t-MDSs. All patients were diagnosed by history taking, clinical examination and laboratory investigations including: CBC, BM aspiration for morphological assessment, and cytomorphological classification according to WHO classification, cytogenetic analysis then IPSS formulation and BM immunophenotyping using monoclonal antibodies to select CD34$^+$ blasts in addition to monoclonal antibodies to detect co-expression of Thy-1,c-Kit or TfR on these CD34$^+$ blasts *Results*. clonal chromosomal abnormalities were detected in 55% (11/20) of *de novo* MDSs, [more frequent in the advanced 71.4% (5/7) than in the initial stages 42.9% (6/13)]. T-MDSs show higher frequencies of karyotype abnormalities 91.7% (11/12) most of them 58.3% (7/12) had abnormality in chromosome 5, 7 or both. Blast cells of almost all cases were myeloblasts, (CD13+, CD33+, HLA-DR+), 59.4% (19/32) had CD34 expression, [50% (10/20) of *de novo* MDSs and 75% (9/12) of t-MDSs]. Most patients with CD34$^+$ blasts were belonging to poor prognosis. Thy-1 and c-Kit was co-expressed with CD34 in 26.3% (5/19) and 57.9% (11/19) respectively. 80% of Thy-1 and 81.8% of CD117 were categorized in intermediate-2 and high risk IPSS indicating poor prognosis. TfR was co-expressed with CD34 in *de novo* MDSs only [15.8% (3/19)]. *Summary and Conclusions*. estimation of co-expression of Thy-1, c-Kit or TfR on CD34$^+$ blast cells using FCM in MDSs patients is an easy and reproducible test that allows evaluating the prognosis of MDSs cases with CD34$^+$ blasts. Co-expression of Thy-1 or c-Kit indicate more immature CD34$^+$ blasts, high risk IPSS classification and poor prognosis while co-expression of TfR indicate more mature CD34$^+$ blasts, low risk IPSS classification and good prognosis. So not only the presence of CD34 but its association with other markers plays a major role in MDSs prognosis.

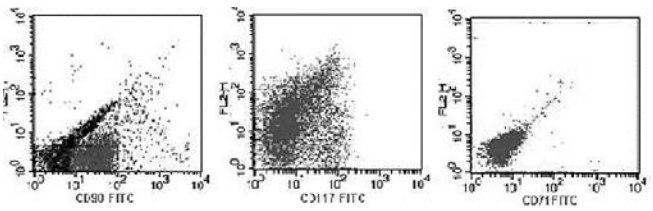

Figure 1. Dotplot display of flowcytometry analysis of a cas

0897

LANGERHANS CELL HISTIOCYTOSIS IN CHILDREN - DIAGNOSIS, TREATMENT AND FOLLOW-UP

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Background. Langerhans cell histiocytosis (LCH) is a rare disease. It is characterized by the clinical heterogeneity of the disease and by the pathogenesis that is still poorly understood. It is regarded as clonal accumulation and proliferation of abnormal bone marrow derived Langerhans cells. *Aims*. To present the experience of University Children's Hospital in Skopje regarding to epidemiological features, diagnosis, treatment and follow-up of LCH in children. *Methods*. We present a retrospective analysis of 54 patients with LCH treated in Department of Hematology/Oncology from January 1977 to January 2009. Analysis of clinical presentation, diagnostic features, treatment and outcomes was carried out. *Results*. During the study period, 54 cases of histology proven LCH (31 males and 23 females) were analyzed. The age at diagnosis ranged from 3 to 192 months. Six of fifty four children presented with unisystemic LCH involving the skin, 8/54 presented single bone lesion, 7/54 had multiple bone lesions and 32/54 presented with multisystem diseases (12 of them had organ dysfunction). All patients had a bone marrow aspirate examined at diagnosis in order to complete the staging of the disease. Because of a very long period of analysis there were different treatment procedures. The most common regimen until 1997 year was combination of Vinblastine and Prednisone. After 1997 year children were treated according to the international studies LCH-I and LCH-II. From 2003 we started to use LCH-III protocol and 6 patients were treated according to LCH-III. Mortality was 90% in group of patients with multisystem disease and organ dysfunction treated before 2003 year. Chemotherapy was unsuccessful in eight patients younger than 2 years of age with multisystem disease and organ dysfunction. They died in the range of 1 to 13 months after the diagnosis. Four children with multisystem disease and organ dysfunction were treated according to LCH III international study. Three of them are still alive with a survival of 12 weeks to 30 months. *Conclusions*. Patients with localized disease and absence of organ dysfunction benefited from standard therapy. More aggressive treatment is necessary for multisystem disease and organ dysfunction. The young children (younger than 2 years of age) with multisystem disease and organ dysfunction had a very poor outcome in our study. LCH-II and LCH-III protocol achieved more successful results than the treatment procedures used before.

0898

THROMBOSIS IN POLYCYTHEMIA VERA ARE LIKELY TO BE RELATED TO HIGHER JAK2V617F ALLELE BURDEN

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Background. Polycythemia vera (PV), one of the Philadelphia-negative myeloproliferative neoplasms (MPD), arises from clonal proliferation of a pluripotent stem cell. In 2005 the JAK2V617F mutation was found in about 90% of patients with PV. Many Authors found some correlations between the presence of the mutation and the clinical course of MPD, however, the results are not all in agreement. Some studies are available regarding the significance of different allele burden in essential thrombocythemia but not in PV. The aim of our study was to compare the JAK2V617F allele burden with ultrasound splenomegaly over 12 cm of cephalocaudad diameter, the occurrence of major thrombosis or hemorrhages at diagnosis or during follow-up and evolution into myelofibrosis, in patients with PV. *Material and Methods*. We studied 72 patients affected by PV (32 males and 40 females, mean age at diagnosis 60.1±14.5 y, median follow-up 7.8 y) diagnosed in agreement with WHO 2008 criteria and, as controls, 9 patients with secondary erythrocytosis (7 males, 2 females, mean age at diagnosis 52.76±17.2 y, median follow-up 1.99 y). The JAK2V617F allele burden was determined with PCR real time, by using Sybr Green as detector and on the basis of these results we stratified our patients in different percentiles of allele burden (<50%, ≥50% and its subgroup ≥80%). Statistical analysis was performed with Student's t test. *Results*. As attended, 0% allele burden was found in controls and in 10 wild type (WT) PV patients. 24 PV had <50% (12.2±10.2%) and 38 ≥50% (79.8±15%) allele burden; within the last group 20 patients had ≥80% (91.7± 6.6%) allele burden. The mean allele burden in the 43 patients with splenomegaly (65.7±30.5%), in the 30 with thrombotic complications (60±36%) and in the 15 evolved into myelofibrosis (77.2±32%) but not in the 17 cases with hemorrhage was higher (respectively p=0.0001, p=0.02 and p= 0.0003) than in patients without complication (splenomegaly 25.6±26.2%, thrombosis 42.8±33% and myelofibrosis 43.7±32.4%). Stratifying the patients, the significance is confirmed for thrombosis in patients with allele burden ≥50% (83.6±16.5 and 75±12.5, p= 0.03) and in a greater way in patients with allele burden ≥80% (93.4±5 and 87.7±6.7, p=0.02) *Conclusions*. In agreement with the literature, most of our PV patients carry JAK2V617F mutation; about half of them had an allele burden >50%. Higher JAK2V617F allele burden were associated with splenomegaly, thrombosis and evolution into myelofibrosis. In particular, thrombosis are more likely to be related to high (>50%) JAK2V617F allele burden. The JAK2V617F allele burden evaluation has a significant value in the prognosis of patients with PV.

0899

LEUKOCYTOSIS IS A RISK FACTOR FOR RECURRENT THROMBOSIS IN PATIENTS WITH POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA

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Background. There is evidence that leukocytosis is associated with an increased risk of first thrombosis in patients with polycythemia vera (PV) and essential thrombocythemia (ET). Whether it is a risk factors for recurrent thrombosis too is currently unknown. *Aims*. In order to investigate the impact of leukocytosis on the risk of recurrent thrombosis in patients with PV and ET, we carried out a multicenter retrospective cohort study. *Patients and Methods*. We recruited 253 patients with PV (n=133) or ET (n=120), with previous arterial (70%) or venous major thrombosis (27.6%) or both (2.4%), and not receiving cytoreduction at the time of thrombosis. The median leukocyte count at that time was 10.2×10^9/L (range 3.1-24.9). The total observation time after thrombosis was 1,602 pt-years (median 5.5). The relative risk of recurrence was estimated as a hazard ratio (HR) using a Cox proportional hazards regression model. The HR was adjusted using recurrence as the dependent variable and selecting as covariates gender, diagnosis (PV or ET), age at the time of the initial thrombosis (>60 or <60 years), presence of one or more vascular risk factors, history of remote thromboses, type of first thrombosis (arterial or venous), leukocytosis at the time of the first thrombosis, and type of treatment following thrombosis. *Results*. Thrombosis recurred in 78 patients (30.7%); after adjustment for potential confounders, age >60 years at the time of the first thrombosis was an independent predictor of recurrence (HR, 1.83, 95%CI 1.12-2.96) and recurrence was prevented either by antiplatelet treatment (HR 0.38, 95%CI 0.18-0.76) and cytoreduction (HR 0.44, 95% CI 0.27-0.70). The patients with a leukocyte count in the highest quartile $>12.4\times10^9$/L at the time of their first thrombosis showed an increased risk for arterial recurrence in respect to all the remaining patients (multivariable HR, 1.95, 95%CI 1.0.3-3.66).The increased risk for arterial recurrence associated with leukocytosis was confirmed among patients <60 years (HR 3.07, 95%CI 1.18-7.96). Leukocytosis was not significantly associated with the risk for recurrence among the patients >60 years neither predisposed to venous recurrences. After the second thrombosis the risk for a further thrombotic event was analyzed by multivariable analysis of the 78 patients having had a recurrence. After adjustment for the potential confounders aforementioned, the hazard for a third thrombotic event between the patients with previous leukocytosis and those without was still increased, but without reaching the statistical significance (HR 2.73, 95%CI 0.86-8.59). *Conclusions*. In the younger patients with PV or ET leukocytosis at the time of first thrombosis is associated with an increased risk of future arterial thrombotic events. It can be suggested that leukocytosis could be an important tool of stratification of the patients not only among the low risk individuals without history of thrombosis but also among the young high risk individuals with a previous history of thrombosis.

Chronic lymphocytic leukemia and related disorders - Biology II

0900

EVALUATION OF ZAP-70 EXPRESSION BY MEAN FLUORESCENCE INTENSITY T/B RATIO IS A MORE USEFUL PREOGNOSTICATOR THAN PERCENTAGE OF POSITIVE CELLS IN CHRONIC LYMPHOCITIC LEUKEMIA

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Background. Expression of the T-cells constitutive ZAP-70 protein by chronic lymphocitic leukemia (CLL) cells has been the focus of many studies in the last few years, due to its ability to stratify indolent and more aggressive disease subsets. Although the strength of ZAP-70 as independent negative prognosticator was demonstrated by several studies, concerns about its use derive from a lack of multicentric standardization of flow-cytometric protocols. Analyses in clinical settings are usually performed according to two methods, respectively evaluating the percentage of CLL cells expressing ZAP-70 compared to isotypic control (ISO-method), or to autologous T-cells (T-method). Of note, while the two methods yield concordant results in most patients, a fraction of cases may be discordant as for evaluation of ZAP-70 positivity. Moreover, either method suffers of an operator dependent variability, mainly related to subjectivity in cursor placement to determine the percentage of ZAP-70+ cells. *Aims*. To compare the ISO- and T-methods with the expression of ZAP-70 evaluated as Mean-Fluorescence-Intensity (MFI) Ratio between gated T and CLL cells (T/B-Ratio-method), and to assess the prognostic significance of the three approaches. *Methods*. Cytometric files relative to ZAP-70 determination according to the three readouts were retrospectively reviewed with BD-DiVa software on a cohort of 173 patients (test set), all with complete clinical and biological prognostic assessment and time-to-treatment (TTT) available. Findings were then validated in an independent cohort of 187 cases from a different institution (validation set). In the two cohorts, ZAP-70 assessment was accomplished using different antibody combinations and different instrumentations for data acquisition. *Results*. ZAP-70 expression was reviewed in the test set by applying the ISO- and T-methods. Utilizing in both cases the 20% of ZAP-70+ cells, selected as the optimal cut-off with prognostic relevance also in this series, 29 (ISO-method) and 63 (T-method) ZAP-70+ cases were defined. By applying the TRatio-method, a value of 3.0 was identified as the optimal prognostic cut-point. According to this value, 75 ZAP-70+ cases (i.e. with Ratio<3.0) were identified in the test set. Univariate analyses resulted in a better separation of ZAP-70+ vs ZAP-70- CLL patients utilizing the T/B-Ratio-method (p=6.8e-06), compared to T- (p=1.94e-05) or ISO- (p=4.35e-3) methods. In multivariate analyses with RAI stage, β2microglobulin, IGVH, FISH, CD38 and CD49d, ZAP-70 was selected as independent risk factor, irrespective of the readout employed for evaluation of ZAP-70 expression; however, the prognostic impact of ZAP-70 appeared stronger when the T/B-Ratio-method was applied (significative hazard ratio=2.2 vs 1.8 with the ISO- or T-methods). To confirm these findings, we analyzed the 187 cases of the validation set with T-method (cut-off=20%) and TRatio-method (cut-off=3.0). Analyses yielded 99 (T-method), and 66 (Ratio-method) ZAP-70+ cases. Univariate analyses also on this cohort resulted in a better separation with T/B-Ratio-method (p=9.8e-07) than T-method (p=3.15e-05). *Conclusions*. We suggest to evaluate ZAP-70 expression in routine settings using the TRatio-method given the operator and laboratory independent feature of this approach. We propose the 3.0 Ratio value as optimal cut-off to discriminate ZAP-70+ (TRatio less than 3.0) from ZAP-70- (Ratio more/equal than 3.0) cases.

0901

MALDI-TOF MS PROFILING TO EXAMINE THE PROTEIN CONTENT IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) is characterized by the expansion of monoclonal mature B lymphocytes with an extremely heterogeneous clinical course possibly due to the acquisition of specific chromosomal abnormalities, the mutational status of immunoglobulin variable heavy chain gene (IgVH), and the expression of CD38 and ZAP-70 proteins. However, these prognostic tools have yet to be implemented on a widespread basis due to combinations of cost, test availability, and validation of their clinical specificity. *Aims*. To provide insights into the proteomic complexity of CLL, identifying proteins potentially of prognostic value, and thus offering insight into the underlying biology of the malignant B cell. *Methods*. A panel of highly purified neoplastic cells (>92%) from 25 untreated, newly diagnosed CLL patients in Binet A was investigated. We used the proteomic technologies MALDI-TOF MS profiling to examine the protein content of CLL B cells. Highly purified B lymphocytes were subjected to cell lysis, followed by sub-fractionation of nuclear, microsomal and water-soluble components. An aliquot of this last fraction underwent a desalting/concentration step over ZipTip C18 and peptide/protein profiles were analyzed using a VoyagerDE PRO MALDI-TOF mass spectrometer (PerSeptiveBiosystems). Separate spectra were obtained for a restricted mass-to charge (m/z) range (1000-25000 Da) in linear mode geometry, by applying an acceleration 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 by presenting as a percentage of the total peak area (individual peak area/total peak area per cent). A hierarchical clustering analysis, with Pearson correlation as similarity metrics and an average linkage as a cluster method, was also performed using Cluster 3 and TreView software. *Results*. Clustering analysis, comprising the CD38 values, [i.e. CD38> 30% (5 cases) versus CD38<30% (20 cases)], separated two well distinct branches. Furthermore, a student's t test applied to CD38 this two groups, allowed the identification of 14 differentially expressed ion signals with statistical significance (p<0.05). The same analysis was conducted using immunoglobulin variable IgVH values,(i.e. mutated (20 cases) versus unmutated (5 cases). Differently to CD38 results, clustering analysis by IgVH mutational status distinguished two not well separated major branches. Nevertheless, applying the student's t-test we have identified 7 differentially expressed ion signals with statistical significance (p<0.05). Moreover, comparing 14 and 7 differentially expressed ion signals achieved by two supervised analyses, 3 m/z values were shared by the two ion signal list (1256, 69; 1311, 55; 4939, 28 m/z values). *Conclusions*. With the delimitation of the number of cases analyzed, these preliminary results demonstrated 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 might give complementary information concerning the molecular mechanism linked to the variable clinical course of the disease.

0902

CHARACTERIZATION OF 22Q11 DELETION IN CLL PATIENTS

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Background. Several prognosticly relevant genomic aberrations have been described in chronic lymphocytic leukemia (CLL). Deletions in 13q14, 17p13, 11q22 and gains on chromosome 12 are present in approximately 80% of cases. However, the genome of CLL patients contains many additional genomic imbalances. Array-based comparative genomic hybridization (arrayCGH) enables detailed whole-genome screening of chromosomal aberrations. *Aims*. We analysed genome of CLL patients using oligonucleotide arrayCGH. In particular, we focused on searching for novel genomic alterations and aberrations poorly characterized so far

which could have a prognostic value. *Methods*. Peripheral blood samples from 40 CLL patients were collected. All commonly used molecular prognostic markers were assessed. Cytogenetic data (FISH or CpG/IL-2-induced metaphase cytogenetics) documented that all samples included to the study harboured at least one chromosomal aberration. DNA isolated from lymphocytes was hybridized on Human Genome CGH Microarray 4 x 44 K (Agilent) and compared to human reference DNA. To provide detailed analysis of the 22q11 deletion, clonal immunoglobulin recombinations detection according to BIOMED-2 protocol and supplementary sequencing was performed. *Results*. Chromosomal imbalances described by cytogenetic methods (FISH and caryotyping) were confirmed by arrayCGH in all cases. Additional aberrations were found in majority of samples (87,5%). Among them, the deletion of 22q11 locus ranging in size up to approximately 0.77 Mb was observed most often. This locus encodes several genes including lambda immunoglobulin light chain (IgL) subgenes. The presence of other coding genes (e.g. miR-650, VPREB1, PRAME) contradistinguishes IgL region from kappa light chain and heavy chain immunoglobulin loci. Gunn *et al.* (2008) previously characterized the minimally deleted region of 22q11 in CLL as 0,34Mb (containing PRAME, ZNF280A, ZNF280B and GGTLC2) and described PRAME (preferentially expressed antigen in melanoma) as a candidate gene with prognostic impact in CLL. We detected this recurrent deletion in 7 (17.5%) patients from our cohort, two cases exhibited biallelic loss. Detection of clonal immunoglobulin recombinations by PCR and sequencing enabled us to determine properly the deletion range and revealed that it corresponds to lambda variable rearrangement. Another six patients with rearranged lambda immunoglobulin gene have deletion smaller than published minimally deleted region, not including PRAME. This was represented by decreased intensity of one or two probes on the arrays thus cannot be reliably designated by statistical algorithm. Altogether, more than 30% patients (13/40) have IgL rearrangement. The deletion of specific genes localized within IgL depends on the subgene rearrangement. In our cohort, PRAME is deleted in about 50% of patients with IgL rearrangement. Another interesting gene miR-650 located between V3-9 and V2-8 lambda variable subgenes is deleted in majority of patients (92%). Based on these results, we assume physiological origin of 22q11 deletion arising from lambda immunoglobulin rearrangement. *Summary*. Recurrent deletion 22q11 is frequently detected in CLL patients. We suggest that deletion is caused by physiological process of lambda immunoglobulin variable region rearrangement. However, the possible pathogenetic or prognostic impact of PRAME or miR-650 deletion/expression cannot be excluded and further analysis is required.

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0903

IDENTIFICATION OF PEPTIDE SEQUENCES BINDING SPECIFICALLY TO THE BCR OF B-CLL CELLS

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Chronic lymphocytic leukemia of the B-cell type (B-CLL) is the most common leukemia in the Western hemisphere with an annual incidence of 3/100 000 people. Increasing evidence suggests that signals from the haemopoietic microenvironment including chronic antigenic stimulation of the B-cell receptor (BCR) causes a deficiency of apoptosis of B-CLL cells and thus may play an important role for expansion of the leukemic cell clone in the patient. To test the hypothesis of antigenic stimulation of B-CLL cells, we employed a phage display technique to identify peptide sequences binding to the antigenic BCR of leukemic cells. We were able to identify two pentamer peptide sequences with very similar consensus sequences binding to the antigenic B cell receptor of tumor cells from 12 of 15 CLL patients. Phages carrying the identified peptide sequences precipitated BCR immunoglobulins from different B-CLL tumor cell lysates (6 of 12), while no BCR immunoglobulins were precipitated from lysates of healthy B-cells (n=3), Mantle Cell Lymphoma cells (n=2) or Burkitt-Lymphoma cell lines (n=5). Phages carrying a random peptide sequence did not precipitate BCR immunoglobulins from B-CLL cell lysates (n=12). Furthermore, phages carrying the identified peptide sequences activated intracellular signalling in B-CLL cells, while phages carrying random peptide sequences did not. These data support the assumption that specific antigenic stimulation of the BCR may be involved in the selection and expansion of the malignant cell clone in B-CLL.

0904

HEAT SHOCK PROTEIN 27 GENE-EXPRESSION: A NOVEL PROGNOSTIC MARKER IN CLL

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Background. Heat shock protein 27 (Hsp27, HSPB1) is a member of the small heat shock protein family and inhibits key effectors of the apoptotic machinery. While Hsp27 is expressed in many cell types and tissues, it is absent in all lymphoid cells as well as most lymphoma cells. In previous microarray-analyses, we found Hsp27 to be differentially expressed in the two prognostic subgroups of chronic lymphocytic leukemia (CLL). *Aims*. To evaluate Hsp27 gene-expression as prognostic marker for CLL. *Methods*. Hsp27 gene-expression was assessed in 113 CLL patients diagnosed at our institution using quantitative real-time PCR. Gene expression data were correlated with clinical data and CLL risk factors. Peripheral blood mononuclear cells (PBMC) of CLL patients were analyzed as well as CD19-sorted cells from normal controls and selected CLL patients. *Results*. Hsp27 gene-expression ranged from 0.25 - 27.1 (median: 2.15; mean: 3.318) compared to normal PBMC (set as 1). Expression correlated with LPL-expression (p=0.003) and mutational status (p=0.012). High Hsp27 gene-expression (>3.318) was significantly associated with unmutated IgVH-genes (p=0.007) and shorter time to first treatment (median 40 months vs. 128 months; p=0.008) [Figure 1: Kaplan Meier estimation showing time to first treatment in patients with high and low Hsp27 expression (cut off: 3.318)]. Binet-stages B and C showed significantly higher Hsp27 gene-expression compared to Binet-stage A (p=0.023). Hsp27 levels were higher in CD19$^+$ selected CLL cells compared to CD19 negative cells, healthy donor (HD) and low expression group showing similar values. In contrast, expression values differed up to 2 logs in the high expression group. *Conclusions*. Hsp27 represents a novel prognostic marker for CLL with the potential of a novel therapeutic target.

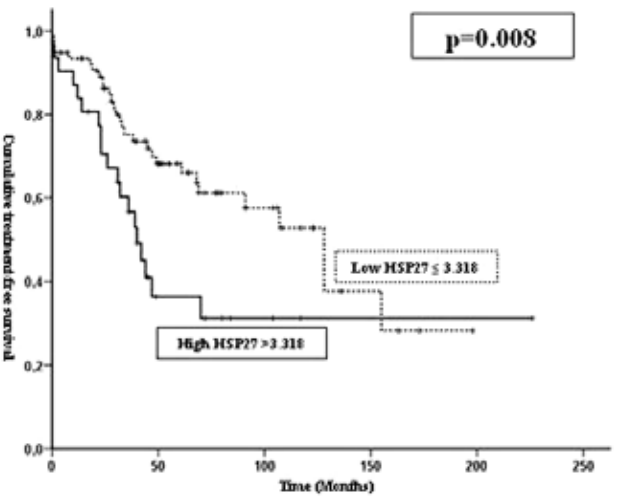

Figure 1. Time to first treatment.

0905

QUANTITATIVE ANALYSIS OF REPETITIVE ELEMENTS METHYLATION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The identification of reliable prognostic factors useful in predicting patient outcome and planning therapeutic strategies is a crucial task to better define the clinical heterogeneity of B-cell chronic lymphocytic leukemia (B-CLL). Gene-specific hypermethylation and global hypomethylation have been previously reported in B-CLL. However, the relationship between aberrant global DNA methylation patterns and clinical and biological risk factors remained unclear. Global hypomethylation of repetitive genomic sequences, such as long interspersed nuclear elements-1 (LINE-1), Alu and satellite α DNA (SAT-α DNA) has been reported to be associated with chromosomal instability in human cancer. *Aims.* In order to investigate the role of global DNA methylation in B-CLL we quantified the methylation levels of Alu, LINE-1 and SAT-α and correlated them with the major biological, molecular and cytogenetic markers known to predict clinical outcome in B-CLL. *Methods.* A quantitative bisulfite-PCR Pyrosequencing method was used to evaluate the methylation patterns of Alu, LINE-1, and SAT-α in a panel of highly purified (>90%) peripheral mononuclear CD19+ cells from 7 healthy donors and 77 B-CLL untreated patients in early stage disease (Binet stage A). FISH, cytofluorimetric and IgVH mutation analyses were performed in all cases. Thirty-three cases carried the 13q14 deletion, 22 of which as the sole abnormality; biallelic 13q14 deletion was identified in 6 cases. Both 11q23 and 17p13 deletions were detected in 12 patients, whereas trisomy 12 was found in 19 patients as a sole exclusive abnormality. ZAP-70 and CD38 expression resulted positive in 29 and 35 cases, respectively, whereas IgVH genes were found to be unmutated in 48 patients. *Results.* The DNA from B-CLLs showed significant ($p<0.001$) decreased levels of methylation of all three types of repetitive elements when compared with controls (median 21.4%5mC vs. 25.9 %5mC, 66.8 %5mC vs. 85.7 %5mC, and 85.7%5mC, vs. 88.2 %5mC, for Alu, LINE-1 and SAT-α, respectively). Concerning the major cytogenetic abnormalities, a statistically significant correlation ($p<0.001$) was found between Alu, LINE-1 and SAT-α hypomethylation levels and the presence of the 17p13 deletion (Alu: 16.8.%5mC, LINE-1: 50.2 %5mC, SAT-α: 52.6%5mC) compared with patients without this lesion (22.4 %5mC, 68.6%5mC, and 85.0 %5mC, respectively). Notably, we found a statistically significant association between the methylation levels of Alu and LINE-1 as well as between Alu and SAT-α DNA and LINE-1 and SAT-α. (Pearson's correlations coefficient rho=0.64, 0.78 and 0.66, respectively; $p<0.001$). Finally, we did not observe any statistically correlation between global DNA methylation level and prognostic parameters such as IgVH mutation status, CD38 or ZAP-70 expression. *Conclusions*. Our study, based on a quantitative approach, extended previous evidence in global methylation in B-CLL and demonstrated a significant hypomethylation associated with high risk patients carrying the 17p deletion. Further studies on larger series would be useful to define whether global methylation levels may have a prognostic relevance in terms of B-CLL clinical outcome.

0906

CHRONIC LYMPHOCYTIC LEUKEMIA CELLS RESCUE THEMSELVES BY INCREASING THE ANTI-APOPTOTIC EFFECT OF BONE MARROW STROMA

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Background. Chronic lymphocytic leukemia (CLL) cells are characterized by their ability to resist apoptosis. The *in vivo* enviroment seems to be responsible for the prolonged life span, because this biologic feature is lost *in vitro*. *Aims*. Our aim was to characterize the interaction between CLL cells and nourishing stroma and to study the effect of stroma on CLL cell survival. *Methods*. Sorted CLL cells from 21 patients were bred on allogenous, normal bone marrow stromal cells (BMSCs) or in culture medium. CLL cell survival was determined after 84 hours of culture by cell counting and propidium iodide/Annexin V staning. Cytokine secretion of BMSCs was measured by multiplex bead assay and mRNA expression in BMSCs and CLL cells by qRT-PCR. CLL cells were immunophenotyped before and after the cell culture. *Results*. BMSCs rescued CLL from apoptosis. In the presence of CLL cells BMSCs secreted antiapoptotic cytokines such as IL6 and IL8 more intensively and up-regulated ICAM-1 and CD40 mRNA expression. The expression of CXCL12 remained unchanged and VCAM1 was slightly increased. In turn, CLL cells bred on BMSCs up-regulated significantly the expression of CD18, CD49d - ligands for the critical adhesion molecules. *Conclusions*. These results indicate the manipulation of BMSCs by CLL cells in favour of apoptosis resistance.

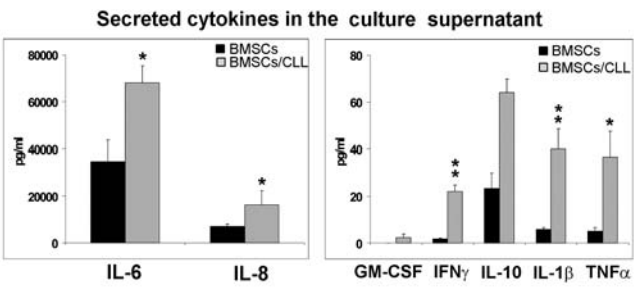

Figure 1.

0907

RELATIONSHIP BETWEEN THERAPY AND CLONAL EVOLUTION OF TP53 ABNORMALITIES IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Although TP53 abnormalities are present at low proportion of CLL patients, their prognostic value is unequivocal. Patients with p53 inactivation are refractory to DNA damaging agents and it was suggested recently that chemotherapy may select resistant clones with inactivated p53. Non-genotoxic therapeutic approaches acting independently on p53 pathway are therefore recommended for patients with TP53 abnormalities. Currently, the monoclonal antibody alemtuzumab is the drug of choice. *Aims*. We studied the relationship between administration of therapy and clonal evolution of TP53 mutation and/or deletion by consecutive monitoring of these abnormalities in patients with originally intact TP53 gene. *Methods*. The deletions at the TP53 locus (17p13.1) were detected using an interphase FISH; mutations in the gene were analyzed by yeast functional analysis (FASAY) coupled to sequencing of templates from yeast colonies bearing mutated TP53 gene. *Results*. Repeated investigation of TP53 status was performed in 112 patients, which manifested no abnormality in this gene in original investigation (median follow-up 29 months, range 4-61 months). 55 patients were treated by at least one course of standard chemo and/or immunotherapy between analyses. In twelve cases a novel TP53 abnormality was

identified; all of them belonged to the subgroup of treated patients. In eight patients the novel TP53 mutation was accompanied by 17p deletion, three cases harboured sole mutation (in one patient FISH result was not available). Surprisingly, five of the patients with novel abnormality were treated with alemtuzumab between the investigations; in two patients alemtuzumab was the only treatment used, with no genotoxic agents administered. Novel TP53 aberrations had crucial impact on patients´ outcome: nine patients died in relation to disease within 1 to 37 months after abnormality detection (median survival 7 months). In contrast, no patient acquired a TP53 abnormality in the subgroup without therapy in the period between investigations. Median time of follow-up was comparable within both subgroups: untreated 27 months; range 4-57 months; group with acquired abnormality 22 months; range 7-51 months. It rules out the possibility that clonal evolution was observed due to a prolonged follow-up. Theoretically, the clonal selection of TP53 defects might be caused not only by the treatment itself, but also by the disease aggressivity (e.g. a more intensive proliferation of the cells). In this respect we have to point out that the proportion of patients with disease progression was lower in the untreated subgroup than in the treated subgroup (61% vs. 89%). *Summary*. TP53 abnormalities were selected during the course of the disease in a significant proportion of patients; they further contributed to disease aggressiveness. Although the exact biological mechanisms, which are involved in the acquisition of TP53 abnormalities, are currently unknown, the therapy plays obviously an important role in their selection. Our data surprisingly suggests that monoclonal antibody alemtuzumab may also contribute to selection of cells with TP53 mutations, probably by providing open niche for proliferation of more aggressive clone after eradication of original CLL population. This issue should be further analysed.

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0908

IGVH MUTATION STATUS AND ZAP-70 EXPRESSION AS INDICATORS FOR MOLECULAR PROFILING SIGNATURE IN B-CLL PATIENTS FOR PROGNOSTIC CLASSIFICATION

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Background. Chronic lymphocytic leukemia (CLL) is a heterogeneous disease characterized by a highly variable clinical course. IgVH mutational status and ZAP-70 protein expression have shown to be strong associated and to offer important prognostic information. *Aims*. Our aim was to determine gene expression profiles of 46 CLL patients divided into three classes: the first (n=26) with mutated IgVH and ZAP-70-, the second (n=12) with unmutated IgVH and ZAP-70+, and the third (n=8) included CLL patients with unmutated IgVH and ZAP-70-, or mutated IgVH and ZAP-70+ respectively. Exploration of microarray data was evaluated in order to define prognostic biomarkers and the biological pathways related to B-CLL. *Methods*. We determined gene expression profiles using Affymetrix HG U133 Plus 2.0 in $CD19^+$ leukemic cells. Probe-level data were pre-processed using robust multi-array average (RMA). Differential expression analysis was performed using Statistical Analysis for Microarray (SAM). Selected genes were grouped according to their biological pathways using GenMAPP (Gene Map Annotator and Pathway Profiler). Finally, patients were clustered in groups with similar expression signature using cluster analysis (K-means, Euclidean distance). *Results.* Statistical analysis revealed 154 differentially expressed probe-sets in the first (mutated IgVH and ZAP-70-) vs the second (unmutated IgVH and ZAP-70+) group, corresponding to 88 genes annotated in public databases (probe-sets monitoring the same gene were selected multiple times). Interestingly, six genes were associated to the following biological pathways: MAPKsignaling (heat shock 70kD protein 8 HSPA8), B cell receptor signaling (ZAP-70, CKLF-like MARVEL transmembrane domain containing 3 CMTM3, dual adaptor of phosphotyrosine and 3-phosphoinositides DAPP1), Matrix Metalloproteinasis (transcription factor 20, TCF20), Apoptosis (X-linked inhibitor of apoptosis XIAP) and T cell receptor signaling (ZAP-70). In particular, ZAP-70, HSPA8, CMTM3 were significatively underexpressed while XIAP, TCF20 and DAPP1 were overexpressed in the first class of patients in comparison to the second class, respectively. No differentially expressed genes were identified in the comparison between the first and the third (mutated IgVH and ZAP-70+ or unmutated IgVH and ZAP-70-) class and between the second and the third class of patients respectively. Based on the expression of the 88 genes identified in the comparison between the first and the second class of patients, the 8 patients of the third class were divided in two clusters: 5 subjects were more similar to the first class, while 3 subjects appeared to belong to the second one. In particular, cluster analysis revealed that the 46 patients were better partitioned in two rather than in three classes, based on their expression profiles. *Summary and Conclusions.* In summary, our preliminary data revealed different gene expression signatures in B-cell chronic lymphocytic leukemia prognostic subgroups of patients, defined by IgVH mutational status and ZAP-70 expression. The functional pathways related to: MAPK signaling, B cell receptor signaling, apoptosis and T cell receptor signaling may ultimately influence CLL biology. Gene expression profiling studies are in progress on larger series of CLL patients in order to assess the association of the molecular signature, based on the identified genes and their pathways, with respect to prognostic information.

0909

SENSITIZATION OF LEUKEMIC CELLS TO FLUDARABINE BY MONOCLONAL ANTIBODY RITUXIMAB - IDENTIFICATION OF RESPONSIBLE GENES

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Background. Chronic lymphocytic leukemia (CLL) represents an incurable disease with a highly variable clinical course. Modern therapies, especially combined regimens of monoclonal antibodies and chemotherapeutics, may prolong progression-free survival and even overall survival in a subset of patients. We have recently reported (Cejkova *et al.*, Eur J Haematol, 2009;82:133-42) that monoclonal antibody rituximab sensitizes a significant proportion of CLL cultures *in vitro* to the subsequently administered fludarabine, regardless the presence of the high-risk genetic abnormalities, i.e. aberrations of the tumor-suppressors ATM and TP53 (which, however, influences the primary response to fludarabine on its own). *Aims*. The aim of the study was to define differentially expressed genes in CLL cultures sensitized by rituximab to fludarabine as opposed to cultures refractory to the sensitization. The selected genes might serve as the markers of sensitivity (or resistance) to the combination of these drugs and could further elucidate the mechanism of rituximab's activity in CLL cells, which is poorly understood. *Methods*. Sixty primary CLL cultures were tested *in vitro* for their viability using WST-1 metabolic assay. Fludarabine was administered for 48h as a single agent (25; 6.25; 1.6 and 0.4 μg/mL) or in parallel on the cells pre-treated for 72h with rituximab in a standard *in vitro* dose (10 μg/mL). Twenty-one samples were significantly sensitized by rituximab ($p<0.01$). Seven the most prominently sensitized samples were subsequently taken for the microarray analysis and compared with eight non-sensitized cultures. In each culture the treated cells were compared with control cells on the microarray (Agilent Technologies, 4x44K microarray). *Results*. We observed down-regulation of several genes in sensitized samples, while the same genes were up-regulated in non-sensitized samples. The opposite effect has not been, surprisingly, observed. Among the identified genes were the following interesting candidates: metallothionein 2A (MT2A), which has been reported in drug efflux function; ribonucleotide reductase M2 subunit (RRM2), connected with drug resistance and topoisomerase II α (TOP2A), reported to influence a response to therapy. *Summary and Conclusions.* The obtained data point to very interesting genes, which might be responsible for the observed sensitization effect. Their relevance will be further validated on the larger cohort of samples by using quantitative real-time PCR.

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0910

THE NOVEL TYPE II CD20 ANTIBODY GA101 MEDIATES SUPERIOR B CELL DEPLETION IN WHOLE BLOOD FROM HEALTHY VOLUNTEERS AND B-CLL PATIENTS

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Background. GA101 is a novel fully humanized IgG1-type monoclonal antibody that binds with high affinity to the extracellular part of the human CD20 antigen. In contrast to rituximab and other CD20 antibodies currently in development, GA101 recognizes a CD20 Type II epitope, resulting in enhanced direct cell death induction and reduced CDC induction. Through glycoengineering GA101 also mediates enhanced induction of effector-cell-mediated ADCC. *Aims*. In order to integrate the different mechanisms of action (ADCC-, CDC- and direct cell death-inducing mechanisms) in one assay format and to reflect the *in vivo* situation in peripheral blood, autologous *ex vivo* B-cell depletion assays with whole blood from healthy donors and B-CLL patients containing natural effector cell populations, human complement and physiological concentrations of human IgG were performed. These assays were used to determine the reduction in the number of CD19 positive B cells after incubation with GA101 in whole blood in direct comparison to rituximab. *Methods*. Heparinized blood of healthy volunteers or B-CLL patients was incubated with different antibodies at different concentrations for 24 h before FACS staining was performed. The B cell (CD19 positive cells) to T cell (CD3 positive cells) ratio (CD45 gate) was used to calculate the percentage of antibody dependent B cell killing, setting the untreated control to 0% and the putative complete depletion of B-cells as 100%. *Results*. Whole blood assays with blood from a panel of 10 healthy donors showed that in comparison to rituximab GA101 was 10-25 fold more potent in terms of EC50 values and 1.5-2.5 fold more efficacious in terms of absolute B cell depletion. The efficacy of whole blood B cell depletion by GA101 was significantly different from that of rituximab in all samples tested. In whole blood isolated from B-CLL patients, GA101 mediated superior B cell depletion both in terms of potency and absolute efficacy of B cell depletion in 8/11 patients in direct comparison to rituximab. In 3/11 patients neither GA101, Rituximab or alemtuzumab mediated B cell depletion. The analysis of additional B-CLL samples is currently ongoing. *Summary and Conclusions*. The whole B cell depletion assays performed in this study show that GA101 mediates superior depletion of normal B cells from blood of healthy volunteers as well as of malignant B cells from blood of B-CLL patients in a majority of samples in comparison to rituximab and alemtuzumab. It can be hypothesized that the B cell depletion in these whole blood assays reflects the *in vivo* situation in peripheral blood most closely. Further studies are foreseen to dissect the contribution of the different ADCC-, CDC- and direct cell death-inducing mechanisms to the killing of B cells from whole blood. In addition, it will be of interest to investigate why a proportion of samples showed no B cell depletion at all irrespective of the antibody used.

0911

IN VITRO CYTOTOXICITY AND ZNF331 ARE RELATED TO RESPONSE AND RELAPSE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) TREATED WITH FLUDARABINE, CYCLOPHOSPHAMIDE AND MITOXANTRONE (FCM)

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Background. The use of fludarabine-based polychemotherapy in patients with CLL has improved the response rate with a fraction of patients achieving complete remission (CR) without evidence of minimal residual disease (MRD). Abnormalities in p53 are associated to worse response to therapy, but there is still a proportion of non-responding patients in whom the mechanism of resistance is unknown. Once CR is achieved, the evolution of responding patients is strongly associated to the persistence or reappearance of MRD, but the underlying mechanisms are not defined. *Aims*. Analyze *in vitro* cytotoxicity, and gene expression profile of CLL patients treated with FCM to identify other parameters related to response and relapse. *Methods*. Clinical results of FCM treatment have been published (Bosch *et al.*, Clin Cancer Res 2008). In addition to standard parameters (cytogenetics and MRD), we have analysed *in vitro* cytotoxicity by a colorimetric assay at 48h of FCM incubation (41 patients), and gene expression profile of purified B cells (>98% after magnetic selection) at diagnosis from 22 patients (14 CR, 6 partial response (PR), 2 failure (F)) by hybridizing to HGU133 Plus 2.0 arrays. Supervised analysis according the type of response and the presence of relapse was conducted with Dchip v2.6. Low density arrays of selected genes (according the magnitude of fold change and the homogeneity within groups) were studied in purified B cells of 48 patients (13 CR-MRD-, 17 CR-MRD+, 18 PR or F) and analysed using Statminer software. *Results*. *In vitro* cytotoxicity correlated with clinical response [CR (n=23): 88% ±6, PR (n=15): 83%±11, F (n=3): 63% ±14)] (p<0.001). Gene expression at diagnosis was highly similar, independent of the type of response achieved, with differences observed in growth, development and cell proliferation pathways. The number of genes (269) differentially expressed between CR and other responses showed a median fold change of 1.7 (percentile 10: 1.48, percentile 90: 2.26). Patients in lasting CR (n=8) differentially expressed 159 comparing with patients who relapsed (n=6). The median fold change was 1.8 (percentile 10: 1.53, percentile 90: 2.8). Low density arrays included 25 genes for long lasting CR versus relapse, and 67 for treatment response. The median of fold change of the selected genes was 2.38 (percentile 10: 1.92, percentile 90: 5.1). Relapse was associated with basal expression ZNF331 (a reactivation of p53 and induction of tumour cell apoptosis), whilst no gene for type of response was identified. *Summary and Conclusions*: In addition to well known parameters for response and relapse, at diagnosis low *in vitro* cytotoxicity is associated to worse response to treatment without identifying genes predicting the response of treatment. In contrast, ZNF331 expression is associated to higher risk of relapse after FCM treatment, identifying a new potential marker for risk of relapse after treatment.

0912

EFFECT OF ZAP-70 ON MIRNA EXPRESSION PROFILE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CML) is known to show a highly variable evolution. ZAP-70 has been found to be one of the most relevant prognostic parameters in CLL. In order to find out the role of ZAP-70 in CLL, we have optimized a methodology for expressing ZAP-70 in CLL cells (Van Bockstaele *et al.* Leukemia 2008). In this way we are able to compare CLL cells only differing in ZAP-70 expression. *Aims*. We wished to explore if introducing ZAP-70 in ZAP-70 negative CLL cells could influence the miRNA expression profile. Moreover a comparison with miRNA expression profiles with prognostic relevance described previously was made. *Methods*. Isolated mononuclear cells from a CLL patient were electroporated three hours after thawing, as previously described, and were stimulated with anti-IgM 16 hours later. After 3 and 24 hours of stimulation CD19$^+$ cells were selected and RNA was isolated. miRNA expression profiles were measured using the Megaplex reverse transcription format of the stem-loop primer-based real-time quantitative PCR (RT-qPCR) method (Applied Biosystems). The Megaplex reaction provides simultaneous reverse transcription of 450 mature miRNAs. *Results*. Hsa-miR-34a was significantly upregulated after both 3 and 24 hours. Hsa-mir-429 was upregulated after 3 hours, but this effect disappeared after 24 hours. Hsa-mir-624 was downregulated after 24 hours but no difference was observed after 3 hours. *Summary*. Hsa-miR-34a is a well known miRNA in CLL and has been described as an adverse prognostic factor in line with our observation as a ZAP-70 induced miRNA. However, it has been shown that p53 may upregulate hsa-mir-34a which further leads the cell into apoptosis and cell cycle arrest. In analogy with the p53 data, one would not expect ZAP-70 to upregulate hsa-mir-34a, as this would rather be associated with good prognosis. Why the opposite is true, remains to be elucidated. Other miRNAs previously described in prognostic profiles in CLL were not differentially expressed in our experiment.

0913

ZAP-70 HAS HIGHER EXPRESSION IN LYMPH NODES AND BONE MARROW COMPARED TO PERIPHERAL BLOOD IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is characterized by highly variable clinical presentation and course with variable affection of different lymphoid organ compartments. There is well documented both intraclonal and interclonal variability among B-CLL cells with respect to different microenvironment in major lymphoid compartments as we have previously shown (Jaksic *et al.*, Blood 2004). ZAP-70 molecule with significant role in cell signaling and is very important negative prognostic parameter. However its expression is evaluated mainly in peripheral blood which is considered as mostly inert compartment compared to bone marrow and lymph nodes where proliferative centers were documented fueling B-CLL proliferation and progression. *Aims*. To evaluate whether there is difference in ZAP-70 expression in B-CLL lymphocytes associated with distinct microenvironment in major lymphoid compartments. *Methods*. Samples from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) representing major compartments in B-CLL were taken on the same day by conventional technique. We have measured the expression of ZAP-70 by flow cytometry by previously published method using ZAP-70, CD3, CD56, CD5 and CD19 markers (Letestu, Cytometry 2006). ZAP-70 expressions was expressed as percentage of ZAP-70 positive B-CLL cells according to ZAP-70 expression in T-lymphocytes (%/T-Ly) and as T/CLL mean fluorescence intensity (MFI) ratio. Results were analyzed by paired tests. *Results*. Samples were taken from 42 typical B-CLL patients, median age was 70yr, there were 55% males, mean β-2 microglobulin was 3.9 mg, mean TTM was 9.3, there were 22% of patients with lymphoma-like tumor distribution (TD<0.5). There were 17, 16 and 8 patients in Binet stages A, B and C respectively. ZAP-70 expression as T/CLL ratio (mean ±s.d.) was 4.9±3.7, 2.9±2.1 and 1.7±0.8 for PB, BM and LN respectively (p<0.00001). Results for percentage of positive B-CLL cells (%/T-Ly) were at the same level of statistical significance (p<0.00001) with median percentage in PB 19.7%, in BM 62.3% and in LN 92.9% showing the lowest expression in peripheral blood and the highest expression in lymph nodes. *Summary and Conclusions*. We have shown that B-CLL lymphocytes taken from different lymphoid compartments have significantly different ZAP-70 expression supporting the importance of lymph node and bone marrow compartments in B-CLL pathogenesis.

0914

THE DISTRIBUTION OF XRCC1-399 AND XPD-751 GENOTYPES IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH SECONDARY TUMORS AND RICHTER TRANSFORMATION

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Background. The clinical course of chronic lymphocytic leukemia (CLL) is associated frequently with secondary solid tumors development and transformation to Richter syndrome (RS), represented in most cases by diffuse large B-cell lymphoma. Molecular indicators of the risk as well as mechanisms of the second neoplasm's development are unknown. Some data suggests that several polymorphisms in DNA repair genes have impact on cancer susceptibility. X-ray repair cross-complementing group 1 (XRCC1) and Xeroderma pigmentosum D (XPD) are most extensively studied in cancer. *Aims*. To evaluate association of XRCC1 Arg399Gln and XPD Lys751Gln polymorphisms with tumors development and transformation to RS in patients with CLL. *Methods*. Polymorphisms were genotyped using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP). Genomic DNA was amplified in PCR using primers designed by Seedhouse *et al.*, 2002, followed by enzymatic digestion of PCR products. The digested products were resolved on 3% agarose gel and analyzed. *Results*. We examined the frequency of 2 polymorphisms in 140 CLL patients divided into three groups: with RS (22), with secondary solid tumor (22) and control group of patients with CLL alone (96). Subgroup of 35 patients with overall survival (OS) more than 3 years was picked out the control group. Patients of these groups were comparable by gender and age. However, all patients with RS were previously treated and most of them had B stage at diagnosis (72.7%) in contrast to the other groups (31.4-36.4%; p<0.001). The distributions of the XRCC1 Arg399Gln as well as XPD Lys751Gln polymorphisms in observed CLL cohort did not differ from healthy Caucasians (Table). Neither the XRCC1-399 nor the XPD-751 genotype distributions showed any differences in CLL patients with secondary tumors. We also found no correlation between XPD-751 genotypes and development of RS. However, the proportion of CLL patients with RS homozygous for XRCC1 Gnl399 allele was higher than in the control group (χ^2 = 5.55) as well as in the subgroup of patients with OS>3y (χ^2 = 5.33). At the same time the frequency of XRCC1 heterozygotes among CLL patients with RS was significantly lower in comparison with two other groups. Since RS developed in previously treated patients only, we examined the XRCC1-399 and XPD-751 genotype distribution in previously treated patients from control group. Those patients as well as previously treated control group patients with OS>3y were uncommon homozygous for XRCC1 Gnl399 allele (9.1% and 7.4% correspondingly) in comparison with RS patients (30%; p<0.01). *Summary*. Our results suggest about possible role of DNA repair mechanisms, specifically XRCC1 and XPD gene polymorphisms in Richter syndrome development in CLL.

Table 1.

	CLL patients				
	Control group		With solid tumors, n = 22	With RS, n = 22	All patients n = 140
	All, n = 96	OS>3y, n = 35			
XRCC1 Arg399Gln p = 0.063					
Arg/Arg	34 (37.8)	9 (28.1)	8 (44.4)	9 (45.0)	51 (39.8)
Arg/Gln	47 (52.2)	21 (65.6)	9 (50.0)	5 (25.0)	61 (47.6)
Gln/Gln	9 (10.0)	2 (6.3)	1 (5.6)	6 (30.0)	16 (12.6)
XPD Lys751Gln p = 0.600					
Lys/Lys	36 (38.7)	14 (40.0)	5 (23.8)	5 (22.7)	46 (33.8)
Lys/Gln	47 (50.3)	18 (51.4)	14 (66.7)	15 (68.2)	76 (55.9)
Gln/Gln	10 (11.0)	3 (8.6)	2 (9.5)	2 (9.1)	14 (10.3)

0915

WHAT IS THE ROLE OF BONE MARROW PATHOLOGICAL EXAMINATION IN CLL? EXTENSIVE HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR EXAMINATION IN A SERIES OF 137 PATIENTS

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We analyzed bone marrow biopsy (BMB) samples from 137 patients with CLL and explored associations with clinical features and outcome, CD38 expression, and IGHV mutation status. Patient group: M:F=84:53, median age: 66 years, Binet stage-A/B/C: 111/20/6. Peripheral blood immunophenotype: (1) $CD38^+$: 48/134 cases (2) IgG^+: 20/131 cases. Mutated/unmutated IGHV genes (M-IGHV/A-IGHV): 86/51 cases. Formalin-fixed, decalcified, paraffin-embedded sections of BMBs were examined: (1) morphologically (H&E); (2) immunohistochemically (ABC technique). The following antibodies were used: CD20, CD79a, CD3, BCL-2, CD5, CD23, SIg/CIg (k/l/m/d/g/a), CD27, myeloperoxidase, PGM1 (CD68), glycophorin-C, CD61. All cases showed neoplastic small lymphocytic infiltration (20-95% of BM cellularity) by $CD20^+CD79a^+$ $CD5^+CD23^+$cells. Medium- or large-sized cells were admixed in 6 and 2 cases, respectively. A moderate number of monocytoid-like cells with clear cytoplasm were identified in 6 cases. Six cases exhibited lymphoplasmocytoid differentiation in a small proportion of the neoplastic population. Finally, two additional cases showed pronounced plasmacytic differentiation which was proven (by immunohistochemistry) of identical heavy and light isotype restriction as the CLL neoplastic lympho-

cytic population. Three patterns of neoplastic lymphocytic infiltration were identified: (1) interstitial: 59 cases; (2) nodular/nodular+interstitial: 51 cases; (3) diffuse: 27 cases. Moderate-to-significant reduction of the granulocytic series was observed in 60/137 cases. The remaining cases showed hyperplastic granulocytic series with a left shift and, occasionally, dysplastic changes of mature forms. Hyperplasia of the erythroid and megakaryocytic series -often with dysplastic changes- with normal/decreased hemoglobin levels and platelet counts was observed in 76/137 and 99/137 cases, respectively; the remaining cases showed moderate-to-significant reduction of either series. Significant correlations were identified between: (i) nodular infiltration and M-IGHV genes (p<0.001); (ii) diffuse infiltration and shorter time-to-progression (p=0.05). In conclusion, BMB examination: (1) permits identification of the rare CLL subtypes with plasmocytoid and/or plasmacytic differentiation (WHO); (2) allows evaluation the BM stroma and the hematopoietic marrow and provides important evidence for CLL-associated hematopoietic autoimmunity. Finally, the favorable prognosis of the nodular pattern of infiltration in CLL may be interpreted in view of its association with M-IGHV genes.

0916

CLLU1 GENE EXPRESSION LEVELS PREDICTS VH MUTATIONAL STATUS IN 90% OF CLL PATIENTS

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CLLU1 is a gene identified by differential display performed in CLL samples with mutated (MU) versus unmutated (UN) VH monoclonal sequence. Initials results using a variety of Q-PCR protocols suggested that CLLU1 overexpression occurs in unmutated CLL patients. Recenly it has been shown that CLLU1 expression level is a stable and inherent feature of CLL clone. CLLU1 gene function is unknown and until now no protein product of the various mRNA splice variants of this gene has been identified. *Aims*. A) To validate a new Q-PCR methodology for the determination of CLLU1 levels in CLL samples and its correlation to VH mutational status. In MCL, B-ALL, MM, tonsilar B, and peripheral blood T cells CLLU1 levels were also determined. B) To analyze if CLLU1 mRNA is inducible upon CLL cell activation *in vitro*. *Methods*. Samples from 69 CLL patients (MU 31 pts, UN 38), 8 MCL (4 MU, 4 UN), 10 B-ALL (5 Ph^+), 5 MM, B cells from 3 tonsils and T cells from 4 healthy subjects were analyzed for CLLU1 expression. Briefly 1μg of RNA was reversed transcribed to cDNA and Q-PCR of CLLU1 gene and β2 microglobulin (control gene) was performed using the CLLU1 profile Quant kit (Ipsogen). Absolute levels of CLLU1 and β2m mRNA are determined and results are expressed as CLLU1/β2m ratio (x10-5). Monoclonal IGVH mutation analysis was performed in all CLL and MCL samples. Two CLL samples with low levels of CLLU1 expression were *in vitro* stimulated with anti-IgM+IL-4 or CD40L+IL-4 for 1-3 days, and examined for levels of CLLU1/β2m and CLLU1/ABL expression. *Results*. In 31 MU CLL pts mean level of CLLU1/,2m was 82, and in 38 UN was 2153 CLLU1 levels in UN pts were in average 26 times higher compared to MU CLL pts levels. Using the cutoff value of CLLU1/β2m of 100, in 90% of all 69 CLL pts VH mutational status could be predicted: 28 of 31 MU had levels less than 100 and 35 of 38 UN CLL pts had levels over 100. No correlation between percentage of VH mutations and CLLU1/β2m ratio was found. MCL CLLU1 average levels was 2.5 with no difference between UN and MU samples. In B-ALL cases, MM, normal tonsilar B cells and T cells were 0.53, 3.23, 4.1, 0.57 respectively. *In vitro* stimulation of MU CLL cells for 1-3 days did not upregulate expression of CLLU1. Anti-IgM/CD40L/IL-4 *in vitro* stimulation of tonsilar B cells and PHA activation of T cells did not increase CLLU1 expression levels. These results were verified by determining CLLU1/ABL ratio. *Conclusions*. Over expression of CLLU1 in the majority of UN CLL pts identifies a unique lineage and disease specific marker, not expressed in other normal cells or B cell malignancies irrespective of VH mutational status. Stable levels of CLLU1 expression during *in vitro* CLL activation suggests that this is a disease/patient specific marker. These results imply that, although this gene function is totally unknown, CLLU1 has a crucial role in the pathogenesis of CLL and it should be further explored.

0917

CD72 EXPRESSION PATTERNS IN ZAP70 POSITIVE AND NEGATIVE CHRONIC LYMPHOCYTIC LEUKEMIA: DIFERENTIAL EXPRESSION OF GENES RELATED WITH BCR SIGNALLING

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Background. Absence of mutations in IgVH genes or higher number of ZAP70+ cells (as a surrogate marker) in chronic lymphocytic leukemia (CLL) B-cells defines a patient group with a poorer clinical course. These features relate to the role of BCR signalling in the proliferation and survival of CLL B-cells, establishing a link between these markers and the biology of CLL prognostic subgroups. *Aims*. Identify additional molecular mechanisms related to the biology of distinct CLL prognostic subgroups. *Methods*. Imunomagnetically purified $CD19^+$ cells from 3 unmutated and 3 mutated CLL cases were independently pooled and used to generate the transcription profiles, using serial analysis of gene expression (SAGE). Percentage of $ZAP70^+$ and $CD72^+$ cells were evaluated by flow cytometry on gated $CD19^+CD5^+$ cells in 24 CLL samples. Positive cases for ZAP70 and CD72 were defined using a cut-off of 30% and 40% positive cells, respectively. Real time PCR was used to quantify the expression levels of RELB, β-Catenin (CTNNB1) and AKT1 on 15 $CD19^+$ enriched (purity >90%) CLL samples. *Results*. SAGE analysis revealed a significant higher level of CD72 transcripts in unmutated samples, a specific B cell surface glycoprotein known to transmit both positive and negative signals in BCR signalling. In line, median percentage of $CD72^+$ cells was significantly higher in $ZAP70^+$ cases compared to ZAP70- cases (82% and 39%, respectively, p=0.0029). Furthermore, percentages of CD72 and ZAP70 were positively correlated (r=0.5930 and p=0.0009). Interestingly, $ZAP70^+$ cases were restricted to $CD72^+$ cases (n=11, [+/+]), whereas 6 $ZAP70^-$ cases were $CD72^+$ [-/+] and 7 were CD72- [-/-]. No differences among these 3 groups were observed in regard to laboratory parameters (white blood cells, total lymphocytes, lymphocyte percentage, haemoglobin, haematocrit and platelet number). Transcripts for RELB, CTNNB1 and AKT1, all related to BCR-mediated proliferation and survival, were expressed at significantly higher levels in ZAP70+CD72+ samples (n=7), compared to ZAP70-CD72+ (n=4). Interestingly, CTNB1 and AKT1 transcripts (but not for RELB, p=0.095) were expressed at significant higher levels in ZAP70-CD72- samples (n=4), compared to ZAP70-CD72+ samples. *Conclusions*. We have shown that the expression of CD72 and $ZAP70^+$ on CLL cells correlate and that CD72 may have a potential role on signaling mechanisms related to CLL B-cell proliferation and survival. In this way, higher levels of the evaluated transcripts on ZAP70-CD72-, compared to ZAP70-CD72+ cases, indicates that in the absence of ZAP70 expression, CD72 may act as a negative regulator of the BCR pathway. Finally, higher expression of these transcripts on $ZAP70^+CD72^+$, compared to ZAP70-CD72+ cases, further corroborates the proposed role of ZAP70 on BCR-mediated proliferation and survival. This work was supported by FAPESP, CNPq and FINEP.

0918

DIFFERENT CXCR4, CXCR3, CXCR5, CCR7 AND KI-67 EXPRESSION IN PERIPHERAL BLOOD, BONE MARROW AND LYMPH NODES IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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Background. B-cell chronic lymphocytic leukemia has highly variable clinical presentation and course with variable involvement of different lymphoid compartments which translates into variable tumor distribution. Important role in lymphocyte trafficking and homing have adhesion molecules and chemokine receptors. We have previously shown that there is significant variability regarding adhesion molecule expression between lymphoid compartments with significant association with clinical presentation and course (Jaksic *et al.*, Blood 2002). Important role in trafficking and homing as well as regulation of expression of adhesion molecules have been documented in *in vitro* models for chemokine receptors. This points to need to evaluate whether there is an intraclonal variability due to distinct microenvironment *in vivo*, as well as whether different chemokine receptor profile correlates with cell proliferations.

Aims. to evaluate and compare the expression of various chemokine receptors as well as marker of proliferation Ki-67 on B-CLL lymphocytes taken from various lymphoid compartments, ie peripheral blood (PB), bone marrow (BM) and lymph nodes (LN). *Methods*. Samples were taken from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) representing major compartments in B-CLL, by conventional techniques on the same day. We have measured expression of CXCR3, CXCR4, CXCR5, CCR7 and Ki-67 on $CD5^+CD19^+$ cells by flow cytometry and we have expressed the results as percentage of positive cells and mean fluorescence intensity (MFI). Results were analyzed by paired tests. *Results*. samples were taken from 21 typical B-CLL patients (median age 69 years, males 56%). Mean β-2 microglobulin was 4.5 mg, mean TTM was 10.3, with 35% of patients with lymphoma-like tumor distribution (TD <0.5). There were 7, 10 and 4 patients in Binet stages A, B and C respectively. Median CXCR3 expression was 15.8, 9.7 and 17.3% for PB, BM and LN respectively (p<0.01 PB and LN vs. BM). Median CXCR4 expression was 51, 17 and 12.3% for PB, BM and LN respectively (p<0.01 PB vs. BM and LN). Median CXCR5 expression was 94.1, 87.7 and 64.5% for PB, BM and LN respectively (p<0.01 PB and BM vs. LN). Median CCR7 expression was 98.2, 96.6 and 72% for PB, BM and LN respectively (p<0.01 PB and BM vs. LN). Mean Ki-67 expression (MFI) 0.89, 0.76 and 1.39 for PB, BM and LN respectively (p<0.01 PB and BM vs. LN). CXCR4 expression was higher in PB compared to BM and LN, CXCR3 expression was lower in BM then in PB and LN, CXCR5 and CCR7 expression was higher in PB and BM compared to LN. Ki-67 expression was highest in lymph nodes. *Summary and Conclusions*. There is a significant intraclonal variability for several chemokine receptors regarding different lymphoid compartments. This chemokine receptor profile inversely correlates with proliferative status of B-CLL cells in respective compartments.

Chronic lymphocytic leukemia and related disorders - Clinical II

0919

HIGH ACTIVITY OF SINGLE-AGENT OFATUMUMAB, A NOVEL CD20 MONOCLONAL ANTIBODY, IN FLUDARABINE- AND ALEMTUZUMAB-REFRACTORY OR BULKY FLUDARABINE-REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA, REGARDLESS OF PRIOR RITUXIMAB EXPOSURE

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Background. Patients with fludarabine-refractory chronic lymphocytic leukemia (CLL) have poor prognosis, and this is particularly true of those also refractory to alemtuzumab (FA-ref) or with bulky (>5 cm) lymphadenopathy (BF-ref) where salvage therapy, including intensive chemoimmunotherapy, has limited activity (20-26% overall response rate [ORR]). Historically, monoclonal antibodies (mAbs) evaluated in this population have no activity; results from a retrospective analysis showed an overall response of 0%, median time to treatment failure of 2 months and median overall survival (OS) of 6 months with mAb salvage therapy (Tam, Leuk Lymphoma 2007;48:1931). Ofatumumab is a unique human mAb that targets a membrane-proximal small-loop epitope on the CD20 molecule and elicits more potent *in vitro* complement-dependent cytotoxicity of B-cell lines and primary CLL cells compared with rituximab (Teeling, Blood 2004;104:1793; Teeling, J Immunol 2006;177:362). *Aims*. To determine whether prior rituximab exposure impacted the efficacy of ofatumumab in patients with FA-ref or BF-ref CLL.

Table 1. Efficacy outcomes by rituximab exposure.

Prior rituximab	FA-ref (n=59)			BF-ref (n=79)		
	n	ORR, % (95% CI)	Median PFS, mo (95% CI)	n	ORR, % (95% CI)	Median PFS, mo (95% CI)
Any prior rituximab*	35	54 (37, 71)	5.5 (3.7, 8.0)	43	44 (29, 60)	5.5 (3.8, 6.4)
FR†	19	53 (29, 76)	4.6 (2.8, 6.4)	27	52 (32, 71)	5.6 (2.5, 7.4)
FCR‡	16	50 (25, 75)	4.6 (2.3, 6.4)	16	44 (20, 70)	5.6 (2.1, 6.6)
No prior rituximab	24	63 (41, 81)	7.1 (4.8, 8.7)	36	50 (33, 67)	6.4 (4.0, 8.0)

*Patients received ≥1 prior rituximab-containing regimen at any time before study entry; †Patients refractory to fludarabine in combination with rituximab (FR), with or without other drugs; ‡Patients refractory to fludarabine in combination with rituximab and cyclophosphamide (FCR), with or without other drugs.
FA-ref=fludarabine- and alemtuzumab-refractory; BF-ref=bulky fludarabine refractory; ORR=overall response rate; PFS=progression-free survival

Methods. Patients with FA-ref or BF-ref CLL received 8 weekly infu-

sions of ofatumumab followed by 4 monthly infusions (Dose 1, 300 mg; Doses 2-12, 2000 mg). Premedications included paracetamol, antihistamine and glucocorticoid. The primary endpoint was ORR (1996 NCI-WG criteria) over 24 weeks assessed by an Independent Review Committee (IRC). Secondary efficacy endpoints included progression-free survival (PFS) and OS. Results from the planned interim analysis of this ongoing, international pivotal study are reported. *Results*. At the interim analysis, 138 treated patients had FA-ref (n=59) or BF-ref (n=79) CLL; 63% had Rai stage III/IV disease at screening and the median number of prior treatments was 5. The ORR (99% confidence interval [CI]) based upon IRC assessment was 58% (40, 74%) in the FA-ref group and 47% (32, 62%) in the BF-ref group. Median PFS (95% CI) was 5.7 months (4.5, 8.0 months) and 5.9 months (4.9, 6.4 months) in the FA-ref and BF-ref groups, respectively, and median OS (95% CI) was 13.7 months (9.4, [upper limit not yet reached] months) and 15.4 months (10.2, 20.2 months), respectively. Prior to receiving ofatumumab, 59% of FA-ref and 54% of BF-ref patients had been treated with a rituximab-containing regimen. Both ORR and median PFS were similar in patients with prior rituximab exposure compared with the overall study population (Table). Response rates and median PFS were also similar in patients refractory to fludarabine in combination with rituximab, with or without cyclophosphamide. *Conclusions*. The activity of ofatumumab was independent of prior rituximab treatment; high response rates were observed irrespective of previous exposure to rituximab-containing regimens, including patients whose disease was refractory to fludarabine-based regimens that contained rituximab.

0920

MULTICENTER EXTERNAL VALIDATION OF A NOMOGRAM AND A SCORE FOR PREDICTING OVERALL SURVIVAL IN CLL: A STUDY ON 757 PATIENTS

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Background. B-cell chronic lymphocytic leukaemia (B-CLL) is a heterogeneous disease with highly variable clinical course. In a recent report (Wierda *et al.*, Blood 2007,109:4679), a prognostic nomogram and a risk score were proposed to predict overall survival by using 6 clinical and routine laboratory parameters: age, β2 microglobulin, absolute lymphocyte count, sex, Rai stage and number of involved lymph node areas. Prognostic models developed in a single centre in a particular population may not perform equally well when applied to other populations in other centres. *Aims*. We wish to validate the prognostic power of the nomogram and risk score in a multicentric Italian population. *Methods*. We collected 757 complete records from a sample of 1109 patients from 6 centres. We used a nomogram and a prognostic score calculated according to the original publication to estimate median overall survival, 5-year and 10-year survival probability; a score stratified Cox model to estimate relative risk of death; univariate and multivariate Cox models to verify independent prognostic power of all 6 clinical and laboratory parameters. A p value<0.05 was considered to be statistically significant. Survival was measured from diagnosis until death from any cause. Kaplan-Meier method was used to estimate median survival. Median follow-up was calculated by inverted censoring method. β2 microglobulin value was standardized. *Results*. Median age was 65 years, median β2 microglobulin was 1 time upper reference limit, median lymphocyte count was 11×10^9/L, 58% of patients were male, 8% were in Rai stage III/IV, 19% had 3 or more involved lymph node areas. Median follow-up was 5.5 years (5.1-5.8). There were 102 deaths (13%) during follow-up and 44% of patients were treated. According to the nomogram, estimated median survival was approximately 11 years, survival probability was approximately 80% at 5 years and 50% at 10 years. Real data showed a not reached median survival at 21 years, an overall survival probability of 89% (86-92) at 5-years and of 77% (73-82) at 10 years. Using the simplified prognostic index score the agreement with real data at 5-years estimate was better then that obtained with the nomogram, but not satisfactory at 10-years estimate. Relative risk of death was in acceptable agreement. All 6 parameters were significant predictors in univariate models, but β2 microglobulin and lymphocyte count were not independent predictors in multivariate models. *Conclusions*. We could not confirm the independent predictive power of β2 microglobulin and lymphocyte count in our CLL population. Stratification with the simplified prognostic index score was able to correctly estimate relative risk of death, but not absolute value of survival probability. Median survival, 5- and 10-years survival probability was underestimated by using both the nomogram or the prognostic index score. The performance of the 6 parameters prognostic model may depend on the population on which it was developed. Further multicentric studies are needed to evaluate its general applicability.

0921

THE PROGNOSIS OF CLINICAL MONOCLONAL B CELL LYMPHOCYTOSIS DIFFERS FROM PROGNOSIS OF RAI 0 CHRONIC LYMPHOCYTIC LEUKAEMIA AND IS RECAPITULATED BY BIOLOGICAL RISK FACTORS

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Background. Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic monoclonal expansion of $<5.0\times10^9$/L circulating CLL-phenotype B-cells. Since the introduction of the new IWCLL guidelines, it has been a matter of debate whether the 5.0×10^9/L CLL-phenotype cell cut-off for CLL diagnosis has a clinical rationale for the definition of MBL. The relationship between MBL and Rai 0 CLL, as well as the impact of biological risk factors on MBL prognosis, are unknown. *Aims*. i) to define whether clinical features at diagnosis, biological profile, and outcome of cMBL are distinguishable from Rai 0 CLL; ii) to identify clinical or biological variables at diagnosis that best predict the risk of evolution from cMBL to CLL requiring treatment. *Methods*. Out of 460 B-cell expansions with CLL-phenotype, 123 clinical MBL (cMBL) were compared to 146 Rai 0 CLL for clinical and biological profile and outcome. *Results*. cMBL had better humoral immune capacity and lower infection risk, lower prevalence of del11q22-q23/del17p13 and TP53 mutations, slower lymphocyte doubling time, and longer treatment-free survival. Also, cMBL diagnosis was a protective factor for treatment risk. Despite these favourable features, all cMBL were projected to progress, and lymphocytes $<1.2\times10^9$/L and $>3.7\times10^9$/L were the best thresholds predicting the lowest and highest risk of progression to CLL. Although IGHV status, CD38 and CD49d expression, and FISH karyotype individually predicted treatment-free survival, multivariate analysis identified the presence of +12 or del17p13 as the sole independent predictor of treatment requirement in cMBL (HR: 5.39, 95% CI 1.98-14.44, p=0.001). *Conclusions*. In this study we confirm that the 5.0×10^9/L cell cut-off can be used to distinguish cMBL from CLL. More importantly, however, we document that: i) cMBL and Rai 0 CLL differ in some biological features and outcome; and ii) cMBL who are destined to progress to symptomatic CLL/SLL requiring treatment can be identified by biological risk factors. Overall, these data show that cMBL has a more favourable clinical course than Rai 0 CLL. Since the biological profile can predict treatment requirement, stratification based on biological prognosticators may be helpful for cMBL management.

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SUCCESSFUL TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA SHOWING 17P DELETION WITH FLUDARABINE, CYCLOPHOSPHAMIDE AND ALEMTUZUMAB

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B-CLL patients with p53 gene deletions [del(17)(p13.1)] or p53 point mutations have an inferior survival and appear resistant to standard chemotherapy. Alemtuzumab (A) is of potential interest in the treatment of p53-deleted B-CLL as its cytotoxicity is predominantly mediated by mechanism that should not involve the p53 pathway. A is relatively ineffective in pts with substantial lymph node enlargement. This consideration limits the usefulness of A monotherapy in CLL pts with p53 defects, as lymphadenopathy is common among such cases. In an attempt to overcome the limitations of A monotherapy in p53-deleted

CLL a protocol was developed in which the three agents, fludarabine (F), cyclophosphamide (C) and A are given in combination to B-CLL patients showing this high risk genetic profile. The FCC regimen consisted of F 40 mg/m^2/d orally on days 1-3, C 250 mg/m^2/d orally on days 1-3 and A 20 mg sc on days 1-3. This combination was repeated on d 29 for up to 6 cycles. Untreated or relapsed/refractory B-CLL patients carrying a p53 deletional status detected by fluorescent *in situ* hybridization (FISH) were selected to be enrolled in this phase II, open-label, single-centre trial. Chromosome abnormalities were detected by FISH testing at study entry from peripheral-blood or bone marrow cells. Only patients showing 17p with a clone size > 20% were enrolled. Mutational status of the variable region of the immunoglobulin heavy chain (IgVH) gene was also determined. Antimicrobial prophylaxis comprised trimethoprim/sulfamethoxazole (960 mg, every other day) and acyclovir (400 mg, twice daily). In the event of clinical or laboratory cytomegalovirus (CMV) reactivation, acyclovir was replaced by oral or intravenous ganciclovir (5 mg/kg/day). Currently, 14 pts have been enrolled in this trial. Median age was 55 years (range 45-72), 9/14 (64%) were male, 13/14 (93%) were in Binet stage B or C. Seven (50%) pts were untreated. The median time from diagnosis to study entry was 22 (range 3-108) and 59 (range 18-99) months for untreated and pretreated pts respectively. In the 7 pre-treated pts median number of prior regimens was 2 (range 1-3). IgVH unmutated was observed in 11 (79%) pts. The majority of patients had lymphadenopathy and/or splenomegaly. The ORR was 86%, with 7 (50%) pts achieving CR, 5 (36%) pts a PR. One remained in SD, while 1 showed progression of the disease. MRD negativity was achieved in 5/14 (36%) pts. Higher number of CR [6/7(87%)] and MRD- CR [4/7(57%)] were observed in untreated pts. A total of 69 cycles were assessable for toxicity. One patient showed a reactivation of a B virus hepatitis. In one patient therapy had to be stopped because of pneumonia sustained by Nocardia. The last patient showing a fungal pneumonia sustained by Aspergillus terreus was successfully treated with voriconazole. There were three subclinical CMV reactivations successfully treated with oral valganciclovir. Grade III-IV neutropenia episodes were observed in 40% of the administered courses while grade III-IV thrombocytopenia episodes were detected only in 8.6% of cycles. Ten pts are in continuous response after a median follow up of 15 months (range 2-27). In conclusion, results of this 4-weekly regimen with F, C and A suggest that this combination is effective in CLL pts with an high risk genetic profile such as del 17p.

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LOW DENSITY LIPOPROTEIN RECEPTOR PROTEIN 4 (LRP4) RS2306029 SINGLE NUCLEOTIDE POLYMORPHISM PREDISPOSES TO TRANSFORMATION OF CHRONIC LYMPHOCYTIC LEUKEMIA TO RICHTER SYNDROME

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Background. The rationale of the study stems from two considerations: i) mechanisms and risk factors of CLL transformation to Richter syndrome (RS) are largely unknown; ii) the identification of a CD38 single nucleotide polymorphism (SNP) as RS risk factor suggests that the host genetic background may be relevant for RS development (Aydin S *et al*. Blood 2008;111:5646). *Aims*. To explore the role of the host genetic background in RS transformation. *Methods*. The study was based on a consecutive series of 331 CLL, of which 21 had transformed to RS. Using an educated guess approach, SNPs were selected according to the following criteria: i) reported association with CLL prognosis; ii) minor allele frequency >5% in Caucasians. Accordingly, 44 SNPs from 44 genes were genotyped on germline DNA extracted from peripheral blood granulocytes by SNP-minisequencing. Primary endpoint of the study was cumulative risk of transformation measured from date of CLL diagnosis to date of biopsy showing that RS transformation had occurred, death or last follow-up. The association between SNPs and risk of RS transformation was actuarially assessed by univariate log-rank analysis considering the minor allele as acting either in a dominant or a in a recessive fashion. Cox proportional hazard regression was used to build multivariate models for survival analysis. False discovery rate (FDR) was used to control for multiple statistical testing. *Results*. Univariate log-rank analysis identified LRP4 rs2306029, a SNP affecting the low density lipoprotein receptor protein 4 gene, as the sole SNP associated with RS transformation. CLL who carried LRP4 rs2306029 TT variant genotype displayed a higher risk of transformation (5-year risk: 14.1%) compared to patients carrying the LRP4 rs2306029 CT/CC genotypes that contained the wild type allele (5-year risk: 4.7%) (HR:4.08; p<0.001) (Figure 1). The association between LRP4 rs2306029 and RS transformation remained significant also after correction for multiple comparisons by FDR (q=0.040). Other variables at CLL diagnosis associated with an increased risk of RS were advanced Binet stage (p<0.001), lymph node size >3 cm, LDH elevation (p<0.001), CD38 expression (p=0.010), ZAP70 expression (p=0.017), unfavorable FISH karyotype (p<0.001), IGHV homology >98% (p<0.001), usage of IGHV4-39 (p<0.001), and stereotyped HCDR3 (p<0.001). None of the clinico-biological categories associated with an increased risk of RS was enriched with the LRP4 rs2306029 TT variant genotype (p>0.050 in all instances), suggesting that LRP4 rs2306029 TT is not a surrogate of other RS risk factors. Multivariate analysis selected LRP4 rs2306029 TT as an independent predictor of RS (HR:3.20; p=0.024), along with stereotyped HCDR3 (HR:3.13; p=0.033), IGHV4-39 usage (HR:5.22; p=0.008) and unfavorable FISH karyotype (HR:3.18; p=0.033). LRP4 rs2306029 is a non-synonymous SNP mapping to exon 31 of LRP4 and leading to Ser1554Gly amino acid substitution. In silico analysis with PupaSuite (http://pupasuite.bioinfo.cipf.es/), PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SNPeffect (http://snpeffect.vib.be/) algorithms predicted LRP4 rs2306029 T variant allele to be dysfunctional and lead to altered LRP4 protein. Conclusions. rs2306029 non-synonymous SNP affecting LRP4 may predispose to CLL transformation to RS. Since LRP4 is involved in Wnt signalling and is expressed in CLL, the gene variant may have pathogenetic relevance for RS development.

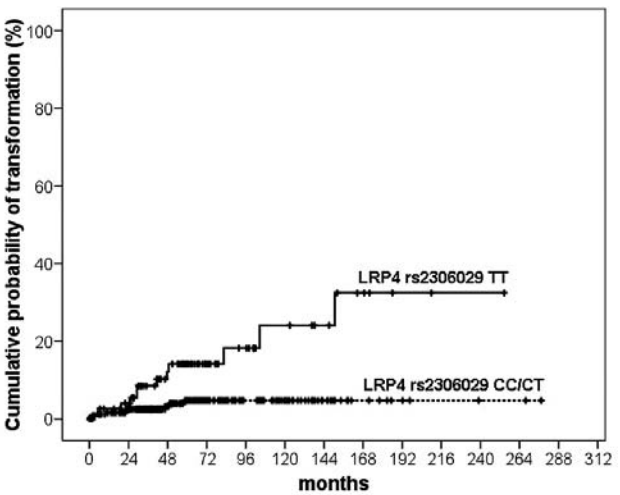

Figure 1. Cumulative risk of trasnformation to RS.

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A PHASE I DOSE-ESCALATION OF LENALIDOMIDE IN PATIENTS WITH RELAPSED OR REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Patients with relapsed or fludarabine refractory CLL have limited treatment options. Lenalidomide is an immunomodulatory drug that demonstrates significant clinical activity in this patient population

and in CLL patients with poor prognostic features including high risk cytogenetics and/or bulky disease. A phase II trial initiated in the relapsed/refractory setting to compare the safety and efficacy of lenalidomide at 25 mg/d with 10 mg/d resulted in 5 cases of tumor lysis syndrome (TLS). Therefore, in an effort to determine a safe dose and schedule the protocol was amended to a stepwise dose-escalation. Herein we present an interim safety data from the amended protocol. *Methods*. Eligible patients had good creatinine clearance (≥60 mL/min), received prior therapy with an alkylating agent and failed or progressed within a year of completing a fludarabine-based regimen. Lenalidomide was started at 2.5 mg daily dose. Intra-patient dose escalation to 5 mg/d occurred after 28 days with further dose escalations in 5 mg increments performed every 28 days, in 6-patient cohorts, until maximum tolerated dose escalation level (MTDEL) was defined, or the 25 mg/d dose level was attained. Patients continued on therapy until disease progression. TLS prophylaxis with 300 mg/d allopurinol and oral hydration were started 3 days before lenalidomide and continued for 3 cycles, with close monitoring for early signs of TLS (Cairo-Bishop Grading), particularly at drug initiation and dose escalations. *Results*. For 30 patients enrolled, median age was 66 years (range 50-76); 23 patients (76.7%) had bulky disease (LAN ≥5cm), and had failed a median 4 prior therapies (range 2-14): 13 patients (43.3%) were refractory to fludarabine and 6 (20.0%) had failed alemtuzumab. Grade 3-4 adverse events (AEs) were consistent with previous studies of lenalidomide in similar patient populations, and included thrombocytopenia (16.7%) and neutropenia (63.3%), of which 3% were febrile neutropenia. Ten patients (33%) developed tumor flare (3 cases were grade 3) at a median dose of 2.5 mg/d and most commonly during the first cycle. Laboratory TLS occurred in 1 patient at the 2.5 mg/d dose level and resolved without drug interruption. There are 14 patients currently on study and 16 patients discontinued therapy. Reasons for discontinuation included lack of efficacy in 7 patients, of whom 5 did not reach the 10 mg/d dose, and 5 patients developed AEs (at 10 mg/d: 1 AIHA, not attributed to lenalidomide and 1 PE in patient with DVT history and on antithrombotic prophylaxis; at 2.5 mg/every other day: 2 Grade 4 thrombocytopenia and 1 Grade 3 neutropenia). At last follow up lenalidomide was deemed tolerable up to the 15 mg/d dose level and the MTDEL had not been reached. Further escalation to the 20 mg/d dose is under investigation and anticipated for presentation. *Conclusions*. A preliminary safety analysis of lenalidomide stepwise dose escalation was found to be tolerable with MTDEL not yet reached at the 15 mg/d dose level. Close monitoring and prophylaxis were effective in the prevention, early detection and treatment of TLS in this heavily pretreated patient population with bulky disease.

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RELATIONSHIP BETWEEN CYTOGENETIC ANOMALIES AND BIOMARKERS IN BINET STAGE A PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA AT DIAGNOSIS: PRELIMINARY RESULTS OF A PROSPECTIVE, MULTICENTER 0-CLL1 GISL STUDY

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Background. The clinical heterogeneity of chronic lymphocytic leukemia (CLL) requires parameters to stratify patients into prognostic subgroups to adapt treatment ranging from 'watch and wait' to allogeneic stem cell transplantation. Different parameters such as lymphocyte doubling time, β-2 microglobulin, CD38 and ZAP-70 expression, immunoglobulin variable heavy chain (IgVH) mutation status and genetic abnormalities have been integrated in clinical practice. By using fluorescence *in situ* hybridization (FISH), cytogenetic abnormalities can be found in approximately 80% of patients. *Aims*. In the present study, we performed FISH analysis to detect the major cytogenetic alterations in a series of patients in Binet stage A included in the prospective multicenter O-CLL1 GISL study started in April 2007. *Methods*. Molecular markers characterization and FISH analyses were previously reported (Cutrona *et al*. Haematologica, 2008; Fabris *et al*. GCC, 2008). *Results*. Up to date, 275 patients have been enrolled in the trial and FISH data concerning trisomy 12 and 13q14, 17p13, 11q23 deletions were available in 192 patients. At least one abnormality was found in 131/192 (68.2%) patients. The most frequent abnormality was del(13)(q14) (100/192, 52%), followed by trisomy 12 (25/192, 13%) (in one case accompanied by 17p13 deletion), del(17)(p13) (6/192, 3%) and del(11)(q22.3) (10/192, 5%). 13q14 deletion was found as a sole abnormalities in 90 patients; in the remaining cases, it was combined with del(17)(p13) (3 pts), trisomy 12 (1 pts) and del(11q)(22.3) (6 pts). Among patients with 13q14 deletions, 71 were monoallelic, 7/100 biallelic, whereas 22 showed combined biallelic and monoallelic deletion patterns. We also analyzed the relationship between each single abnormality and CD38 and ZAP-70 expression, and IgVH mutational status. In particular, the CD38 percentages were 8.4±1.8 (mean value±SD), 19.5±2.8, 45.1±7.1, 30.3±8.4, 52.9±9.9 for del(13)(q14), normal, trisomy 12, del(11)(q22) and del(17)(p13) FISH alterations (p<0.0001), respectively. The percentages of IgVH mutations significantly (p<0.0001) correlated with cytogenetic alterations; namely, 5.3±0.4 for cases with del(13q14), 4.6±0.6 in normal, 1.8±0.6 in trisomy 12, 0.2±0.1 in del(11)(q22) and 1.6±1.7 in the 4 cases with del(17p13). Similarly, a significantly (p=0.0005) lower mean value of ZAP-70 expression was accounted in del(13q14) (29.7±2.3) as compared with normal cases (34.6±3.2), trisomy 12 (43.8±5.4), del(11)(q22) (55.9±9.7) and del(17)(p13) (54.8±11.8). Based on a scoring system in which 1 point was assigned to each unfavorable biomarker (i.e. CD38, ZAP-70 or IgVH mutational status) we stratified our series in three different groups from 0 to 3 according to the absence or presence of one, two or all three biomarkers. Similarly, cytogenetic abnormalities were clustered in 3 risk groups [i.e. low del(13)(q14) and normal; intermediate (trisomy 12); and high risk del(11)(q22) and del(17)(p13)] as suggested by others. Interestingly, 72/77 cases scoring 0 for the biomarkers, gathered in the low FISH group. Conversely, out of the 13 cases with high FISH risk, 10 cluster in scoring 2-3. *Conclusions*. Our preliminary results indicate that in Binet stage A CLL patients at diagnosis cytogenetic abnormalities with an expected negative clinical impact are relatively few (16/192, 8%) but significantly associated with prognostic biomarkers which negatively predict the clinical outcome in CLL.

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BETA2-MICROGLOBULIN IS A BETTER PREDICTOR OF TREATMENT-FREE SURVIVAL IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA IF ADJUSTED ACCORDING TO GLOMERULAR FILTRATION RATE

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Background. Even in the era of newer and sophisticated prognostic markers, β2-microblobulin (B2M) remains a simple but very powerful predictor of treatment-free survival (TFS) and overall survival (OS) in patients with chronic lymphocytic leukaemia (CLL). However, B2M levels are heavily influenced by the patient's glomerular filtration rate (GFR). *Aims*. To evaluate whether GFR-adjusted B2M (GFR-B2M) had improved prognostic value compared to unadjusted B2M in a cohort of over 450 consecutive CLL patients from two separate institutions. *Methods*. Inclusion in this study was base purely on the confirmed diagnosis of CLL as defined by the WHO. Institutional databases from Hospital Sant Pau (HSP), Barcelona, and Birmingham Heartlands Hospital (BHH)

were screened for patient information relating to age; gender; Binet stage; B2M level at diagnosis; creatinine level at diagnosis; time and type of CLL-specific treatment; and overall survival. Results on the IgVH mutation status, genomic abnormalities as detected by FISH; CD38 expression and ZAP-70 expression were available in a proportion of patients. Standard clinical criteria were used for defining the initiation of therapy in all patients. GFR was estimated using the modification of diet in renal disease equation: GFR (ml/min/1.73m^2) = 186.3 x Serum Creatinine (mg/dL)-1.154 x Age (y)-0.203 x 1.212 if black, x 0.742 if female. For the purpose of this study we devised the so-called GFR-adjusted B2M (GFR-B2M) using the following equation: GFR-B2M = B2M (mg/L) x GFR (ml/min) / 100. Receiver operating characteristic (ROC) curves were generated in order to identify the most appropriate cut-off levels for both B2M and GFR-B2M. The area under the ROC curves was also calculated to evaluate the suitability of both B2M and GFR-B2M as predictors for treatment-free survival (TFS). The primary endpoints for comparison were TFS and overall survival (OS). The prognostic impact of each covariate was explored in the HSP cohort and validated in the BHH cohort. *Results*. By multivariate analysis, we observed a significantly shorter TFS in patients who were ZAP-70+ (p<0.001), with increased GFR-B2M (p<0.001), and del(11q) or del(17p) as detected by FISH (p<0.001). These results were validated in the BHH cohort. When OS was evaluated by multivariate analysis, age of 65 years or older (p<0.001) and poor risk FISH abnormalities (p<0.001) had a confirmed adverse prognostic impact, but the predictive value of GFR-B2M was lost in the validation analysis. In all survival models, B2M did not attain independent significance unless GFR-B2M was eliminated from the analysis. *Conclusions*. GFR-B2M is a better predictor of TFS than unadjusted B2M in CLL patients.

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FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB IN FIRST-LINE TREATMENT OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): RETROSPECTIVE ANALYSIS OF CZECH CLL STUDY GROUP

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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) based on the results of CLL-8 study (Hallek *et al*., 2008). Although higher dose of rituximab (500 mg/m^2 from 2nd cycle) was used in CLL-8 as well as landmark phase II study (Keating *et al.*, 2005), it is purely empirical and ideal rituximab dosing remains unknown. Furthemore, 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. *Patients and Methods*. Between October 2002 and May 2008, we treated 107 pts with active CLL (68% males, median age, 60 years [range, 27-75]) by FCR regimen as first-line therapy at five centers cooperating within Czech CLL Study Group. 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 40mg/m^2 p.o. d1-3) and cyclophosphamide (250 mg/m^4 i.v. or p.o. d1-3). Rituximab was administered i.v. on day 1 of each cycle at the dose of 375mg/m^2 in all cycles (n=87) or 500mg/m^2 from 2nd cycle (n=20). Treatment was repeated every 4 weeks. Antimicrobial prophylaxis and growth factors were not routinely used. Low/intermediate/high risk according to modified Rai staging was present in 1/72/27%. IgVH mutation status and FISH aberrations were available in 85% and 79% of pts. IgVH genes were unmutated in 74%; according to hierarchical model, del 13q was present in 31%, trisomy 12 in 9%, del11q in 26% and del17p in 8%. *Results*. At the time of analysis (February 2009), the median observation time was 22.3 months (mo). Median number of FCR cycles was 5. The overall response rate/complete response rates were 92/47%. Median PFS was 30 mo; median overall survival (OS) was not reached. Patients with unmutated IgVH genes had significantly shorter PFS (p=0.0051). Small numbers of pts in each FISH aberration group precluded a meaningful statistical analysis. Four out of 7 pts with del 17p responded to FCR (1x CR, 3x PR). Patients treated with lower dose of rituximab (375mg/m^2) did not have significantly different ORR, CR and PFS from those treated with 500 mg/m^2 (p=0.79, p=0.24 and p=0.65). Grade III/IV neutropenia occured in 22/14% of cycles and thrombocytopenia grade III/IV in 5/3% of cycles. Serious infections occured in 1% of cycles only. G-CSF was administered in 54% and recombinant erythropoietin in 13% of pts. *Conclusions*. Treatment of CLL patiens 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. Further studies are needed to address the question whether lower dose of rituximab (375mg/m^2) in FCR yields the same therapeutical efficacy as 500 mg/m^2.

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0928

LENALIDOMIDE ASSOCIATED TUMOR FLARE REACTION CORRELATES WITH CLINICAL RESPONSE IN PATIENTS WITH RELAPSED OR REFRACTORY B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Lenalidomide is an immunomodulatory agent currently under investigation for treatment of chronic lymphocytic leukemia (CLL) and lymphoma. Tumor flare (TFR) is a unique clinical feature observed in CLL patients receiving lenalidomide, and may be related to its antitumor activity. TFR is characterized by painful, tender enlargement of disease involved lymph nodes, spleen and/or liver along with low grade fever. The underlying mechanism and impact of TFR on treatment outcomes remain unclear. To better understand its clinical significance, efficacy results from our phase II trial of patients with relapsed/refractory B-CLL treated with lenalidomide were correlated with the incidence, severity, and duration of TFR. *Methods*. Forty-five patients were divided into 2 groups: A) lenalidomide 25 mg/day for 21 days of a 28-day cycle without TFR prophylaxis (n=29); B) lenalidomide 10 mg/day with dose escalations in 5 mg increments every 1-2 weeks until maximum dose of 25 mg/day was achieved (n=16). Also, during days 1-14 of cycle 1, group B received oral prednisone (20 mg/day for 5 days followed by 10 mg/day for 5 days) as TFR prophylaxis. *Results*. For 45 patients evaluated, median age was 64 years, median baseline ALC was 43.6×10^9/L, and 29 patients (64%) had Rai stage III/IV disease. Patients had a median of 3 prior therapies. Clinically significant TFR developed in 30 patients (67%): 20 (67%) grade I, 7 (23%) grade II, and 3 (10%) grade III. Median time to TFR was 6 days (range 0-56) and median time to TFR resolution was 14 days (95% CI: 10-26). In >90% of cases TFR occurred only during the first cycle. For combined groups A and B, 11 patients required TFR treatment consisting of NSAIDs with or without oral morphine; only 3 patients received morphine. No patient discontinued lenalidomide due to TFR. Median age for those who developed TFR relative to those who did not was 61 and 71 years, respectively (p=0.004). TFR occurred in 19 patients (66%) from group A and in 11 (69%) patients from group B. In groups A and B, grade II/III TFR occurred in 9 (47%) and in 1 (9%) patient, respectively (p=0.05), with a median time to onset of 4 and 9 days, respectively (p=0.01). To date 8 patients achieved a complete response (CR) of who 7 had developed TFR. Among 15 patients without TFR only 1 CR (7%) was observed (p=0.24). Although median progression-free survival (PFS) for patients with and without TFR was 19.9 and 19.4 months, respectively, PFS in group A relative to group B was 23 vs. 17.8 months (p=0.74). This difference did not reach statistical significance potentially due to small patient numbers. *Conclusions*. TFR appears to be an acute inflammatory reaction primarily involving tumor bearing sites. Despite a low sample size our analysis suggests that TFR development may correlate with achievement of a CR with lenalidomide. Steroid prophylaxis decreases the severity but not the incidence of TFR. Whether TFR prophylaxis using steroids is effective remains to be determined in a larger cohort of patients.

0929

ABNORMAL SERUM FREE LIGHT CHAIN RATIO AS A PREDICTOR OF ADVERSE OUTCOME IN CHRONIC LYMPHOCYTIC LEUKEMIA: HAS THE TIME COME FOR A SIMPLE PROGNOSTIC MARKER?

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Background. As chronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course, recent studies have mainly focused on ‹dentification of a new and simple molecular and biological prognostic marker. An abnormal serum free light chain ratio (FLCR), which is considered to be a reliable predictor of outcome in plasma cell disorders, is reported in approximately 44% of CLL patients. *Aims*. This retrospective study is planned to assess the prognostic value of FLCR in CLL and to estimate its impact on survival and outcome of this specific group of patients. *Methods*. Quantitative levels of serum FLC were measured nephelometrically in sera collected at presentation or before treatment. A normal sFLCR range [kappa(k)/lambda(l)] was defined as 0,26-1,65. ZAP 70 and CD38 expression were quantified by flow cytometry. Chromosomal abnormalities were determined by interphase fluorescence *in situ* hybridization (FISH) on peripheral blood using multicolor probes for chromosome 12 centromere, locus specific 11q22.3, D13S319 and D13S25 and 17p13.1. Patients were stratified as high, intermediate and low risk based on FISH results. *Results*. A study cohort of 101 patients [median age 62(37-90); M/F: 68/33] were eligible for the current study. Median follow-up of the whole cohort was 28(1.1-234) months. An abnormal FLCR was found in 30 patients (29,7%), while 71 patients (70,3%) presented a normal FLCR. Abnormal FLCR was associated with leukocyte count (p=0.009), atypical lymphocyte morphology (p=0.046), clinical stage (p=0.018), lactate dehydrogenase (p=0.046) and CD38 levels (p=0.005). A significant linear relationship was observed between FLCR and CD38 on logistic regression analysis (p=0.03; OR:1,02). The three genetic risk groups, stratified according to FISH, did not differ significantly with respect to FLCR (p>0.05). Time to first treatment (TTFT) was 54,6% for the whole cohort; 57.4% in normal versus 46.1% in abnormal FLCR groups at the end of median 28(1,1-234) months of follow up (p>0.05). Abnormal FLCR was more frequent in patients requiring treatment (p=0.04). Overall survival (OS) was 49.2% for the whole cohort; 74.7% in FLCR normal versus 36,3% in FLCR abnormal groups, at the end of median 28(1.1-234) months of follow up (p=0.05). ZAP 70, CD38, clinical stage and performance status were identified as independent prognostic parameters for OS in multivariate analysis. *Summary and Conclusions*. This study highlighted the potential prognostic value of FLCR in CLL patients. Abnormal FLCR was found to be predictive for reduced OS. The remarkable association of FLCR and CD38 may reflect FLCR's biological prognostic value. Further clinical efforts should focus on the biological role of FLCR to maximize our understanding of this new and simple candidate prognostic marker. Prospective studies are warranted to validate the adverse impact of FLCR on clinical outcome before it is brought into routine clinical practice

0930

HIGH INTRACELLULAR CONTENT OF CYCLIN-DEPENDENT KINASE INHIBITOR P27KIP1 IN EARLY- AND INTERMEDIATE STAGE B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA LYMPHOCYTES PREDICTS RAPID PROGRESSION OF THE DISEASE

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Background. Previous studies showed that peripheral blood lymphocytes of B-cell chronic lymphocytic leukemia (B-CLL) displayed a high intracellular level of cell cycle inhibitory protein p27Kip1. It has been suggested that high expression of p27Kip1 may confer them survival advantage and be associated with unfavorable prognosis, but prognostic significance of p27Kip1 expression for previously untreated, non-advanced B-CLL patients was not established. *Aims*. We studied a relationship between the intracellular level of p27Kip1 of lymphocytes of early- and intermediate stage B-CLL patients and their spontaneous apoptosis *in vitro*, as well as prognostic significance of p27Kip1 in B-CLL lymphocytes for the risk of disease progression. *Methods*. Intracellular p27Kip1 content of peripheral blood lymphocytes obtained from 48 previously untreated 0-II Rai stage B-CLL patients, analyzed by flow cytometry, was determined as the mean fluorescence intensity (MFI). The *in vitro* viability and spontaneous apoptosis of those lymphocytes after 72-hours culture were also assessed. During the follow-up period (6-71 months, median 59.5), we recorded the time elapsed to the doubling of peripheral lymphocyte count, progression to a higher Rai stage and the appearance of indications for cytostatic treatment. *Results*. p27Kip1 expression was not correlated with initial peripheral lymphocytes count, CD38 expression, cell viability nor spontaneous apoptosis ratio after 72-hours culture. In order to assess the possible prognostic significance of cellular p27Kip1 content for the clinical course of B-CLL, we have divided the patients' group into low expressors and high expressors of p27Kip1. We have chosen the cut-off p27Kip1-dependent MIF value at 270 AU, i.e. in such a way that the differences in the occurrence of clinical progression between low- and high expressors were maximal. We observed that doubling of lymphocyte number, progression to higher Rai stage and indications for cytostatic treatment occurred significantly earlier in patients with high p27Kip1 expression than in the group with low p27Kip1 content. Accordingly to that, the frequency of each of three above-mentioned events during the whole follow-up period was markedly higher in the group with p27Kip1 - dependent MIF above 270 AU as compared with low p27Kip1 expressors. We did not find a prognostic significance of *in vitro* cell viability nor apoptosis as to the risk of disease progression. Conclusions: Our results indicate that elevated intracellular expression of p27Kip1 in peripheral blood leukemic lymphocytes has an unfavorable prognostic value in early- and intermediate stage B-CLL patients as to the risk of disease progression. Further studies are required to elucidate the issue of the relationship between p27Kip1 expression and survival of leukemic cells, and to answer the question about the independency of its prognostic significance.

0931

ALEMTUZUMAB ADMINISTERED BY SUBCUTANEOUS ROUTE IS LESS EFFECTIVE THAN INTRAVENOUS ROUTE FOR FIRST LINE THERAPY OF T-CELL PROLYMPHOCYTIC LEUKAEMIA: RESULTS OF A PILOT STUDY (UKCLL05)

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Background. T-cell prolymphocytic leukaemia (T-PLL) is a rare aggressive post thymic lymphoid disorder with distinctive clinical and pathological features. It is resistant to conventional chemotherapy and has a short median survival of 7 months. Intravenous (IV) alemtuzumab is an effective and well tolerated therapy for this disease, inducing remission in 76% of patients in the relapsed and refractory setting and increasing median survival to 24 months in responders. *Aims*. The aim of this study was to determine the efficacy of subcutaneous (SC) administration of alemtuzumab in previously untreated T-PLL patients. *Methods*. After an initial week of dose escalation, patients were treated with a dose of 30 mg 3 times weekly. Patients could be switched to the IV route if they had local side effects or showed a lack of response. *Results*. Nine patients were enrolled. Male: female Ratio was 2:1; median age was 61 years. All cases were centrally reviewed and confirmed to have a diagnosis of T-PLL. Eight had an immunophenotype typical of T-PLL (CD2, CD3, CD5, CD7 and CD 4 positive). One patient was unusually CD7 negative and also did not have an abnormality of chromosome 14. Seven had a complex karyotype and 8 had chromosome 14 abnormalities. Patients were treated on trial for a median of 7 weeks (range 3-13 weeks). 55% required a change to IV treatment due to a lack of response. This resulted in an increase in overall response (OR) from 33% (1 CR, 2 PR) to 44%.(2 CR, 2 PR). Five patients required addition of pentostatin to alemtuzumab at a dose of 4 mg/m^2 IV weekly to augment response. This increased (OR) to 77% (1 additional CR and 2 PR). 5 patients had haemopoietic progenitor cell transplants (HPCT) as consolidation; 1 autologous and 4 allogeneic. Median overall survival was 20 months (range 1-26 months) and disease free survival was 12 months (range 0-20 months). The treatment was well tolerated; however 2 patients had skin reactions to the SC injections. One patient required treatment for CMV reactivation and 2 patients had Grade 4 haematologic toxicity which was related to disease and not treatment. Four patients remain alive and well in CR with a follow up of between 12 and 23 months, of whom 3 had allogeneic HPCT. The study was terminated early as a data safety monitoring review felt that SC treatment was not efficacious and patients should not be offered SC treatment. 5. *Conclusions*. This pilot

study suggests that the SC route of administration of alemtuzumab is not as effective as IV in the treatment of T-PLL; a series of 16 patients receiving 1st line therapy with IV alemtuzumab has shown an OR of 94% with 88% CR. It is therefore advisable that IV treatment should be used. Pentostatin is an effective agent to augment response. Allogeneic HPCT should be used as consolidation therapy when possible.

0932

INCLUSION OF TOTAL BODY CT SCAN IN THE INITIAL WORK-UP OF CLL PATIENTS WITH EARLY-STAGE ON CLINICAL GROUNDS: PRELIMINARY RESULTS OF A PROSPECTIVE, MULTICENTER O-CLL1- GISL STUDY

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Background. The clinical staging systems proposed by Rai and Binet represent the backbone for assessing prognosis in patients with Chronic Lymphocytic Leukemia (CLL). However, staging systems are not devoid of some limitations, among the most significant of which is the lack of recognition of early-stage patients who will progress. Unlike the guidelines for assessing the response to therapy for most other types of non-Hodgkin's lymphomas, the widely-used NCI-WG guidelines for patients with CLL do not incorporate use of computed tomography (CT) scans in the algorithm. However, two recent retrospective study challenged this notion. highlighting the importance of prospective validation of CT scans before routine inclusion in CLL work up. *Aims*. In the present study, we investigated whether total body CT scan allowed to individuate among Binet stage A CLL patients, included in the prospective multicenter O-CLL01 GISL study, cases in more advanced stage and whether this subgroup showed a different expression of clinical and biological prognostic markers. *Patients.* Up to date, 275 patients have been enrolled in the trial started in April 2007 and total body CT scan were available in 87 patients. Fifty-two patients (60%) were male and the median age was 61 years (range, 33 to 71 years). All patients are in Binet stage A, while 83 patients were at low risk (0-I stages) and 4 at intermediate risk (II stage) by Rai classification. LDH was elevated in 11.5% of cases and B2-microglobulin in 24%. Twenty-eight patients (33%) were IgVH unmutated, 31 patients (36%) had a high ZAP-70 expression, 17 patients (20%) were CD38 positive (>30%). Fluorescence *in situ* hybridization (FISH) data are available in 61/87 cases; the most frequent abnormality was del(13)(q14) (29 pts 33%), followed by trisomy 12 (5 pts, 6%), del(17p13) (4 pts 5%) and del(11q22.3) (2 pts 2%), 21 cases (24%) 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. *Results*. Considering total body CT scan, 22 out of 83 analyzed (25%) patients were converted into Binet stage B. Notably, 64% were male, LDH was elevated in 18% of cases and B2-microglobulin in 18%, 41% were IgVH unmutated, 27% had a high ZAP-70 expression, 27% were CD38 positive, 4,5% 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 and ZAP-70 expression and cytogenetic abnormalities were observed between Binet A cases and Binet B. According the Rai classification 14/83 (17%) low risk patients became at intermediate risk with the integration of total body CT scan. Also this subset of patients did not show a statistically different expression of all prognostic markers, but a higher rate of cases with elevated B2-microglobulin (p=0.003), than patients at low risk. Finally, total body CT scan allowed to early individuate a second neoplasia in 2 cases (lung cancer 1 pt, renal cell carcinoma 1 pt). *Conclusions*. In line with literature information, our preliminary data indicate that the integration of total body CT scans in the clinical staging allowed to individuate among Binet A CLL cases on clinical grounds 25% of cases with a more advanced stage. Although a more advanced stage did not correlate with both clinical and biological variables reflecting bad prognosis. A longer follow-up will allow to demonstrate whether the inclusion of total body CT scan in the initial work-up of patients with early-stage on clinical grounds provide relevant prognostic information.

0933

CLL WITH BURKITT-TYPE TRANSLOCATION: A SUBGROUP WITH AN AGGRESSIVE DISEASE COURSE

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Background. Balanced translocations are uncommon in chronic lymphocytic leukemia (CLL), and their significance remains poorly understood. *Aims*. The aim of the present study was to identify the characteristics of CLL patients with a Burkitt-type translocation. *Methods*. Clinical and cytogenetic files from patients referred to Belgian and French institutions between 1990 and 2009 for cytogenetic characterisation of CLL, and displaying a t(8;14)(q24;q32) or a variant translocation, were reviewed. FISH was performed to confirm involvement of MYC gene and to analyze other prognostically significant aberrations, i.e. those affecting 17p, 11q, 12 and 13q. IgVH mutational status was additionally assessed. *Results*. 16 patients displayed a t(8;14) or variant (representing < 0.5% of CLLs referred during the period of study). These were mainly males (ratio M/F: 14/2) with a median age of 69 years (range 46-84). Six patients presented with Binet stage A, 3 with stage B and 7 with stage C. Lymphadenopathy and splenomegaly were present in 9/16 and 10/15 patients, respectively. Anemia and thrombocytopenia were present at diagnosis in 3 and 4 patients, respectively. In 3/10 patients, a monoclonal paraprotein was observed. Morphology was compatible with "typical" CLL in 2 cases, "atypical" CLL in 9 cases (i.e. presence of occasional prolymphocytes (< 10%) in 4, and CLL/PL (10-55%) in 2 cases) and PLL in 3 cases (i.e. > 55% prolymphocytes). Morphological data could not be obtained for 2 cases. Immunophenotypical data matched a Matutes-Catovsky score of > 3 in all but one cases. CD38 was expressed (i.e. > 30%) in 9/15 patients. The Burkitt-type translocation was the sole aberration in 2 cases. The translocation most frequently involved IGH, but variant cases involving the IGK or IGL locus were observed in 4 and 4 cases, respectively. The Burkitt-type translocation was associated with one other abnormality in 7 cases. Complexity (> 3 changes including the t(8;14) or variant) was observed in 7 cases. Associated recurrent aberrations included del(11q) (n=6), trisomy 12 (n=5) cases and del(6q) (n=2). VH was mutated in 6 and unmutated in 3 cases. V3 and V7 family were used in 6 cases and 1 case, and V3-21 was never used. There were no recurrent VDJ combinations. Follow-up data was available in all patients over a median period of 23.5 months (range 1-168). Median time to treatment (TTT) was 0.5 months (range 0.5-36). 11/16 received therapy; 4 were refractory and none of the remaining achieved complete remission. Disease-related death occurred in 8 patients, whereas 6 are still alive and 2 are lost to follow-up. *Conclusions*. CLL with Burkitt-type translocation represents an extremely rare entity with an aggressive disease course, an advanced stage at presentation, adverse cytogenetic findings (i.e. del(11q), complexity), short TTT and poor response to therapy.

Myeloma and other monoclonal gammopathies - Biology II

0934

AN EXPERIMENTAL STUDY FOR PERIPHERAL NEUROPATHY ASSOCIATED WITH WALDENSTRÖM'S MACROGLOBULINEMIA*

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Waldenström's macroglobulinemia (WM), is a lymphoplasmacytic lymphoma characterized by IgM production. Neuropathy is a common complication of WM, conferring considerable morbidity but its precise pathophysiology remains obscure. In our previous study an animal model of WM has been described (Tsingotjidou *et al.*, 2007, in press). In the current study an effort has been made to develop the WM-related neuropathy in non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice. For this reason, freshly obtained bone marrow biopsies from WM patients were implanted intra muscularly into the hindlimbs of 10 SCID mice. The animals were monitored for neuropathy with histological and immunohistochemical evaluation along with behavioural tests in the mid- and the end- of the experiment period. Possible sensory neuropathy was estimated with hot-plate and touch-response tests; grip strength and body suspension tests were used to assess for motor neuropathy. Following different survival periods (from 2 to 8 months) electrophysiological data were also obtained to assess possible chances in conduction velocity along the sciatic nerve from the experimental animals just prior to their sacrifice. The light microscopy of semi thin (1 μm) toluidine blue stained sections of affected sciatic nerves revealed areas with reduced number of larger diameter axons and an increase in the density of smaller myelinated and/or unmyelinated axons. These changes are evidence of a neuropathic process and were not present in controls. Immunohistochemical detection of different molecules in the sciatic nerves is under investigation. Based on the hot plate and touch response tests it can be concluded that the developed sensory neuropathy selectively affected the fiber-neural endings responsible for the finer feeling of touch, the Aδ fibers of the peripheral nerves. The results from the grip strength and the body suspension tests test show that the WM implanted mice develop a motor neuropathy since the values of the above mentioned tests are lowered at the experimental animals. Electrophysiological data have been standardized by testing the control animals. For this reason the motor (MNCV) and the sensory (SNCV) nerve conduction velocity of the sciatic nerve of the left hind limb were measured. For the control animals the MNCV was estimated to be 35 ± 1.60 m/s, close to the values estimated by other researchers. Also, in the control animals the SNCV was estimated to be 25.20 ± 0.55 m/s. Upon completion of the planned experimental period, the conduction velocities of the affected nerves will be measured and compared to the established measurements of the control animals.

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Reference

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0935

HGF AND IGF-1 POTENTIATE SDF-1 MEDIATED MYELOMA CELL MIGRATION THROUGH ACTIVATION OF PAK

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Background. Multiple myeloma (MM) is characterized by the accumulation and dissemination of malignant plasma cells in the bone marrow, a process that requires cell migration. Both SDF-1, IGF-1 and HGF is known to induce MM cell migration and by exploring such processes it could be possible to find new therapies targeting the MM cell and the interaction with the BM environment. *Aims*. SDF-1, IGF-1 and HGF are all potent mediators of migration *in vitro*, and we wanted to study the effects of combinations of these cytokines on migration in myeloma cell lines and patient samples. *Methods*. Migration was studied in a Transwell migration assay and cells were transfected with siRNAs using electroporation. Receptor expression and cell viability was determined using flow cytometry and protein detection was done with Western blot. *Results*. We could show a strong synergistic effect on migration when combining SDF-1 with either HGF or IGF-1; this synergy was dependent on an SDF-1 gradient. However, both HGF and IGF-1 potentiated SDF-1- induced migration irrespective of where they were added, to the upper, to the lower or to both compartments in the migration assay. In JJN-3 cells, which secrete large amounts of HGF, SDF-1- induced migration was reduced when the c-Met inhibitor PHA665752 was added to the cells, demonstrating cooperative activity between autocrine HGF and exogenous SDF-1. HGF and IGF-1 did not affect the level of SDF-1 receptor expressed on the cell surface. There was a clear correlation between degree of migration and PAK activation both in INA-6 and IH-1 cells and in primary myeloma cells. Downregulation of Pak 1 and Pak 2 with siRNA resulted in lower cell migration than in control cells, in agreement with a role for Pak in cytokine-induced myeloma cell migration. *Conclusions*. HGF and IGF-1 potentiated SDF-1 -mediated myeloma cell migration through activation of Pak. The study suggests that PAK could be a target in treatment of multiple myeloma.

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SPARC GENE EXPRESSION IN MULTIPLE MYELOMA PATIENTS AND ITS REGULATION BY CYTOTOXIC TREATMENTS IN MYELOMA CELL LINES

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Background. SPARC (Osteonectin) is molecule of extracellular matrix with important cell growth, attachment and migration functions. In oncology its role is under intensive investigation since SPARC may support the tumour growth, but may also block its progression acting as tumour suppressor all this depending on the type of neoplasm. We have recently shown that humoral SPARC in MM patients can serve as biomarker. In this study we looked at the SPARC gene expression by MM cells in patients and myeloma cell lines. *Aims*. We have analyzed the expression of SPARC gene in bone marrow (BM) and peripheral blood (PB) of healthy controls, MM patients and 2 myeloma cell lines: Thiel and NCI H929. *Methods*. We includeed 35 MM patients and 17 controls into the study. Expression of SPARC and internal housekeeping gene GAPDH was analyzed using quantitative PCR (Taqman gene expression assaysR) in unfractionated mononuclear cells (MNC) from BM and PB and in cell lines grown in standard cultures. Cell lines were in addition treated with bortesomib (BORT), dexamethasone (DEXA), thalidomide (THAL) and cyclosporine (CSA) in different pharmacological concentrations to evaluate its effect on SPARC expression and rate of viability measured by MTT assay. *Results*. SPARC gene was higher expressed in MM samples compared to controls but only for PB MNCs values reached statistical significance (p=0.0078, Mann-Whitney). We could not demonstrate correlation between expression values in BM and PB. By the disease stage, SPARC was higher in active disease and lower in Durie-Salmon stage 3. In resistant cell line NCI-H929 (MMSET-IgH+), SPARC was basally low expressed but treatment with THAL and CSA increased its expression, whereas decreased cell viability. In Thiel cell line there was higher basal level of SPARC expression, whereasTHAL and CSA suppressed the expression parallel to reduced viability. BORT treatment in Thiel cells strongly reduced viability with little effect on SPARC expression. Finally, DEXA only slightly suppressed SPARC expression in both cell lines without significant effect on cell viability. *Conclusions*. Expression of SPARC gene is increased in active myeloma disease with the decline in the expression by disease progression. *In vitro* results for resistant and non-resistant myeloma cell lines showed different pattern of SPARC gene expression and different response to anti-myeloma drugs. These results warrant further research into the biology of SPARC in MM with future attention to tumour-stroma interaction of marrow microenvironment.

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GLOBAL MICRORNA EXPRESSION PROFILING IN MULTIPLE MYELOMA IDENTIFIES DEREGULATED PATTERNS ASSOCIATED WITH DISTINCT MOLECULAR GROUPS

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Background. The recent discovery of microRNA (miRNA) genes, encoding for a class of small non-coding RNAs involved in the regulation of cell cycle, survival and differentiation programs, has added a further level of complexity to normal and cancer cell biology. Impaired miRNA expression has already been demonstrated in a number of solid tumors and, more recently, in some hematological disorders; nevertheless, to date only little evidence of miRNA expression/deregulation in multiple myeloma (MM) has been reported. *Aims*. To characterize miRNA expression profiling of MM plasma cells (PCs) and integrate miRNA expression data with other molecular features of MM patients (IGH translocations and other genetic abnormalities). *Methods*. A CD138 positive PCs isolation was performed from bone marrow biopsies of 38 newly diagnosed MM, 2 plasma cell leukemia patients, and 3 healthy donors. Total RNA was extracted using Trizol reagent. The miRNA expression data were generated on Agilent miRNA microarrays V2 (representing 723 human mature miRNAs from the Sanger miRBase v10.1), and normalized using the AromaLight Bioconductor package. Hierarchical agglomerative clustering was performed by means of DNA-Chip Analyzer (dChip) software, using Pearson's correlation coefficient and average linkage as distance and linkage methods, respectively. Differentially expressed miRNAs were identified using the "Significant Analysis of Microarrays" (SAM) algorithm. The expression of some miRNAs was validated by means of quantitative real time RT-PCR, using specific TaqMan® microRNA assays (Applied Biosystems). *Results*. An unsupervised analysis of the samples based on the most variably expressed miRNAs across the dataset grouped the PCs from healthy donors separately from MM PCs; among the pathological samples, the most striking finding was that the seven patients with t(4;14) (TC4) were tightly clustered, as were four out of the five samples with translocated MAF genes (TC5). A partial grouping of the TC2 cases (mostly hyperdiploid) was also observed, whereas the TC1 [showing t(11;14)] and TC3 (mostly expressing Cyclin D2) samples were dispersed along the dendrogram. A multiclass supervised analysis of the miRNA expression between the members of the 5 TC groups highlighted specific miRNA signatures, in particular characterizing the TC4 and TC5 groups (upregulation of *miR-34b**, *miR-150*, *miR-1*, *miR-155*, *miR-133a*, *miR-133b*, *miR-155**). A less consistent miRNA signature was observed in t(11;14) cases and TC2 group, whereas we could not identify any TC3-specific miRNA signature. None of the differentially expressed miRNAs in patients with specific IGH translocations maps to the rearranged chromosomal regions. Additionally, no differentially expressed miRNAs were associated with the occurrence of neither 13q14 deletion nor 1 q gain. *Conclusions*. Our data on miRNA expression profiling in a representative panel of MM patients revealed a global deregulation of miRNA expression in myeloma cells, and identified miRNA signatures associated with MM molecular subgroups. These data extend previous limited data and represent the basis for further investigations aimed at functionally characterizing specific miRNAs in MM.

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THE COMPARISON OF SERUM LEVELS OF SELECTED BIOMARKERS AND CYTOGENETIC CHANGES IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a malignant neoplasm of plasma cells characterized by production of monoclonal immunoglobulin detectable in the serum and/or urine and various levels of organ damage. In MM chromosomal abnormalities and biochemical markers have biologic and prognostic significance. Some less usual deductable biochemical parameters appear to be very promising as possible markers of neoplastic transformation and also as the markers of advanced stage, progression and monitoring of treatment response in patients with MM. *Aims*. In the present study we compare serum levels of 12 selected biomarkers and cytogenetic changes in 128 MM patients examined at time of diagnosis. *Methods*. We used conventional cytogenetics and FICTION method with locus specific probes 1q21/1p36 (Kreatech, MP Biomedicals, CA, USA), RB1, IgH, IgH/CCND1, IgH/FGFR3 and centromeric probes for chromosomes 7, 9, 11, 15, 17 (Abbott-Vysis, Downers Grove, IL, USA). M-FISH and CGH (MetaSystems, Altussheim, Germany) were also applied in order to determine other chromosomal abnormalities. For evaluation of serum levels of analysed parameters were used following methods: radioenzymatic assay (thymidine kinase), ELISA (β2-microglobulin), radioimmunoanalysis (ICTP, PINP), enzymoimmunoassay (sIL-6R, sVCAM, sICAM-1, sOPG) and quantitative enzymatic immunoassay (sHGF, sVEGF, syndecan-1/CD138 and sFas). Mann-Whitney's test was used for statistical evaluation. *Results*. Significantly higher levels of β2-microglobulin (p=0,010) were consistent with complex karyotype. Gains of 1q21 significantly correlate with higher levels of sIL-6R (p=0,038), but significantly lower levels of sIL-6R (p=0,009) correlate with t(11;14). Lower levels of OPG were associated with deletion of RB1 gene (p=0,013), as well as lower levels of PINP with t(4;14) (p=0,031). Serum levels of selected biomarkers did not correlate significantly with polyploidy. *Conclusions*. This preliminary analysis shows that cytogenetic changes have effect on cytokine network in multiple myeloma, especially on one of key-role factors (sIL-6R) in pathogenesis of multiple myeloma. Important prognostic factors such as HGF and syndecan-1/CD138 are not influenced by analysed cytogenetic changes in our cohort of patients.

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NON-PROTEASOME TARGETS OF PROTEASOME INHIBITORS BORTEZOMIB AND CARFILZOMIB

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Background. Carfilzomib is a next generation proteasome inhibitor that is structurally and mechanistically distinct from bortezomib and is currently undergoing clinical investigation in multiple myeloma, non-Hodgkin's lymphoma, and solid tumors. The mechanisms of action of carfilzomib and bortezomib differ due to their unique pharmacophores: bortezomib is a peptide boronate while carfilzomib is a peptide epoxyketone. Phase 1 and 2 studies with carfilzomib suggest a clinical safety profile that has commonalities and distinctions compared to bortezomib (Blood 112, abstract 865). In particular, the painful peripheral neuropathy that is commonly observed with bortezomib (Velcade® package insert) appears to be less severe and possibly less frequent with carfilzomib, raising the possibility that non-proteasome mechanisms may underlie this toxicity. *Aims*. To gain insight into the unique clinical toxicities of bortezomib and carfilzomib, we evaluated their propensity to act as inhibitors of non-proteasomal enzymes. *Methods*. We executed a two-pronged approach consisting of candidate and proteomic strategies to identify the non-proteasomal targets of bortezomib and carfilzomib. Identified targets were validated in human peripheral blood mononuclear cell (PBMC) lysates and IC50 values were determined using FP-biotin, a serine hydrolase-specific fluorophosphonate active site probe. *In vivo* inhibition of the targets was determined in rats following a single dose of each either carfilzomib or bortezomib. To evaluate the contribution of the pharmacophore to target inhibition, a matrix of nine inhibitors consisting of three peptide backbones (derived from bortezomib, carfilzomib, and MG132) coupled to three pharmacophores (boronate, epoxyketone, or aldehyde) were tested. *Results*. In a screen of candidate cysteine, aspartyl, metallo-, and serine proteases, bortezomib significantly inhibited the serine proteases cathepsin G (CatG) and chymase, while carfilzomib did not inhibit these enzymes. IC50 values for these targets were determined in PBMC cell extracts and were found to be 2 and 256 nM for chymase and CatG, respectively. To obtain a global inhibition profile of the serine hydrolases that are targeted by bortezomib, single dimension mass spectrometry analysis was performed on FP-biotin-reactive proteins from PBMC cell extracts. In addition to CatG and chymase, bortezomib was found to inhibit cathepsin A (CatA) and dipeptidyl peptidase II (DPP II) with 0.5 and 410 nM IC50 values respectively. Furthermore, CatG activity in the spleen was inhibited by 60% in bortezomib-treated rats but was unaffected in carfilzomib-treated

animals. Finally, using the panel of inhibitors with equivalent amino acid chains but varying pharmacophores, we observed that the boronate versions of the inhibitors displayed potent activity towards CatG, CatA, and chymase, while the epoxyketone versions were inactive. *Conclusions*. These data demonstrate that non-proteasomal enzymes, CatG, CatA, DPP II and chymase, can be targeted by bortezomib at physiologically relevant concentrations (bortezomib Cmax = 312 nM). Carfilzomib does not inhibit non-proteasomal targets in our assays. Inhibition of these non-proteasomal proteases is dependent upon the boronate pharmacophore; epoxyketone-containing proteasome inhibitors were inactive. *In vivo* inhibition of rat splenocyte CatG together with literature reports (Trends Immuno 28:541-550; Trends Neurosci 26:496-500; British J Pharm 150:176-185) supports the hypothesis that off-target activity of bortezomib may be linked to toxicities such as neuropathic pain.

0940

CXCR4 AND CD43 CO-EXPRESSION IN PLASMA CELL MALIGNANCIES

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Background. Bone marrow (BM) microenvironment has been envised as a "trap" for circulating multiple myeloma (MM) precursors, which expand and differentiate to malignant plasma cells within the BM, invading the peripheral blood only in the terminal stage of the disease, i.e. plasma cell leukemia (PCL). While several adhesion molecules have been identified that promote the binding of malignant plasma cells to BM accessory cells and extracellular matrix (i.e. CXCR4), little is known on molecules able to prevent such interactions, by eventually favoring the escape of tumor cells from the BM "trap". Previous studies have indicated that the CD43 sialoglycoprotein functions as an anti-adhesion molecule by providing a repulsive barrier around cells due to its extended conformation and negative charge of sialylated residues. *Aims*. We investigated CXCR4 expression and its association with the expression of the CD43 sialoglycoprotein on bone marrow plasma cells of MM and PCL patients. *Methods*. We have analyzed the bone marrow blood of 40 patients with MM and 8 with PCL. 26 out of 40 MM patients presented a IgG component, while the remaining 14 were IgA. On the basis of the staging criteria, 23/40 patients were in stage I-II and 17/40 in stage III. We evaluated CXCR4 and CD43 expression by flow cytometry. Mean fluorescence intensity ratios (MFIRs) were calculated by dividing the mean fluorescence intensity for CXCR4 and CD43 by the mean fluorescence of the respective non-specific isotype control. *Results*. 23/40 MM patients (stage I-II) showed high CXCR4 expression (median MFIR 15.2; range: 10.3-50.4) while 17/40 (stage III) showed lower CXCR4 expression (median MFIR 6.5; range: 5.1-9.5). All MM patients were CD43 negative. By contrast all PCL patients were CD43 positive (MFIR 7.8; range: 6.2-10.1), while CXCR4 expression was like MM stage III patients group (MFIR 6.2; range: 4.8-8.9). *Conclusions*. Our results that malignant plasma cells from MM lack surface CD43 as opposed to PCL in which CD43 expression was found at a high cellular density, indicate that CD43 expression may be related to the biological and clinical progression of MM. Moreover the possible prognostic role of CXCR4 in MM warrants further clinical investigation on a larger series of patients even on the basis of future therapeutic strategies.

0941

NOVEL WHOLE CELL ASSAYS TO MONITOR PROTEASOME ACTIVITY IN MULTIPLE MYELOMA

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Background.The proteasome is a multicatalytic complex involved in the regulation of key cellular processes including cell cycle control and apoptosis. Proteasome inhibitors have shown particular efficacy in Multiple Myeloma (MM). We have previously profiled the three main components of proteasome activity using assays based on cell lysates (>5×10^6 cells). This strategy has now been adapted for use in a smaller number of whole cells (1×10^4) to facilitate clinical studies. *Aims*. To develop an assay to profile proteasome activity in small numbers of whole cells for application to clinical investigations. *Methods*. Fluorogenic substrates Z-Leu-Leu-Glu-AMC and Succ-LLVY-AMC, specific for the Peptidyl Glutamate Peptide Hydrolysing (PGPH) and Chymotrypsin-Like (CT-L) proteasome activities respectively were modified by adapting the side chain of glutamic acid as a methyl ester to aid cell entry. These modified substrates have been used with both MM cell lines and clinical samples, where increased fluorescence is directly proportional to proteasome activity. The effects of three proteasome inhibitors, BZ-Leu-Leu-Leu-COCHO (BZLLL), MG132 and PS341, were investigated in the U266 MM cell line, and correlated with initiation of apoptosis using the Enzchek® caspase-3 assay, with confirmation by western blotting. *Results*. The level of CT-L activity was uniformly reduced by each inhibitor to approximately 50% of control (p=0.037). In contrast, PGPH activity was reduced to 29.8±2.7%, 47.3±12.8% and 15.4±0.6% of control (p≤0.019) with BZLLL, PS-341 and MG132 respectively. Addition of BZLLL and PS-341 to whole cell preparations also induced a dose-dependent increase in caspase-3 activity. Maximal inhibition of CT-L and PGPH substrates was observed at 9.9±0.8 hours and 9.4±2.6 hours respectively with 10 μM BZLLL, 7.8±0.6 hours and 7.7±0.6 hours respectively with 10nM PS-341 and 8.5±0.9 hours and 6.7±1.1 hours respectively with MG132. *Conclusions*. Modified fluorogenic substrates specific for the CT - L and PGPH proteasome activities have been shown to efficiently permeabilise whole cells. Together with cell - permeable proteasome inhibitors, these have been applied to the accurate profiling of proteasome activity in small numbers of intact cells, thus facilitating their application to investigations with clinical samples.

0942

GROWTH FACTORS, HGF, IL-6, AND IL-3 DIFFERENTIALLY REGULATE DIFFERENTIATION OF HUMAN MULTIPLE MYELOMA MESENCHYMAL STEM CELLS IN RELATION TO BONE METABOLISM

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Background. Along with the progression of multiple myeloma (MM), myeloma-induced bone destruction is the result of an increased function of osteoclasts, which is not accompanied by a comparable increase of osteoblast activity. Various growth factors involved in MM progression and an individual factor differentially affects the balance between osteoblasts and osteoclasts.

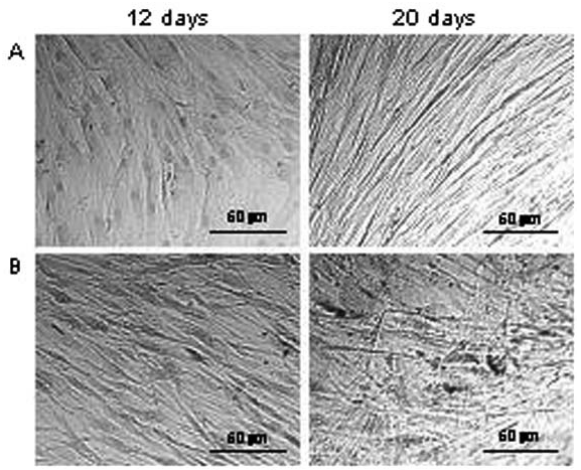

Figure 1. Osteogenic induction of hMM-MSCs. After being cultured in MSC-culture medium (A) or osteogenic medium (B) for 12 and 20 days. Osteogenic induction was detected by Alizarin R staining (ARS).

Aims. To investigate various growth factors or cytokines effect on either osteoblast or osteoclast differentiation of multipotent human mesenchymal stem cells (hMSC) obtained from MM patients. *Methods*. To investigate growth factors affecting osteoblast differentiation of mesenchymal stem cells, osteoblast differentiation related genes (RUNX2, OPG, and ALPL) and osteoclast differentiation related genes (TGF-1, MIP-1, and RANKL) were examined using immunostain, RT-PCR and expressions of

genes related to Wnt pathway by western blot analysis. In addition, effect of osteoblast differentiation by treated with growth factors in co-culture with MM cells was examined by ALP and Alizarin red staining. *Results*. HGF, IL-6 and IL-3 stimulated osteoclast differentiation via osteoblastic factors were down-regulated but osteoclastic factors were up-regulated in hMSC. MM cells treated with rhHGF were reduced β-catenin phosphorytion and activation of DKK1 expression. In this study, blockage of Wnt signaling pathway in MM cells was deregulated osteoblast differentiation on hMSC. Osteoblast differentiation was regulated by different time points after treated with growth factors in hMSC via regulation of different expression patterns accordingly. In addition, When hMSC alone or co-culture with MM cells, osteoblast differentiation was reduced with co-cultured with MM cells. *Conclusions*. We suggested that Wnt signaling pathway was critical in osteoblastic differentiation on hMSC via abrogated Wnt mediated signaling pathway by activation of HGF expression in MM cells. Moreover, when compared with hMSC alone or co-cultured with MM cells, those growth factors were synergistically reduced osteoblast differentiaton in co-cultured with MM cells.

0943

IMMUNOHISTOCHEMICAL EVALUATION OF SARCOGLYCANS AND INTEGRIN IN GINGIVAL EPITHELIUM OF MULTIPLE MYELOMA PATIENTS WITH BISPHOSPHONATE-INDUCED OSTEONECROSIS OF THE JAW

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Background. Osteonecrosis of the jaw (ONJ) is an adverse outcome associated to bisphosphonate treatment. However, it is not known whether the ONJ lesion originates in the bone, or whether it may initiate in the oral mucosa. *Aims*. Aim of our study was to characterize the distribution of different mucosal proteins after bisphosphonate administration and to evaluate the structural damage of the mucosal in ONJ patients. In particular we evaluated changes in basement membranes and in expression of costameric proteins (vinculin-talin-integrin system), laminin, and type IV collagen.

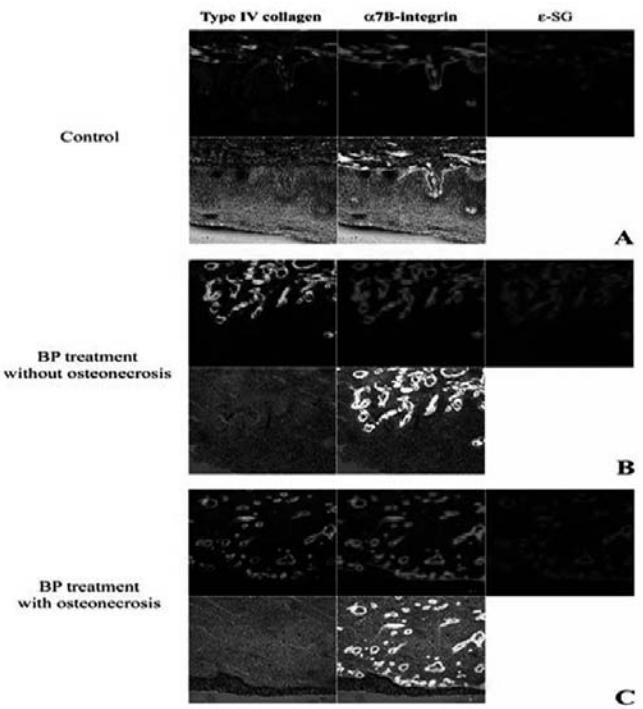

Figure 1. Compound panel showing immunohistochemical finding.

Methods. Samples of human gingival epithelium were obtained from patients treated with zoledronate which no showed the osteonecrosis and from patients treated with bisphosphonates which showed the osteonecrosis. These samples were compared with gingival epithelium of control subjects which had undergone odontoiatric surgery for other reasons. Patients treated with bisphosphonates were affected by Multiple myeloma. Immunohistochemical study was made using confocal laser scanning microscopy. *Results*. All tested proteins were almost absent in basal lamina and oral mucosa of subjects treated with bisphosphonates without osteonecrosis, whereas in oral mucosa with necrosis, they showed a clearly detectable staining pattern of the same proteins, specifically in basal lamina, but less in comparison to control samples. Moreover, in oral mucosa with lesion, a massive neoangiogenesis was detectable. The triple immunofluorescence reaction, performed between type IV collagen, integrin, and ε-sarcoglycan antibodies, in control subjects, showed in fact a clear normal pattern of all proteins in basal lamina, which is perfectly delineated; besides, any vascular structure is detectable while the epithelial side is not detectable for these proteins. The fluorescence analysis of same proteins in samples treated with bisphosphonates of subjects that no showed lesions showed an almost absence of protein staining patterns in correspondence of basal lamina, but clearly detectable staining for all tested proteins in correspondence of vascular structures that are numerous. In the sections of subjects treated with bisphosphonates that showed the osteonecrosis, we observed an increase of protein staining patterns on basal lamina and a qualitatively and quantitatively increase of vessels below the basal lamina. Triple fluorescence reaction performed with laminin, α-sarcoglycan, and β-sarcoglycan in control subject showed a normal staining patterns of all proteins with a clear fluorescence on basal lamina. In bisphosphonates treated subjects that no showed lesions, basal lamina fluorescence is absent whereas the vessels are clearly detectable. In ONJ subjects it was possible to highlight newly an increase of staining patterns on basal lamina and a massive increase of vascular structures. Triple fluorescence reactions using vinculin, integrin, and γ-sarcoglycan antibodies showed the same behaviours of other proteins. Finally, in the samples treated with bisphosphonates showing lesion, it was detectable an increase of staining pattern both in the basal lamina and in vessels. *Conclusions*. The increase of these proteins in basal lamina, in concomitance with formation of the lesion, could indicate a compensative behaviour in the remodelling of the gingival mucosa in order to restore the epithelial architecture. The neoangiogenesis, unexpected feature during bisphosphonate treatment, could confirm this compensative role of tested proteins.

0944

SEQUENTIAL EVALUATION OF SERUM HEPCIDIN IN ANEMIC MYELOMA PATIENTS: A PILOT STUDY OF CORRELATIONS WITH MYELOMA TREATMENT, DISEASE VARIABLES, AND ANEMIA RESPONSE

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Background. Hepcidin is a key modulator of iron metabolism and cancer-related anemia. However, its role in hematological malignancies and particularly in Multiple Myeloma (MM), has not been determined. *Aims*. In this pilot study we examined possible correlations of baseline and sequential serum hepcidin values with disease variables and treatment with Immunomodulatory drug-based regimens (IMiDs group) or conventional chemotherapy (conventional group). In addition, analysis included correlations of serum hepcidin with disease response (DR), duration of response (DOR), and anemia response (AR) in a cohort of anemic MM patients. *Methods*. Thirty-four anemic myeloma patients newly diagnosed or at progression, were studied. Eighteen were treated with IMiDs-based regimens and 16 with conventional therapy. Patients at progression should have remained in partial response for at least 6 months before study. Patients under IMiDs maintenance for the last 6 months, patients with active infection and those treated with erythropoiesis-stimulating agents or iron for the last 2 months prior study initiation were excluded. *Results*. In the IMiDs group, hepcidin level decreased significantly between baseline value and second treatment cycle (cycle 2) (p=0.03) followed by stabilization at significantly lower levels, after three cycles (p=0.01). In the conventional group, hepcidin decreased significantly before cycle 2 (p=0.03) but returned to baseline values between cycles 2 and 4 (p>0.05). In the combined groups, baseline hepcidin was positively correlated with ISS score, β2 microglobulin (β2M), creatinine, ferritin and transferrin saturation and inversely with Hb and PLT (p<0.05). In the same groups, hepcidin before cycle 3 was negatively correlated with DR (p=0.018). In the conventional group, serum hepcidin above the upper normal limit before cycle 2 was negatively correlated with AR (p=0.023). In the same group, baseline hepcidin was inversely correlated with DR at 2 months (DR 2) and DOR (p=0.039,

p=0.006, respectively). Overall, hepcidin before cycle 3 was the only independent predictor for DR and DR 2 (p=0.03 for both end-points). Hepcidin before cycle 2 above the upper normal limit was the only predictor for AR (p=0.02). *Conclusions*. These results suggest that serum hepcidin is correlated with important disease variables such as ISS and β2M that reflect tumor activity and clinical outcome in MM, and thus may serve as a surrogate marker for disease activity. Following the initial treatment cycles, hepcidin was strongly correlated with DR in the combined groups. In the conventional but not in IMiDs group, baseline hepcidin was significantly correlated with DR and DOR suggesting that IMiDs-based regimens may overcome the negative impact of baseline hepcidin on response indicators. Patients in the IMiDs group maintained decreased hepcidin levels through cycle 3 in contrast to the conventional treatment group, where hepcidin returned to baseline highlighting the effectiveness of IMiDs-based regimens on disease control. In contrast with other significant disease biomarkers, serum hepcidin has potential as a predictor for disease and anemia response in MM, particularly during the initial phase of treatment. Overall, our results indicate the utility of serum hepcidin to monitor disease response and guide therapeutic decisions in MM.

0945

FGFR3 IS EXPRESSED AND IMPORTANT FOR SURVIVAL IN INA-6, A HUMAN MYELOMA CELL LINE WITHOUT A T(4;14)

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Background. Fibroblast growth factor receptor 3 (FGFR3) might play a role in the survival and proliferation of myeloma (MM) cells. Primary MM cells and MM cell lines (HMCL) with t(4;14) express FGFR3 in 75% of the cases. *Aims*. If FGFR3 plays an important carcinogenic role, one might expect other genetic aberrations than t(4;14) to lead to expression of the gene. We show data suggesting that gene amplification is another mechanism leading to expression of FGFR3. *Methods*. Fluorescence *in situ* hybridization with locus-specific probes for FGFR3 and MMSET was used on metaphase spreads of the t(11;14) INA-6, and array-CGH was used to look at the genomic alterations. Western Blot and qRT-PCR detected the FGFR3 expression. To determine the functional role of the receptor, small-molecule FGFR3-inhibitors SU-5402 and PD-173074 were used in proliferation studies and viability studies. The t(4;14) HMCLs IH-1 was used as a positive control and OH-2 without IGH translocations or FGFR3 expression was used as a negative control. All three HMCLs are IL-6 dependant. FGFR3 was sequenced in INA-6 and IH-1 to look for activating mutations. MMSET expression was also determined by qRT-PCR. *Results*. The FGFR3 was present in INA-6 cells although the cell line is t(4;14) negative. In INA-6 the expression of FGFR3 is dependant on IL-6, and the expression of FGFR3 is decreased when cells are starved for IL-6 over night. The same level of decreased FGFR3 was not seen in IL-6 deprived IH-1 cells. INA-6 has a trisomy of chromosome 4 and there was a duplication of the 4p16.3 locus on one 4p on chromosome 4. The FGFR3 inhibitors, SU-5402 and PD-173074, each reduced proliferation of INA-6 cells, both in the presence and in the absence of aFGF. Similar inhibition was seen in IH-1 cells, a t(4;14) HMCL, whereas OH-2 cells, HMCL without FGFR3 expression, were insensitive to the drugs. The same was also seen when looking at cell viability with flow cytometry. The FGFR3 was not mutated in IH-1 or INA-6 determined by sequencing. MMSET in INA-6 was close to the expression in OH-2 and significantly lower than in IH-1. *Summary and Conclusions*. INA-6 expresses wild type FGFR3 without having a t(4;14). Amplification of the FGFR3 might cause the expression. The FGFR3 level decreases when the cells are deprived of IL-6. The alternative mode of aberrant FGFR3 expression shown in INA-6 cells is supportive of an oncogenic role for this receptor in MM. Since INA-6 it does not express MMSET at a high level, contrary to all the t(4;14) cell lines and, hence, lacks the IGH/MMSET fusion transcript, it might be useful as a model system for investigating FGFR3's role in MM cells independently of the interaction of high levels of MMSET.

0946

ESTABLISHMENT OF AN INTERLEUKIN-6 INDEPENDENT VARIANT (CMA-03/06) OF THE HUMAN MYELOMA CELL LINE CMA-03: BIOLOGICAL AND MOLECULAR CHARACTERIZATION BY A GENOMIC INTEGRATIVE ANALYSIS

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Background. The growth and survival of multiple myeloma (MM) cells in the bone marrow microenvironment is regulated by functional complex interactions between the tumor cells and the surrounding bone marrow stromal cells mediated by adhesion molecules and the production of several cytokines of which interleukin-6 (IL-6) has been identified as the most important. Major advances in the investigation of MM biology were made possible by the availability of human myeloma cell lines (HMCLs). The IL-6-dependent CMA-03 cell line was established in our laboratory from a peritoneal effusion of a refractory relapsed MM patient. By gradually decreasing the IL-6 added to the culture, an IL-6-independent variant, CMA-03/06, could be obtained. *Aims*. To perform a biological and molecular characterization of this novel cell line, and to provide insights into the signaling pathways and target genes involved in the growth and survival of CMA-03/06. *Methods*. The growth, immunophenotypic, cytogenetic and fluorescence *in situ* hybridization (FISH) characterization of CMA-03/06 was performed by means of standard procedures. IL-6 production into the culture media was determined using a high sensitivity IL-6 specific ELISA. Global gene expression profiling (GEP) was performed by means of the GeneChip® Human Gene 1.0 ST Arrays (Affymetrix); the supervised analyses were done using the SAM software version 3.0. Genome-wide profiling data were generated by means of Affymetrix GeneChip® Human Mapping 250K Nsp arrays; copy number (CN) alterations were calculated using the DNAcopy Bioconductor package, based on circular binary segmentation method. *Results*. The addition of IL-6 to the culture medium of CMA-03/06 or co-culture with multipotent mesenchymal stromal cells didn't induce an increase in proliferation of the cells. CD45 expression was considerably reduced in CMA-03/06 cells compared with CMA-03, whereas they were found positive for both chains of IL-6 receptor, CD126 and CD130, almost undetectable in CMA-03. Conventional cytogenetic and FISH analyses didn't reveal differences between the two HMCLs. IL-6 could not be detected in the supernatants from either CMA-03 or CMA-03/06. Western blot analysis revealed the IL-6 induced activation of STAT3 and STAT1 in both HMCLs. GEP analysis of CMA-03/06 compared with CMA-03 allowed the identification of 21 upregulated and 47 downregulated genes, mainly involved in cellular signaling, cell cycle, cell adhesion, cell development, regulation of transcription, apoptosis; many of them are known to be involved in myeloma biology. The genome-wide analysis allowed the identification of about 100 altered chromosomal regions common to both HMCLs, mostly DNA gains. Comparison of CMA-03/06 with CMA-03 evidenced a different CN in only 15 small chromosomal regions, eight of which didn't contain any transcript, whereas few genes were located on the other ones. None of the genes differentially expressed in CMA-03/06 compared with CMA-03 except one were positioned on these regions. *Conclusions*. Our data confirm the IL-6 independence of CMA-03/06 and the absence of an autocrine IL-6 loop, even though the IL-6 pathway is still active. Since the cell line uses other signaling pathways for its growth, it may represent a suitable model for studies concerning the pathways involved in cytokine responses in MM.

0947

INHIBITION OF PROTEASOME SUBUNITS BY CARFILZOMIB IN PHASE 1B CLINICAL TRIAL MEASURED USING A NOVEL SUBUNIT-SPECIFIC ELISA

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Background. Proteasome inhibition has been validated as a therapeutic approach for the treatment of multiple myeloma (MM) and mantle cell lymphoma. However, the contribution of individual proteasome active sites to the clinical efficacy of bortezomib, an approved agent, and next generation proteasome inhibitors (PIs) such as carfilzomib, NPI-0052 and CEP-18770 are largely unknown. The constitutive and immunoproteasome each contain three catalytic sites characterized as chymotrypsin-like (β5 and LMP7), caspase-like (β1 and LMP2) and trypsin-like (β2 and MECL1), which the aforementioned drugs target with varying selectivity and reversibility, depending on the reactive warhead and backbone structure. These differences may contribute to the distinct properties of these molecules observed in the laboratory and the clinic. Our current understanding of proteasome activity relies on fluorogenic substrate assays that do not adequately represent proteasome subunit activities. New assays that can measure subunit activity in cells are needed in order to establish the relationship between proteasome inhibition and anti-tumor activity. *Aims*. To develop a quantitative assay capable of measuring the activity of each catalytic subunit in the constitutive proteasome and the immunoproteasome in human patient samples. To asses proteasome subunit inhibition in samples from patients enrolled in a Phase 1b carfilzomib/lenalidomide/dexamethasone combination clinical trial. *Methods*. We developed a proteasome constitutive/immuno subunit ELISA (abbreviated ProCISE) to measure the activity of individual proteasome subunits. The assay utilizes a biotinylated peptide epoxyketone probe to label and capture each active subunit, and subunit-specific antibodies for detection. Purified proteasomes and whole blood treated *ex vivo* with carfilzomib and bortezomib, as well as samples from patients receiving carfilzomib as part of a Phase 1b clinical trial were analyzed for levels of individual subunit active sites using the validated ProCISE assay. *Results*. The ProCISE assay was validated using commercially available purified constitutive (c20S) or immunoproteasome (i20S) protein and human RBC or PBMC lysates derived from healthy donors. The assay is capable of measuring proteasome concentrations from 0.23-1.49 µg/mL to 25 µg/mL, depending on the subunit. When compared to bortezomib *in vitro* and *ex vivo*, carfilzomib showed greater selectivity for the chymotrypsin-like subunits β5 and LMP7 in both cases, whereas bortezomib displayed significant activity towards β1 and LMP2 subunits. Preliminary data from patients receiving the 15 mg/m^2 dose of carfilzomib as part of a combination Phase 1b trial, showed strong inhibition of the chymotrypsin-like subunits β5 (74%) and LMP7 (84%) after the first dose. The LMP2 and MECL1 subunits of the immunoproteasome were affected significantly less and inhibition of the constitutive subunits β1 and β2 was found to be minimal. *Summary*. ProCISE is a robust and reproducible assay for measuring proteasome activity in both research and clinical settings. Using ProCISE, we demonstrated that carfilzomib potently and selectively inhibited the chymotrypsin-like activity of the constitutive proteasome and immunoproteasome in purified proteasome, *ex vivo* treated blood and samples derived from MM patients receiving carfilzomib treatment. The ProCISE assay is an important new tool for studying the role of the proteasome and its inhibitors in MM biology and therapy.

0948

EXPRESSION OF CD27 ON PLASMA CELLS IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is characterised by a clonal expansion of neoplastic plasma cells (PC). These PCs are mostly CD19$^-$ CD56$^+$, but in a small group of patients clonal CD19$^+$ PCs occur. Light chains clonality assessment is widely used to discriminate between clonal and polyclonal PCs, although especially in low infiltration cases results are not convincing. CD27 is expressed on memory B cells and also on normal CD19$^+$ plasma cells, so one can assume that this marker can be used for confirmation of polyclonal CD19$^+$ PCs in MM as well. *Aims*. The aim of this study was to analyse expression of CD27 on CD19$^+$ PCs in different groups of MM patients and to prove that CD27 can be routinely used to confirm polyclonality of CD19$^+$ PCs. *Methods*. A total of 60 patients were analysed and this group consisted of 15 monoclonal gammopathy of undetermined significance (MGUS) patients; 10 newly diagnosed MM pts; 9 relapsed and/or reanalysed MM pts; 14 transplanted MM pts; and 10 control pts without monoclonal gammopathy. Bone marrow PCs were identified as CD38$^+$CD138$^+$ cells and expression of CD19, CD56, and CD27 was analysed on these PCs. Clonality assessment using kappa and lambda light chains was done in almost all cases. *Results*. When analysed MGUS patients correlation between median of CD19$^+$ PCs (37.1%) and CD19$^+$CD27$^+$ PCs (40.9%) was found (0.95). In MM there was found 1 case with clonal CD19$^+$ PCs, but correlation was higher than in MGUS (0.97), although median of CD19$^+$ PCs (0.9%) and CD19$^+$CD27$^+$ PCs (2.0%) was lower. In subgroup of relapsed and/or reanalysed patients were found 3 cases of clonal CD19$^+$ PCs, median of CD19$^+$ PCs (54.0%) was higher than CD19$^+$CD27$^+$ PCs (42.2%) and correlation was lower than in previous subgroups (0.87). In patients after transplantation was found only 1 case of clonal CD19$^+$ PCs, thus correlation between CD19$^+$ PCs (78.5%) and CD19$^+$CD27$^+$ PCs (80.8%) was similar to MGUS and MM subgroups (0.92). In subgroup of patients without monoclonal gammopathy was found high correlation between CD19$^+$ PCs (63.8%) and CD19$^+$CD27$^+$ PCs (64.7%) as was expected (0.94). Finally, in group of 5 patients with clonal CD19+ PCs was the correlation between median of CD19$^+$ PCs and CD19$^+$CD27$^+$ PCs significantly lower (0.36) than in group 55 patients with polyclonal CD19$^+$ PCs (0.96). *Summary and Conclusions*. In our hands expression of CD27 on CD19$^+$ PCs can discriminate between clonal and polyclonal CD19$^+$ PCs and thus can be potentially used instead of labour intensive light chains assessment.

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Myeloma and other monoclonal gammopathies - Clinical II

0949

LONGER DURATION OF TREATMENT AND MAINTENANCE OF BEST RESPONSE WITH LENALIDOMIDE + DEXAMETHASONE INCREASES OVERALL SURVIVAL (OS) IN PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA

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Background. Lenalidomide is an oral immunomodulatory' drug active in relapsed/refractory multiple myeloma (RRMM). Lenalidomide + dexamethasone for treatment of patients with RRMM is well tolerated and can be given for a prolonged period of time. *Aims*. Statistical data were analyzed to determine whether there is a survival benefit to continuing lenalidomide + dexamethasone therapy after achieving best clinical response. *Methods*. Updated, pooled data from the phase-3 trials MM-009 & MM-010 were analyzed. Median follow-up time for surviving patients was 48 months. Kaplan-Meier (K-M) survival estimates for patients achieving a partial response (PR) or better were compared between patients on continuous treatment (defined as ongoing treatment or discontinuation due to progression) and patients discontinuing treatment due to adverse events, consent withdrawal, or other reasons. Unlike a conventional Cox regression, which is biased in favor of patients who continued treatment, time-dependent Cox models summarize the evidence over a range of landmarks. After testing for potential biases between the two patient groups in the K-M analysis, two time-dependent multivariate regressions using dummy variables as proxies for duration of treatment from study entry were run: one evaluating the potential benefit of continuing on lenalidomide + dexamethasone treatment and one measuring the benefit of an additional lenalidomide cycle. *Results*. The K-M estimate of median survival in patients continuing treatment after achieving PR or better (N=174) was 50.9 months [95% confidence interval (CI): 43.0-NA] vs. 34.95 months [26.4-55.7] in patients discontinuing treatment early (N=38; p=0.0594; Figure 1).

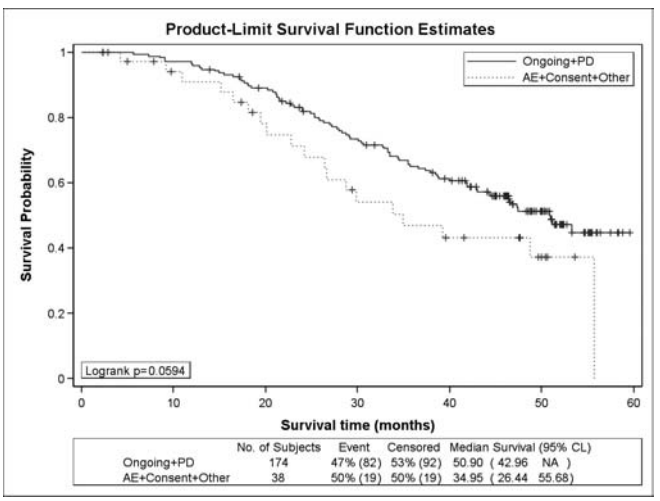

Figure 1. Kaplan-Meier estimates on overall survival.

In order to avoid the potential bias of favorable prognostic features (e.g. age, number of prior therapies, β2-microglobulin (B2M) and best response achieved) between patients on continuous treatment and patients discontinuing treatment due to adverse events, consent withdrawal, or other reasons, Cox proportional hazard regression analyses including lenalidomide + dexamethasone patients with PR or better (N=212) were performed. Longer lenalidomide treatment duration measured as either continued lenalidomide treatment (HR=0.137 [95% CI: 0.045-0.417]; p=0.0005) or an additional lenalidomide cycle (HR=0.921 [95% CI: 0.886-0.957]; p<0.0001) was associated with improved survival outcomes when controlling for other variables. Amongst the statistically significant patient characteristics, more prior anti-myeloma therapies (HR=1.778 [95% CI: 1.126-2.809]; p=0.0136), higher B2M (HR=1.708 [95% CI: 1.085-2.689]; p=0.0208), and a more advanced multiple myeloma stage as measured by Durie-Salmon criteria (HR=1.462 [95% CI: 0.997-2.144], p=0.0515) were all associated with a higher risk of death, whereas the achievement of CR, nCR or VGPR as best response (HR= 0.681 [95% CI: 0.434-1.068], p=0.0942) was associated with a lower risk of death. *Conclusions*. Continued treatment with lenalidomide + dexamethasone has a statistically significant positive impact on overall survival, when controlling for patient characteristics and best response achieved. Ideally, this observation would require further confirmation in form of a randomized clinical trial.

0950

CONFIRMATION OF THE UTILITY OF THE IMWG THROMBOPROPHYLAXIS STRATEGY IN MYELOMA PATIENTS RECEIVING THALIDOMIDE; EXPERIENCE OF A LARGE SINGLE CENTRE INSTITUTION

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Background and Aims. The risk of venous thromboembolism (VTE) is increased in multiple myeloma (MM) patients treated with thalidomide, especially in combination with dexamethasone or chemotherapy (reported as between 3-15%). Various VTE prevention strategies have been used, however the optimal prophylactic regime is unknown. Guidelines have recently been published by the International Myeloma Working Group (IMWG, Leukemia 2008, 22, 414-423), suggesting thromboprophylactic strategies should be used if there are risk factors based upon MM therapy (combination of thalidomide with dexamethasone and / or chemotherapy), disease related (hyperviscosity) or patient-related (previous VTE, central catheter, associated disease, surgery or use of erythropoietin). We have reviewed the impact of VTE prophylaxis in MM patients treated with thalidomide at our institutions over the last 4 years. *Methods*. 83 patients were treated with thalidomide, either in combination with dexamethasone and chemotherapy (n=57); with dexamethasone or chemotherapy (n=14), or with single agent thalidomide (n=12). Median age 63 years (range 41-81), male:female ratio 1.18:1, isotypes as follows; IgG (n=51), IgA (n=14), light chain only (n=13), IgD (n=1), non-secretory (n=2), plasma cell leukaemia (n=2). 71 patients receiving combination therapy were treated at relapse (n=45), induction (n=8) or when refractory to induction therapy (n=18). VTE prophylaxis was as follows; low molecular weight heparin (LMWH, n=17), full dose warfarin (target INR 2.5, n=8), low dose warfarin (n=6), low dose aspirin (n=8), or no thromboprophylaxis (n= 32). Additional risk factors (either individual or MM related) were present in 18 /71 patients that received thalidomide in combination with another agent, with 5/18 patients receiving either LMWH or full dose warfarin. 33 patients received thalidomide as maintenance treatment (single agent), with no VTE prophylaxis given to any patient, although additional risk factors were identified in 12 patients. *Results*. VTE occurred in 3/46 (6%) patients treated with either low dose aspirin/warfarin (n=1), or no thromboprophylaxis (n=2). There were no cases of VTE in patients treated with LMWH or full dose warfarin (n=25). VTE occurred in 1/18 patients with at least 1 additional risk factor as defined by the IMWG, in a patient who had a prior VTE (who did not receive any thromboprohylaxis). VTE occurred in 1/33 (3%) patients receiving thalidomide maintenance (this patient had a prior VTE). *Conclusions*. VTE in MM patients treated with thalidomide in combination with dexamthasone or chemotherapy is reduced by using LMWH or full dose warfarin. There is no role for using aspirin or low dose warfarin in this patient group. The incidence of VTE without adequate thromboprophylaxis is 6%, which is lower than has been reported. Maintenance treatment with thalidomide may be associated with an increased risk of VTE in patients where additional risk factors are identified. Careful risk assessment and assignment of patients requiring thalidomide treatment for MM will inform the appropriate use of anticoagulation strategies where needed, and avoid unnecessary use of anticoagulation in patients at low risk.

0951

CLNICAL EFFICACY OF VEL-CTD (BORTEZOMIB, CYCLOPHOSPHAMIDE, THALIDOMIDE, AND DEXAMETHASONE) REGIMEN IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: A PHASE II STUDY

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Background and Objectives. Bortezomib is a small molecule, dipeptidyl boronic acid proteosome inhibitor and it was largely studied in refractory/relapsed myeloma in the beginning. As a combination with other targeted therapies such as thalidomide or chemotherapeutic agents, the response rate was reported to be up to 50% including 20% complete response. In this study, we assessed the efficacy and safety of the combination with bortezomib, cyclophosphamide, thalidomide and dexamethasone (Vel-CTD) for the patients with relapsed/refractory multiple myeloma. *Design and Methods*. Seventy-three patients who had received at least two cycles of treatment were enrolled. Bortezomib was given at 1.3 mg/m^2 (D1, 4, 8, 11), cyclophosphamide (150mg/m^2 P.O. on D1-4), thalidomide (50 mg/day, daily), and dexamethasone (20mg/m^2 I.V. on D1-5, D15-19). *Results*. There were 37 males (50.7%) and 36 females (49.3%). The median age of patients was 67 years (range, 40~79 years). Median number of the previous treatment regimens were two (range, 1~6). Of total 73 patients, sixty-four patients (87.7%) achieved at least a partial response (PR) including 20 (27.4%) with a stringent complete response (sCR) and 14 (19.2%) with a CR as their best response. Three-year progression-free survival (PFS) was 13.6±6.1% and three-year overall survival (OS) from the start of Vel-CTD was 48.7±9.1%. Patients who achieved a good response at least a PR after four cycles of Vel-CTD showed longer PFS than those with poor therapeutic responses (3yr PFS, 22.7±8.7% vs. 0, p=0.000). Further, patients who achieved a good therapeutic responses also showed significantly longer OS than those with poor responses (3yr OS, 58.1±11.0% vs. 34.7±15.1%., p=0.002). Patients with a good response at least a PR after eight cycles of Vel-CTD also showed longer PFS (2yr PFS 21.3±8.3% vs. 0, p=0.000) and OS (2yr OS, 55.7±1.2% vs. 25.0±2.2%, p=0.006) than those with poor responders. Of 411 evaluable treatment cycles, grade 3 or 4 toxicities included thrombocytopenia (11.5%), neutropenia (4.6%), peripheral sensory neuropathy (4.4%), and thrombosis (0.5%). *Conclusions*. Vel-CTD is an active salvage therapy with manageable toxicity in patients with relapsed/refractory myeloma. However, trials evaluating novel treatment approaches after bortezomib should be offered to reduce the rate of disease progression, particularly in patients who fail to show a good response at least PR after four or eight cycles of therapy.

0952

OPTIMIZATION OF BORTEZOMIB-BASED COMBINATIVE REGIMEN IN ELDERLY RELAPSED MULTIPLE MYELOMA PATIENTS

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Background. Bortezomib-based regimens represents a chance for a longer survival for multiple myeloma patients. Despite of the high efficacy in relapsed patients bortezomib has specific toxicity, which often leads to prematurely interruption of the treatment, especially in elderly patients and/or patients with poor performance status. Therefore further optimization of bortezomib-based regimens is essential. *Aims*. We evaluated the potential of combinative regimen CVD (Cyclophosphamide, Bortezomib (Velcade), Dexamethasone) reduced intensity (CVD senior; 50% reduction) in elderly patients and/or patients with poor status performance with relapsed multiple myeloma. The results were compared with CVD junior regimen outcome used in patients aged <65 years and good status performance. *Patients and Methods*. A total of 71 patients (30xCVD senior vs 41xCVD junior) was evaluated in period from February 2007 until December 2008. Regimen CVD senior: Bortezomib (1.3mg/m^2,day 1 and 15); Cyclophosphamide (50mg p.o. daily) and dexamethasone 20mg (day 1-4, 15-18). Regimen CVD junior: Bortezomib (1.3mg/m^2,day 1,4,8 and 15); Cyclophosphamide (500mg/m^2 i.v. day 1 and 15) and dexamethasone 40mg (day 1-4,15-18). Duration of cycle was 28 days in both regimens. There was a significantly higher age in CVD senior in comparison to CVD junior group - median age 71.1 (49.5-81.7) vs 60.0 years (44.3-77.7), p=<0.001; a trend to higher pretreatment - number of previous treatment lines 1-13.3% vs. 31.7%/2-63.3% vs. 53.7%/3 or more-23.3% vs 14.6% (p=0.165); a trend to lower Karnofski status (80 vs. 90%,p=0.109); a higher frequency of clinical stage 3 according to ISS (37.5% vs 20.6%; p=0.118). Median follow-up from the start of therapy was 13.9 vs. 9.1 months (p=0.030). *Results*. The basic results for regimen CVD senior vs junior are: overall response (ORR=CR+PR) was 39.9% vs. 64.9% (CR 3.6% vs. 13.5%, VGPR 14.3% vs. 16.2% and PR 21.4 vs. 35.1%); stable disease 53.6% vs. 21.6% and progression 7.1% vs. 13.5%. Althought there was achieved better overall response in CVD junior group (p=0.027), there was not significant difference for medians of parameters of survival (TTP/PFS 8.9 vs 11.3 months, OS from the start of therapy 12.3 vs 16.3 months, DOR 7 vs 10.3 months) A higher total dose of bortezomib achieved by patients in each group resulted in a better response in both regimens (a trend for CVD senior-p=0.128; significant for CVD junior-p=0.006). Number of grade 3 or 4 AEs was not significantly different (p=0.301), but grade 4 AEs were not observed in CVD senior group (0 vs 18.5%). Dose of bortezomib was reduced in less frequency in CVD senior regimen [6,7% (2/30) vs 26.8% (11/41)], median number of cycles was similar (5.2 vs 5.1;p=0.86). *Conclusions*. The toleration of reduced intensity regimen by elderly patients is similar as toleration of full dose regimen by younger patients. Furthermore there was no grade 4 AEs in CVD senior group. Duration of treatment and number of cycles obtained was similar. Althought there was a worse overall response in CVD senior group, parameters of survival were not significantly different. The reduced intensity CVD regimen seems to be a good option for elderly patients with poor status performance with well balanced efficacy/toxicity ratio.

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0953

EFFICACY OF PAD REGIMEN AS AN INDUCTION THERAPY BEFORE AUTOLOGOUS STEM CELL TRANSPLANTATION IN VAD OR CTD REFRACTORY MYELOMA

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Bortezomib induces apoptosis of multiple myeloma (MM) cells, and affects their microenvironment. Combination of Bortezomib, Doxorubicin and Dexamethasone (PAD) gives responses in 95% of newly diagnosed MM (Oakervee *et al*. Br J Haematol 2005; 129: 755-62). *Aims, Patients and Methods*. Aim of this study was to evaluate prospectively response to: 1. bortezomib alone (1.3 mg/m^2 on days 1,4,8,11 in a 21-day cycle for up to 8 cycles) in patients with relapsed or refractory MM (bortezomib group) 2. combination of bortezomib (as above), doxorubicin (9 mg/m^2 days 1-4) and dexamethasone (40 mg days 1-4) as an induction therapy before high-dose therapy in VAD or CTD refractory MM patients and as salvage therapy at relapse (PAD group). *Results*. From November 2004 until February 2009 after informed consent was obtained 50 MM patients have been included: 22M, 28F, median age 59yrs (33-78). Thirty nine was IgG, 7 IgA, 4 only light chain, 84% had osteolysis. According to Durie-Salmon staging system 18% was I, 14% II, 68% III and acc. to ISS 52% I, 32% II, 16% III. The median number of prior therapies was in bortezomib group 2.4 (1-5), in PAD group 1.3 (1-2). Median time from diagnosis to bortezomib therapy was in bortezomib group 44 months (13-149) in PAD group 14 months (2-33). Twenty one patients received bortezomib alone for relapse and 30 patients received PAD-21 as induction treatment and 10 for relapse. Median follow-up time since bortezomib administration was 10 months (1-42) (in bortezomib group 16 months and in PAD group 7 months). Median number of cycles received was in bortezomib group 6 (1-18) in PAD group 6 (1-9). Response acc. to IMWG criteria for the patients who received bortezomib alone was 31.6% (nCR 10%, PR 22%). Median time to progression for responders was 6 months. For MM patients who achieved less than PR after 4 cycles of VAD or CTD and than received PAD as induction therapy response rate was 76.3% (CR 14.3%, nCR 14.3%, PR 47.7%, SD 19.0%, PD 4.7%). In 8 of these patients ASCT was performed and 4 are in process of stem cell collection. Five of 6 evaluable patients with relapsed MM showed partial remission after PAD therapy. *Conclusions*. In relapsed myeloma the rate of response to bortezomib alone is 31%, with median duration of response of 6 months. In VAD or CTD refractory MM patients PAD as induction therapy before high-dose therapy offers response rate of 76%.

0954

INCORPORATION OF THE BONE MARKER CARBOXY-TERMINAL TELOPEPTIDE OF TYPE-1 COLLAGEN (ICTP) INTO A COMBINED ISS-ICTP SCORE IMPROVES THE PROGNOSTIC SIGNIFICANCE OF THE ISS IN SYMPTOMATIC MULTIPLE MYELOMA

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Background. The prognosis of patients with newly diagnosed symptomatic multiple myeloma (MM) has been improved, but the outcome is still highly variable. Several prognostic markers, including parameters of tumor burden and cytogenetics were adopted to identify high-risk patients. Recently the International Staging System (ISS), including the parameters β2-microglobulin (β2M) and albumin, was introduced for patients with symptomatic MM. In previous studies the bone resorption marker carboxy-terminal telopeptide of type-1 collagen (ICTP) was identified as a sensitive and specific marker of increased bone resorption and as a strong prognostic factor for overall survival (OS) in MM. *Aims*. Since bone disease is a hallmark of MM, we investigated the prognostic impact of the bone resorption marker ICTP in combination with ISS, β2M, albumin and deletion of chromosome 13 (del(13q14)) and high-dose therapy (HDT) in 100 patients with newly diagnosed symptomatic MM. *Methods*. ICTP was detected by a commercially available radioimmunoassay (Orion Diagnostica, Espoo, Finland). The prognostic factors del(13q14), albumin and β2M were evaluated, using previously established cut-off values to dichotomize risk groups for survival analysis. ISS was calculated from albumin and β2M according to the cut-off values given in the original publication by Greipp *et al.* 2005. *Results*. β2M alone, albumin alone, ISS, del(13q14) and ICTP were significant prognostic factors for overall survival. In contrast to ICTP, lytic bone lesions, as detected by conventional radiography, were not an independent prognostic factor for OS (p=0.289). In a multivariate analysis, ICTP was the most powerful prognostic factor (log rank p<0.001, hazard-ratio: 9-fold increase). Furthermore ICTP clearly separated two subgroups with a good and a worse prognosis within each of the three ISS stages (ISS I: p=0.027, ISS II: p=0.022, ISS III: p=0.013). A combined ISS-ICTP score, including β2M (cut off: 3.5 mg/L), albumin (cut off: <3.5 g/dL) and ICTP (cut off: reference limit) significantly (p<0.001) separated four risk groups with a 5-year overall survival rate of 94% in the very low risk group, 65% in the low risk group, 46% in the intermediate risk group and 22% in the high risk group, respectively (Figure 1). In the very low risk group, there was no significant survival benefit with high-dose therapy (p=0.24), while HDT was a favorable prognostic factor in ISS-ICTP risk groups 1-3 (p=0.037, p=0.011 and p=0.012) and in ISS stages I-III (p=0.026, p=0.001 and p=0.052). *Summary and Conclusions*. Our data demonstrate that the inclusion of the bone resorption marker ICTP adds to the prognostic value of ISS.

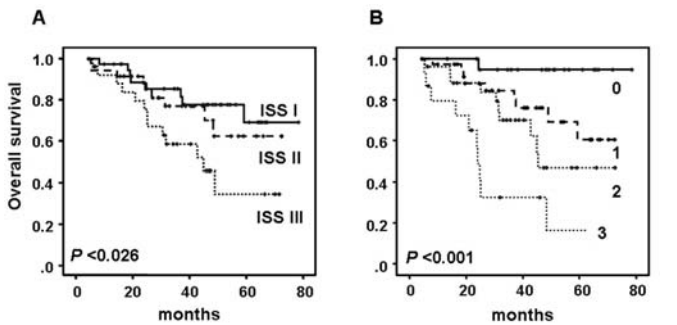

Figure 1. Prognostic value of ISS (A) and combined ISS-ICTP score (B).

0955

A RANDOMIZED PHASE II STUDY (GISL - MM03 TRIAL) WITH ORAL MELPHALAN+ PREDNISONE (MP) VERSUS MELPHALAN, + PREDNISONE + THALIDOMIDE (MPT) FOR NEWLY DIAGNOSED ELDERLY PATIENTS WITH MULTIPLE MYELOMA

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Background. Several papers showed that MPT compared with MP improved response and survival outcomes. However, side effects increase and toxicity, as neutropenia, neuropathy, deep-vein thrombosis, depression and constipation reduce patients compliance. Thus, several patients have to discontinue or reduce the dose of Thalidomide. *Aims*. Aim of our research is to verify these results in a group of 128 patients not eligible for high dose treatment with stem cells support. *Patients and Methods.* The MM03 trial was approved by Ethical Committee of Modena and registered at Osservatorio Nazionale sulla Sperimentazione Clinica dei Medicinali at the end 2004. A total of 128 patients were enrolled between January 2005 to October 2008: 53% entered MPT arm. Median age of patients were 75 years (range: 63-88 yrs), 50% were female, 55% had Durie and Salmon stage II and 45% had stage III. Distribution of M spike were: 67%, 22% and 11% for IgG, IgA and urine light chains. Univariate analysis of variables at baseline did not show any difference between the two arms. In arm A Melphalan was given at a dose of 0.25 mg/kg and Prednisone 60 mg/m^2 for 4 days every 28 days; in arm B Thalidomide was added at a dose of 100 mg every day; a maximum of ten cycles was planned. At the end of induction therapy, 26 patients who had obtained Complete Response, Partial Response, Minimal Response or Stable disease were randomized for maintenance with Dexamethasone 20 mg, day 1-4 every 28 days or Dexamethasone at the same dose plus Thalidomide 100 mg/day every day. Only 14 patients follow this indication and assumed maintenance treatment for 6-12 months; no differences were observed between the 2 arm of maintenance. The use of growth factors, was at treating physicians discretion. Starting from May 2005 enoxiparin was recommended during the first 4 cycles of MPT arm. *Results*. Of 128 patients, 91 patients are evaluable for response while 20 are still on treatment. The response, including MR among all patients who entered protocol were 76%, while 9% had SD and 15% presented PD. Considering separately the two arm, we observed in arm A 12% CR, 43% PR, 9% MR, 3% SD and 23% PD and in arm B 18% CR, 57% PR, 11% MR, 5% SD and 9% PD. A comparison between 55% of CR plus PR in arm A with 75% in arm B shows a p=0.049, showing an advantage for MPT arm. After a median follow up of 14 months, median Overall Survival at 24 months are not still reached. During treatment or progression 24 patients died. Grade III/IV adverse events were: 6 deep-vein thrombosis, all in MPT arm, 1 during enoxaparin prophylaxis, 1 pneumonitis, 6 neutropenia, 3 neurotoxicities, 3 constipation. *Conclusions*. Our results show that the addition of Thalidomide to MP improve number and quality of response. We are still collecting data for a better definition of OS, FFS and toxicity in arm A in comparison with arm B, and they will be presented during the meeting.

0956

SURVIVAL ANALYSIS OF PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA: AN INTERIM REPORT FROM AN INTERNATIONAL ELECTRONIC OBSERVATIONAL STUDY OF BORTEZOMIB

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Background. Multiple myeloma (MM) is associated with significant morbidity and mortality. Recent advances in treatment for MM have led to improved outcome and survival benefits in patients with relapsed refractory MM. Bortezomib (VELCADE®) is a proteasome inhibitor indicated for the treatment of MM. The clinical and health economic outcomes in MM patients in clinical practice are captured in an international electronic VELCADE observational study (eVOBS) - a non-interventional study of patients from Belgium, France, Greece, Russia, Spain, Sweden, and Turkey. The study started enrollment in October 2006. We report interim survival analysis of patients enrolled through 21 November 2008 data cut-off. *Aims*. In this analysis we assess survival in patients with relapsed refractory MM treated with bortezomib in real-life settings. *Methods*. Adults scheduled to receive bortezomib for MM are being enrolled in eVOBS. All bortezomib dosages and concomitant treatments (except investigational therapies) are permitted. Survival data are continuously being collected prospectively for 3 years. In this analysis we report stratified Kaplan-Meier survival analysis and multivariate proportional hazards regression for all patients. Patients still alive at the data cut-off date were censored. The current analysis excluded Spain and Russia due to time lag involved in data availability. *Results*. At baseline a total of 447 patients (58% male, median age 64.5years) were reviewed. At data cut-off date, the follow-up time for the majority (55%) of patients was still less than 6 months. 55% of the patients were censored at 6 months and 76% at 12 months The baseline patient characteristics are comparable to the pivotal clinical study of bortezomib versus dexamethasone in the relapsed refractory setting, APEX (Richardson PG *et al.* N England J Med. 2005; 352:2487-98). However, patients enrolled in eVOBS experienced significant renal impairment compared with patients in APEX study. In eVOBS, bortezomib was initiated as 2nd-, 3rd-, and 4+th-line therapy in 45%, 28%, and 17% of patients, respectively. Overall survival was 87% (95% CI: 83-91) at 6 months, 67% (95% CI: 58-74) at 12 months. Statistically significant association was observed between improved survival and patients aged <70 years, line of therapy, albumin >3.5g/dL and creatinine clearance ≥60 mL/min at baseline. *Conclusions*. This non-interventional observational study provides preliminary estimates on survival of bortezomib treated multiple myeloma patients as seen in real-life clinical practice. The clinical data in this study are still maturing and are expected to provide valuable insight to treatment outcome.

0957

THALIDOMIDE OR BORTEZOMIB IN COMBINATION WITH DEXAMETHASONE AS SECOND-LINE THERAPY FOR REFRACTORY/RELAPSED MULTIPLE MYELOMA: A COMPARISON OF EFFICACY AND TOXICITY

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Background. Therapeutic options for patients with relapsed/refractory multiple myeloma (MM) have expanded in the recent years with the introduction of novel agents such as thalidomide, bortezomib and lenalidomide. Although their efficacy has been demonstrated in clinical trials, there are no studies comparing these new agents in the setting of relapsed/refractory MM. *Aims*. To compare the efficacy and toxicity of thalidomide or bortezomib, in combination with dexamethasone, as second-line therapy. *Methods*. We analyzed a homogeneous population of MM patients refractory or relapsed after first-line therapy, who were treated either with thalidomide or bortezomib plus dexamethasone. Thalidomide was administered orally at doses of 50-200 mg/daily until progression. Bortezomib 1.3 mg/mq was given intravenously on days 1,4,8,11 every 21 days up to 6-8 cycles. *Results*. The analysis included 59 patients treated with thalidomide and 33 with bortezomib. Age, sex, isotype, Durie-Salmon stage, ISS, cytogenetics, prior transplant, status of disease (relapsed or refractory) were well balanced in the two groups. The median time from diagnosis to initiation of thalidomide or bortezomib was 23 and 20 months respectively (p=0.25). Thalidomide was administered at the median dose of 100 mg/day for a median time of 11 months (1-92). Patients treated with bortezomib received a median number of 4 cycles (1-8). In an intention-to-treat analysis, the overall response rate was similar in the two groups (thalidomide 64%, bortezomib 61%, p=0.13), while the CR+VGPR rate was higher in the latter one (14% vs 33%, p=0.03). The median follow-up was significantly longer for patients treated with thalidomide than with bortezomib (25 vs 11 months, p=0.0001). Overall, progression occurred in 80% of patients treated with thalidomide and 90% of those treated with bortezomib. Patients receiving thalidomide had a significantly better progression-free survival (PFS) (13 vs 4 months, p<0.0001) and overall survival (OS) (26 vs 12 months, p=0.01). The proportion of patients who required dose reduction for any toxicity was similar in the two groups (20% with thalidomide, 32% with bortezomib, p=0.16), while the discontinuation rate was significantly higher with bortezomib (8% vs 24%, p=0.03). Patients receiving bortezomib had a higher incidence of anaemia (12% vs 0%, p=0.01), thrombocytopenia (76% vs 0%, p<0.0001), fatigue (36% vs 12%, p=0.006) as compared to the other group. The incidence of peripheral neuropathy (PN) was similar (54% with thalidomide, 58% with bortezomib, p=0.46), with a trend for a higher incidence of grade ≥2 PN in the latter group (p=0.09). Gastrointestinal toxicity was reported in 44% and 40% of patients, respectively (p=0.41), but grade 3 toxicities occurred only with bortezomib (8%). The incidence of thromboembolic events was similar (7% with thalidomide, 6% with bortezomib, p=0.6). Bradycardia was observed only in patients treated with thalidomide (12%). *Conclusions*. Bortezomib, as second-line therapy, yields significantly higher CR+VGPR rate as compared to thalidomide. These favourable initial results, however, do not translate into better long-term outcome. Patients treated with thalidomide, in fact, have significantly longer PFS and OS. The higher rate of early discontinuation for toxicity in the bortezomib group may account for these results.

0958

INCREASED BONE MINERAL DENSITY IN A SUBSET OF PATIENTS WITH RELAPSED MULTIPLE MYELOMA WHO RECEIVED THE COMBINATION OF BORTEZOMIB, DEXAMETHASONE AND ZOLEDRONIC ACID

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Background. Bortezomib is a first-in-class proteasome inhibitor with established antimyeloma efficacy that also showed a beneficial effect on bone metabolism. Bortezomib reduces bone resorption and increases bone formation markers and osteoblast counts in trephine biopsies. However, there are no data for the effect of bortezomib on bone mineral density (BMD) of myeloma patients. *Aims*. To evaluate the effect of the combination of bortezomib plus dexamethasone (VD) and zoledronic acid on BMD of patients with relapsed myeloma. *Methods*. We studied the files of 27 consecutive patients (16M/11F; median age 69.5 years) with relapsed MM who received VD and had BMD before and after 8 cycles of therapy. The median number of previous lines of therapies was 2 (range: 1-5). Bortezomib was given at the standard dose of 1.3 mg/m^2 on D1,4,8,11 of a 21-day cycle, while dexamethasone was given at a dose of 20 mg, p.o., the day of bortezomib administration and the following day. All patients also received zoledronic acid (4mg, iv, monthly). BMD of the lumbar spine (L1-L4, antero-posterior view), and femoral neck (FN) was measured by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-1000 scanner. Evidence of myeloma bone disease at the time of relapse was documented by plain radiography. All patients had measurements of urinary NTX (bone resorption marker) and serum bone alkaline phosphatase (bALP) and osteocalcin (bone formation markers), pre- and every month post-VD. *Results*. Twenty patients achieved an objective response (at least PR), while 2 patients had a minimal response and 5 patients had stable disease after 8 cycles of therapy. Before VD, skeletal survey revealed that 8 patients (29%) had lytic lesions in 1-3 areas (group A), 18 (66%) had lytic lesions in more than 3 areas and/or a pathological fracture (group B), while only one patient had diffuse osteoporosis. DXA measurements showed that 7 patients (25%) had

osteopenia and 14 (51%) osteoporosis at least in one of the evaluated sites at the time of relapse, according to WHO criteria. The combination of VD plus zoledronic acid produced no significant changes in both L1-L4 and FN BMD after 8 cycles of therapy. At the same time, NTX was reduced while bALP and osteocalcin were markedly increased (p<0.001). However, 4 patients (14%) showed at least 10% of increase in L1-L4 BMD (median 16%). All these patients had osteopososis according to DXA measurement, had responded to VD therapy (3 PR and one CR), had received VD as second line treatment and in terms of lytic bone disease 3 patients belonged to group A, while the other one was the only patient who had diffuse osteoporosis. No change in FN BMD was seen in these patients. *Summary and Conclusions*. The combination of VD plus zoledronic acid may increase BMD in a subset of relapsed myeloma patients with low BMD and non-extensive lytic disease who receive this regimen early in their disease therapy. Based on these results, a prospective study evaluating BMD in patients with relapsed/refractory myeloma who receive VD as second line therapy has just started.

0959

COMBINATION OF INTERNATIONAL STAGING SYSTEM AND CYTOGENETICS CAN PREDICT POOR PROGNOSIS IN MULTIPLE MYELOMA AFTER HD-CHEMOTHERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION

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Backgroud. Multiple myeloma (MM) is one of the key hematologic malignancies in which the impact of dose intensity has been demonstrated. High-dose chemotherapy (HD-CHT) followed autologous stem cell transplantation (ASCT) is performed as a standard treatment for newly diagnosed multiple myeloma. Combinations of prognostic factors such as cytogenetics and international scoring system (ISS) may be useful to stratify prognosis after ASCT. *Methods*. We evaluated transplant outcome among 76 consecutive patients (pts) with newly diagnosed MM who underwent single ASCT between February 2000 and October 2007. Median age was 56 years old and median time from diagnosis to ASCT was 5,5 months. *Results*. The ISS stages at diagnosis were 1 (n=28), 2 (n=28) a 3 (n=20). Cytogenetical analyses of bone marrow at diagnosis detected metaphase abnormalities (including 13 chromosome) in 19 pts, and interphase abnormalities in 14 of 49 (29%) patients, deletion of 17p13 in 3, t(4;14) in 8, and t(14;16) in 3 pts. Patients were defined as high-risk if they had any of these abnormatities or the ISS stage 3. Thirty- one pts (41%) were classified as high-risk. All pts were conditioned with HD melphalan (200mg/m^2). With a median follow up of 2,7 years, overall survival (OS) rates 3 years after ASCT in non-high-risk group were 84% and in high-risk group were 46%, respectively (p=0.006). Progression-free survival rates were 29% and 10%, respectively (p=0.066). Although 23pts achieved complete remission (CR) and 15 achieved VGPR after ASCT, 19 of them relapsed. Of these 19pts, survival rates 1year after relapse in each group were 100% and 34%, respectively (p=0.035). Sixty-five percent of the pts in high-risk group died within 1 year after relapse, although they received salvage chemotherapy containing Velcade or Thalidomide. *Conclusions*. The combination of cytogenetics and ISS can predict prognosis of MM after ASCT. A major problem was a poor prognosis among relapsed pts in high-risk group. The limited duration of responses after HD-CHT for pts with poor prognostic factors highlight the need to develop a risk- adapted treatment strategy. New drugs, alogeneic transplant and combination of these are subjects for future investigation. System (ISS) may be useful to stratify prognosis after ASCT.

0960

THE IMPACT OF FRONTLINE TREATMENT ON SURVIVAL OUTCOMES IN MULTIPLE MYELOMA: THE SGH EXPERIENCE

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Background. Multiple myeloma (MM) is a plasma cell malignancy characterized by an indolent clinical course with recurrent remissions 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 sought to 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 2003-2007 with complete clinical data and followed at SGH 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 and differences were compared using the log-rank test. *Results*. 142 patients with complete treatment data were available for review. The median age was 59 yrs. 36 (24.3%), 61 (41.2%) and 41 (27.7%) patients presented with ISS stage I, II and III disease respectively. Distribution of various induction therapy received are shown in Table 1. 58 (39.%) and 46 (31%) patients attained CR and PR respectively after frontline treatment. The median progression free survival was 3.0yrs and independent of response status. Patients who attained CR however, had a significantly better median OS at 9.1yrs (95% CI: 5.7,12.4) compared to those who achieved PR at 5.6 yrs (95% CI: 4.3,6.9) (p=0.01). No significant differences were observed in the response rates among the different frontline regimes, although bortezomib-based induction showed a trend towards more rapid reduction of M-band. *Summary and Conclusions*. Achievement of CR seems to confer a more favorable survival. Application of a Cox regression model is in progress to better understand the contributions of treatment, particularly bortezomib and transplant, as well as other factors in these patients.

Table 1.

	Frequency	Percent
Other alkykating agents	7	4.7
VAD Based	45	30.4
Thal/Dex Based	64	43.2
Melphalan Based	8	5.4
Bortezomib Based	24	16.2
Total	148	100.0

0961

18F-FDG-PET UTILITY IN MULTIPLE MYELOMA. NEW DURIE-SALMON PLUS STAGING SYSTEM. OUR EXPERIENCE

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Background. Multiple myeloma (MM) is a heterogeneous disease. To standardize the therapy approach is essential to correctly characterize it since diagnosis. Increasing use MR, CT, and FDG-PET have conducted to a new MM classification, Durie-Salmon Plus staging system, according to the number of existing lesions in MR and FDG-PET. Previously, x-ray survey was the gold standard, but up to 25% can be negative in the presence of myelomatous lesions, and its sensitivity is markedly decreased in extramedullary lesions. The advantage of FDG-PET compare to CT or MR, is the ability to discriminate between necrotic or scar tissues and new or old active lesions. It is especially valuable in the evaluation of recurrences and non-secreting myeloma. *Aims*. to evaluate the utility of FDG-PET in MM compare to conventional imaging techniques (CIT). *Methods*. Descriptive analysis of 41 patients with MM, MGUS, and plasmocytomas, and imaging results in the diagnosis and treatment. *Results*. 71 FDG-PET, 25 MR and 36 CT in 41 patients also several X-ray series and bone scintigraphies were performed. When compare to CIT, FDG-PET revealed understaging in 15 patients. In four patients initial staging was change from I to III, and five were up-staged from II to III. The other six showed higher number of lesions but without changing the stage. Interestingly one patient was FDG-PET positive, but in complete remission (CR) considering the rest of diagnostic tools. Myelomatous infiltration was histologically proven. Post treatment FDG-PET was negative. FDG-PET was negative in all eleven patients in CR and the four cases in CR after ASCT. In patients with partial response FDG-PET showed a decrease in the number and intensity

uptake of the lesions. There were seven patients with positive consistent RM, CT, bone scintigraphy and FDG-PET, but in this the number and extension of the lesions were lower, been able to discriminate active from necrotic, scar tissue. All patients with active disease were FDG-PET positive, including a postransplant relapse. When MGUS patients were evaluated, they all were FDG-PET negative. Interestingly there was a patient with a left maxilar plasmocytoma in which CT was the one which defined local disease compare to non specific focal activity described in the FDG-PET. *Summary*. It is necessary to integrate the novel diagnostic tools in the diagnosis and follow up of MM. New Durie-Salmon Plus staging system integrates FDG-PET and MR information in the evaluation of MM. It is essential for further prognostic and therapy evaluation. Like previously published in other series, FDG-PET studies are positive in all active MM and plasmocytomas. It significantly increases the number of lesions and extension of the disease in some cases that conducted to changes in the staging and therapy approach of several patients. It also discriminate the nature of non defined lesions, excluding myelomatous infiltration in some cases. When partial response to treatment occur a decrease of FDG-PET uptake was observed in the lesions. Additionally, all cases with complete response and the case of MGUS were FDG-PET negative.

0962

AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN 5 PATIENTS AFFECTED BY POEMS SYNDROME

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Background. POEMS is a multisystemic paraneoplastic syndrome, the acronym refers to Polyneuropathy, Organomegaly, Endocrinopathy, M protein, Skin changes. This disease is progressive and weakening for patients and lead to death generally for neurological problem without therapy. *Aims and Methods*. We treated 5 pts affected by POEMS syndrome with high dose chemotherapy and autologous peripheral blood stem cell transplantation (aPBSCT). Pts were 3 male and 2 female, median age 54ys (range 44-62). All pts had a severe, rapidly progressive sensory-motor peripheral neuropathy, involving extremities, with inability to walk. All pts had M component IgA-lambda and 1 had also M component IgG-lambda, all had plasmacytosis (7-10%) in bone marrow. Endocrinopathy was present in all pts as thyroid disease and in 1 patient as hypogonadotropic Hypogonadism, in 1 pts as hypophysary adenoma, in 1 pts as glucose intolerance. All pts presented melanosis. 2 patient had splenomegaly, and 3 hepatomegaly. A patient had sclerotic bone lesion. One patient had significantly abnormal pulmonary function before aPBSCT. All pts received Cyclophosphamide 1500 mg/m^2 on day 1,3 and Methylprednisolon 250 mg from day 1-4 for 2 cycles and G-CSF 5 mcg/kg/day was added after 2° cycle for mobilization. 3 pts were previously treated with high dose Ig i.v. and steroids in the neurologic unit. Time from diagnosis to aPBSCT was 4 months (range 3-7). Conditioning regimen was HDMel (Melphalan 100 mg/m^2 for 2 consecutive days). *Results*. Engraftment was rapid and sustained. After a median follow-up of 57 months (range 5-69), all pts are alive with slow but progressive improvement in neurological disease, skin changes, organomegaly, performance status and without evidence of plasmacytosis. Negativization of M component was observed in all patients. Pt with sclerotic bone lesion also received radiotherapy. VEGF level was only performed after aPBSCT, but in our results (see Table 1) this parameter was not correlated with clinical and laboratoristics improvement.

Table 1.

	M component	VEGF value (pg/ml) **
Patient 1	Absent	1701
Patient 2	Absent	1653
Patient 3	Absent	1433
Patient 4	Absent	2400
Patient 5	Absent	12024
Median value		1701

**normal range < 1000 pg/ml; doubt 1000-1500 pg/ml; positive >1500 pg/ml.

Conclusions. Our experience confirms that HDMel and aPBSCT is feasible and efficacious and should be the treatment of choice for POEMS, arresting and even reversing the disease course. Early diagnosis is important to obtain best response and improve clinical outcome. aPBSCT might be safety be performed at experienced transplant centres combined to neurological expertise. We did not observe correlation between VEGF level and clinical improvement, but this data should be confirmed in the follow-up to clarify the role of bevacizumab, anti-VEGF antibody, as new therapeutic option for patients who can not perform transplant or relapse after aPBSCT.

0963

CONTRIBUTION OF THALIDOMIDE MAINTENANCE TO LONG TERM RESULTS OF AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH MULTIPLE MYELOMA

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Background. High-dose chemotherapy followed by autologous cell transplantation (ASCT) improves survival in patients with multiple myeloma (MM); however, disease progression after ASCT remains to be problem. Progression free survival (PFS) is shorter in patients who are in partial response (PR) after ASCT compared to the ones achieving complete response (CR). New treatment approaches are required to improve PFS and overall survival (OS). New treatment modalities including maintenance treatment may be beneficial in patients with less than CR. *Aims*. We aimed to analyse contribution to PFS and OS of Thalidomide maintenance treatment in myeloma patients, with a response less than CR after ASCT. *Methods*. Forty six patients [30 male, 16 female; median age 55 (32-71)] who received melphalan 200 mg/m^2 followed by ASCT were enrolled to this study. Median follow-up was 18 (6-65) months. Patients were divided in to three groups; CR,T(-) patients: Patients who achieved CR, 3 months status post transplantation (n=20), who did not require any maintenance; PR,T(+) (n=18): disease status at the third month post-transplant was PR, Thalidomide at 100 mg/day was started at day 100 post-transplantation and PR,T(-) (n=8) patients who were not treated with any maintenance treatment despite a disease response less than CR. *Results*. In CR, T(-) group, 8 patients continued in CR and relapse developed in 12 patients, PFS was 12 (4-65) months. In PR, T(+) group, median Thalidomide maintenance treatment was 12 (4-32) months. The best response under Talidomide treatment was CR in 9 patients, PR in 8 patients, and progressive disease (PD) in 1 patient. During maintenance treatment 10 patients preserved their best disease status, but relapse from CR was observed in 3 patients and PD was identified in 5 patients. PFS was 18(0-48) months. In PR, T(-) group, 4 patients preserved disease status achieved after transplantation, but PD developed in 4 patients. PFS was 12.5 (6-29) months. PFS probabilities were not different between CR, T(-) group (37.0%) and PR, T(+) group (32.9%) (p>0.05). In PR, T(-) group, PFS calculated at first 2 years was lower (46.8%) than PR, T(+) group (74.2%), without statistically significance (p>0.05). Overall survival probability was calculated 85.7%, 94.4%, and 26.7 in CR, T(-), PR, T(+), and PR, T(-) groups, respectively. OS in PR, T(+) patients was not different from CR, T(-) patients (p>0.05), and significantly higher than PR, T(-) patients (p=0.02). *Conclusions*. We found that maintenance treatment with thalidomide after high-dose chemotherapy improves the response rate, and the overall survival in patients with myeloma. In post-transplantation long term period, the patients with PR who received Thalidomide maintenance had a similar PFS and OS ratios to patients in CR. Thalidomide may be a logical approach after post-ASCT setting for treatment of residual disease.

0964

SERUM DKK1 IN SMOLDERING MULTIPLE MYELOMA

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Background. Dickkopf-1(DKK-1) gene and its protein act as Wnt-sig-

nalling inhibitor in Multiple Myeloma (MM). It has been related with inhibition of osteoblastic function and differentiation. Previously, our group has communicated that the serum concentration of this parameter is higher in MM patients than in Monoclonal Gammopathy of Unknown Significance (MGUS), and is related to the bone lesion in MM. The behavior of DKK1 has not been described in the set of Smoldering Multiple Myeloma (sMM). *Aims*. To study serum DKK1 in sMM patients, comparing it with a series of MM and MGUS patients diagnosed at the same time. *Material and Methods*. 64 MM, 36 sMM y 53 MGUS diagnosed at Hospitals of Castilla-León-Spain between 2003 and 2008. Bone involvement in MM patients was evaluated by conventional Rx; patients were classified in two groups: without lyses (osteoporosis or no bone disease) and with lyses (some lytic lesion or pathological fracture no vertebral). Serum samples were collected at diagnosis and stored at -80ºC before three hours. Serum DKK1 was measured by double-sandwich enzimoimmunoassay (EIA), standardized in our laboratory. We also analyzed CTX (β-crosslaps) in Elecsys' analizator and bone Álkaline Phosphatase (bAP), MIP1 α, OPG and sRANKL by EIA. Results are expressed by median of the values. *Statistical methods*. Non-parametric test (Mann-Whitney U, Kruskal-Wallis and Spearman correlation). *Results*. Serum DKK1 was higher in MM patients (32.5 ng/mL) compared to sMM patients (24.4), and also more elevated in sMM than in MGUS patients (20.6), although no comparison was statistically significant. As previously described, the comparison between MM and MGUS was significant (p<0.01). When analyzed separately, serum DKK1 in MM patients without lytic lesion (n=26) or with lyses (n=37) vs. sMM patients, no significant differences were observed. We analyzed possible correlations between serum DKK1 and the other cytokines, chemokines and biochemical markers, all related with bone disease, in each diagnostic group. Only significant direct correlation between DKK! and bAP was observed in MM patients. *Conclusions*. Serum DKK1 in sMM patients showed values between MM and MGUS patients, but the differences were not statistically significant. This would be consistent with a intermediate biological behaviour regarding bone disease.

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0965

ELEVETED SERUM B-LYMPHOCYTE STIMULATOR LEVELS IN DIAGNOSIS ARE RELATED TO DISEASE AGGRESSIVENESS AND ADVERSE SURVIVAL IN MULTIPLE MYELOMA

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Multiple Myeloma (MM) is characterized by the uncontrolled proliferation of monoclonal Plasma Cells. Numerous cytokines participate in the interactions between Plasma Cells and the medullary milleu. B-Lymphocyte-Stimulator (BLyS) is a member of the TNF-superfamily that is important in B-cell differentiation and immunoglobulin production. It has been found of prognostic value in Lymphomas and other Plasma Cell dyskrasias. The aim of the present study was to investigate the relationship of serum BlyS levels in MM patients at the time of diagnosis with disease aggressiveness and survival.88 MM patients were included in our study. Median age was 67 years (42 -84 years). 55% were men, 18,5% were staged I according to Durie and Salmon, 32,5% II and 49% III respectively while 22% were staged I according to ISS, 25% II and 53% III respectively. Myeloma type was IgG in 65,5%, IgA in 19% and light chain in 15,5%. 13% of the patients presented with renal failure, 32% had haemoglobin levels under 10 g/dL and thrombocytopenia (PLT<100×10^9/L) was observed in 4%. Serum LDH and CRP were abnormal in 15% and 47% of the patients respectively. 46% of the patients had bone marrow infiltration over 50% while 28% presented with extensive bone disease. Serum BLyS levels were determined by ELISA (R&D Systems, Quantikine) in duplicate measured in frozen sera drawn at the time of the diagnosis. Values under the median were defined as "low". Statistical analysis was performed by standard methods. Serum BLyS levels ranged from undetectable to 921 pg/mL (median 122 pg/mL). High BLyS levels correlated with low albumin (p=0.037) and high LDH (p=0.012) and had significantly reduced 5-year-survival (50% vs. 82%, p=0.044). No other correlations were found. In conclusion high serum BLyS levels at the time point of the diagnosis are related to aggressive disease and represent an adverse prognostic factor in Myeloma patients. Our findings may be of special interest considering the recent development of anti-BLyS agents.

0966

EPIDEMIOLOGY OF BISPHOSPHONATE USE AND JAW OSTEONECROSIS INCIDENCE IN MULTIPLE MYELOMA PATIENTS IN THE REPUBLIC OF IRELAND

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Background. Osteonecrosis of the jaw (ONJ) is a recently described complication of bisphosphonate therapy. *Aims*. To audit epidemiology of bisphosphonate use and ONJ incidence in multiple myeloma (MM) patients in the Republic of Ireland (ROI). *Methods*. Questionnaires on bisphosphonate use, ONJ incidence and management were sent to the 15 haematology departments in ROI treating MM. *Results*. We received replies from 11 centres. 456 MM patients currently attend these centres with 127 new cases of MM in the last year. 5/11 centres have a written policy on bisphosphonate use. Zoledronic acid is used in 9 centres, pamidronate is used in 7 centres, clodronate is used in 3 centres and ibandronate is used in 1 centre. Patients in 10/11 centres undergo a dental review prior to commencing bisphosphonates. All centres use bisphosphonates for at least 2 years, 6 centres stop at 2 years if MM is stable, 3 reduce to 3 monthly dosing after 2 years and 2 continue monthly dosing indefinitely. 10/11 centres stop bisphosphonates prior to any dental work. 6 centres stop bisphosphonates 1 month prior to dental work, the remainder stopping between 6 weeks and 3 months prior to dental treatment. 6 centres restart bisphosphonates once healing is complete following dental work. 4 other centres specified a time for restarting bisphosphonates ranging from 1-6 months. There have been 24 ONJ cases in MM patients attending these 11 centres. We have data for 21 of these cases. The median age is 62 years (range 43-78 years). The median time since diagnosis of MM at presentation of ONJ was 36 months (range 5-108 months). 14 cases had IgG MM, 3 cases had IgA MM, 4 cases had non secretory MM; none had light chain only MM. 18 patients were on zoledronic acid at the time of diagnosis of ONJ, 3 patients were on pamidronate. The median duration of bisphosphonate use at the time of diagnosis of ONJ was 24 months (range 4-60 months). 13 of the patients were on myeloma treatment at the time of ONJ diagnosis and 8 were on no treatment at that time. 9 patients were on thalidomide, 9 patients were on dexamethasone, 2 patients were on prednisolone, 2 patients were on bortezomib and 1 patient was on melphalan. 9 patients had previously undergone an autologous stem cell transplant prior to ONJ diagnosis. 5/21 cases of ONJ were preceded by dental work. A further case was preceded by a broken tooth. The diagnosis of ONJ was based on clinical findings alone in 6 cases. Radiology was required in the other 15 cases. A histological diagnosis was made in 4 cases. In all cases the bisphosphonate was stopped once ONJ was diagnosed. Antibiotics were administered in 12 cases. Surgery was required in 4 cases. In 2 cases the bisphosphonate was restarted again after ONJ diagnosis. *Conclusions*. There is great variation in bisphosphonate use in MM in ROI. Evidence based national guidelines on bisphosphonate use may help to maximize their benefits in MM whilst limiting their side effects.

0967

THE ASSOCIATION BETWEEN RESVERATROL WITH SIMVASTATIN DECREASES IGM SECRETION IN WALDENSTROM MACROGLOBULINAEMIA

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Background. Waldenström macroglobulinemia (WM) is a distinct B-cell lymphoproliferative disorder characterized by lymphoplasmacytic bone marrow infiltration along with an immunoglobulin M (IgM) monoclonal gammopathy. Despite advances in therapy, WM remains incurable, with 5-6 years median overall survival of patients in symptomatic WM. Therapy is postponed for asymptomatic patients, and progressive anemia is the most common indication for initiation of treatment. Resveratrol (3,4',5-tri-hydroxy-trans-stilbene) is an antioxidant constituent of a wide variety of plant species including grapes. It has gained considerable attention because of its anticancer properties, as shown in solid and hematologic malignancies. Published data show that resveratrol has significant antitumor activity in WM cells line. Moreover, simvastatin, a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor, induced inhibition of proliferation, cytotoxic effect and apoptosis in IgM secreting cell lines as well as in primary CD19$^+$ WM cells. With this background we have treated 4 patients with asymptomatic WM with an association of simvastatin and resveratrol. Aims of this study is to test the efficacy of resveratrol plus simvastatin in asymptomatic Waldenstrom macroglobulinemia. *Methods*. 4 male pts, median age 59.2 yr (range, 42-73) and asymptomatic WM were treated with a schedule containing resveratrol 40 mg/die and simvastatin 20 mg/die for 90 days.At enrollment patients characteristic were hemoglobin level median, 12.1 g/dL, serum β2-microglobulin level median, 2.4 mg/L, and IgM peaks median, 1.8 g/dL. All patients have taken regularly the drugs and there have been no adverse events.CK and LDH serum levels were kept in the normal range. *Results*. In all patients a reduction of more than 50% of the IgM peak was observed after 3 months of therapy and is still maintained at 6 months of foll. *Conclusions*. Our data demonstrate clearly that the association between resveratrol with simvastatin decreases IgM secretion in Waldenstrom macroglobulinaemia and and can be useful in asymptomatic or low risk patients not having any adverse effects.

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THE CLINICAL SIGNIFICANCE OF TIMP-1, TIMP-2, MMP-2, MMP-9, OSTEOPROTEGERIN, SRANKL, AND ISS IN MULTIPLE MYELOMA PATIENTS

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Background. Multiple myeloma (MM) is an incurable disease and the outcome highly variable. The ISS is the most useful system based on serum β2-microglobulin and albumin levels. However, this system does not include the evaluation of bone involvement by the disease that is characteristic in MM patients. The OPG/ RANKL system is a critical regulator of bone metabolism. Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases that are inhibited by tissue inhibitors of metalloproteinase (TIMP). MMP-9 is secreted by MM cell lines and MM cells, and may enhance bone absorption in a direct paracrine fashion. Objectives: a) to compare levels of sRANKL, OPG, MMP-2, MMP-9, TIMP-1, TIMP-2 between newly diagnosed multiple myeloma (MM) patients and healthy controls; b) to study the relationship between these parameters and diverse stages of disease according the ISS; and c) to evaluate the relationship between these parameters and bone disease. *Methods*. We studied 38 newly diagnosed and untreated MM patients (24males). The median age was 61years-old (range 39-91). The International Myeloma Working Group criteria and ISS were used to classify patients. Bone involvement was graded according to standard X-ray evaluation into two scores: low score including patients with no lesions, until two bones involved or diffuse osteoporosis and high score including patients with lesions in more than two bones or presence of bone fracture. Ten healthy volunteers were used as controls. MMP-2 and MMP-9 were determined by PAGE gelatin zymography from plasma as previously described. MMP-9 was also measured by ELISA using commercial assay. TIMP-1 and TIMP-2, OPG and sRANKL concentrations were also measured by ELISA. Comparisons among groups were analyzed by ANOVA and the correlation by the Spearman's correlation coefficient. $p<0.05$ were considered to be statistically significance. *Results*. Patients with MM had elevated TIMP-1 and TIMP-2 values compared with controls ($p<0.0001$). Plasma OPG concentration was significantly higher in MM patients compared with controls ($p=0.0012$). No significant difference was found between plasma sRANKL, pro-MMP2, pro-MMP9 and MMP-9 (detected by ELISA) in MM patients and controls. Plasma TIMP-1 correlated positively with OPG (r: 0.64, $p<0.0001$). There were a clearly statistical difference between TIMP-1 and TIMP-2 values and different staging groups ($p=0.0002$, $p=0.0046$, respectively). Plasma levels of OPG were significantly higher in stage III in comparison to the stage I and II ($p=0.012$, $p=0.032$), while no significant difference was found between stage I and II. None statistic significantly relationship was found between patients with low or high score and the measured parameters. For sRANKL, MMP-2 and MMP-9, no difference between patients and controls was detected and no increase of levels was demonstrated with the progression of the disease. We also found a positive correlation between TIMP-1 and OPG, TIMP-1 and serum calcium, and TIMP-2 and serum calcium. None of parameters that we analyzed were influenced by bone disease status. *Conclusions*. We described for the first time in the literature that the values of TIMP-1 and TIMP-2 are increased in MM patients compared with controls and there is a strong relationship between the prognostic staging (ISS) and the levels measured. So, the evaluation of TIMP-1 and TIMP-2 can be considered a prognostic marker in newly diagnosed MM patients.

0969

EVALUATING THE POTENTIAL USE OF RISK CATEGORISATION IN THE MANAGEMENT OF MGUS

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Background. Monoclonal gammopathy of uncertain significance (MGUS) is an increasingly common condition requiring lifelong follow-up due to the risk of progression to a plasma-cell malignancy. Currently, many patients remain under follow-up by haematologists in secondary care clinics. In recent years, a risk stratification model has been proposed which may enable targeted follow-up of high-risk patients.[1] *Aims*. To evaluate the potential use of risk categorisation in the follow-up of MGUS. *Methods*. In the Swansea/Neath Port Talbot region of Wales (population approximately 350,000), all patients with a paraproteinaemia attending haematology clinics between 2003 and 2007 were identified. Clinic letters and laboratory reports were used to ascertain patient diagnosis and obtain results of routine follow-up investigations. MGUS patients who had not progressed to a malignant condition and had undergone serum free light chain (FLC) ratio investigation were identified and risk categorised into four risk groups based on the presence of risk factors (low-risk= 0 risk factors, high-risk= 3 risk factors).[1] The risk factors are: non-IgG MGUS, M Paraprotein>15g/L and an abnormal FLC ratio (<0.26 or >1.65)[1]. A random sample of 40 patients from the high-intermediate and low risk groups, with a follow-up of >2 years, were further investigated to find the number of clinic visits during their follow-up. *Results*. 374 patients had MGUS at presentation, corresponding to 61% of identified paraproteinaemias (n=617). Mean age at presentation was 71 years (SD±12). 342 MGUS patients attended haematology clinics on more than one occasion. The median follow-up was 3.4 years, with a total follow-up of 1292 person-years. Progression to active myeloma/ plasmacytoma occurred in 3.8% (13 cases) whereas progression to other plasma-cell related malignancies occurred in 0.9% (3 cases). In addition, 6 patients progressed to asymptomatic myeloma (indolent/ smouldering myeloma). A group of 147 MGUS patients who had not progressed to malignancy were risk categorised (Figure 1). 31% of patients had no risk factors (low-risk), 47% had 1 risk factor (low-intermediate risk), 21% had 3 risk factors (high-intermediate risk) and 1 patient had all 3 risk factors (high-risk). On average, the frequency of clinic visits was equivalent to one visit every 19 weeks for low-risk patients and one visit every 18 weeks for the high-intermediate risk patients. *Summary and Conclusions*. Using the recently proposed MGUS risk stratification model, we have found that the majority of MGUS patients attending haematology clinics on a frequent basis are in the lower risk categories, which are associated with a low rate of MGUS progression.[1] Furthermore, on average, patients belonging to the lowest risk group (with no risk factors) are being followed up almost as frequently as patients in the high-intermediate risk group (with 2 risk factors). Since routine secondary care follow-up of patients belonging to the lowest risk group may not be necessary[1], implementation of the risk categorisation model would

reduce the number of patients followed-up in clinics and hence reduce costs while helping to free up resources. Lower risk patients may benefit from the introduction of a new system of follow-up e.g. nurse-led clinics.

Reference

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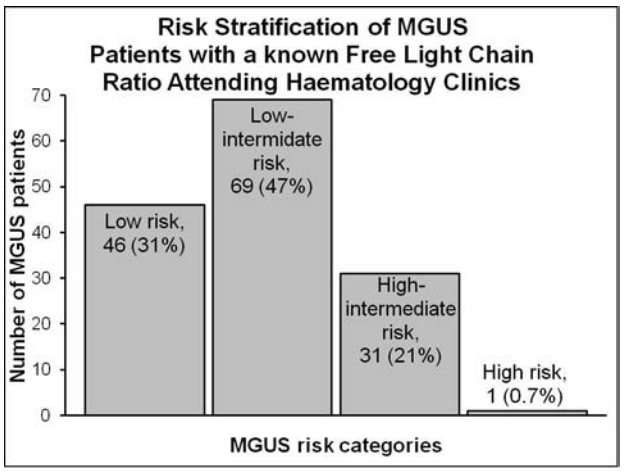

Figure 1. Number of MGUS patients in each risk category.

Non-Hodgkin lymphoma - Clinical III

0970

EFFICATIOUS BUT INSIDIOUS. A RETROSPECTIVE ANALYSIS OF FLUDARABINE-INDUCED MYELOTOXICITY USING LONG-TERM CULTURES -INITIATING CELLS IN 100 FOLLICULAR LYMPHOMA PATIENTS

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Background. Fludarabine alone or in combination has been recognized as effective treatment in patients with follicular lymphoma (FL), but can also induce a substantial long-term myelotoxicity. The mechanism of this toxicity has never been studied. *Aims*. To elucidate which compartment of hematopoietic precursors is damaged or not after different chemotherapy. *Methods*. Hundred FL patients were analyzed in this retrospective study. Median age was 55 (32-75) years, 40% had bone marrow involvement and 21 patients were relapsed (1-4 previous course of therapy; 85%immuno/chemotherapy). Myelotoxicity was assessed by cultivation of two types of hematopoietic progenitor cells: colony forming units- granulocyte-macrophage (CFU-GM) and long-term cultures-initiating cells (LTC-IC). The pre-treatment amounts of CFU-GM and LTC-IC were related to age, sex, and stage of disease, bone marrow involvement and previous therapy. The post-treatment comparison of CFU-GM and LTC-IC was performed among different regimens: fludarabine-based (R±FND), procarbazine-based (R±COPP) and R±CHOP-like therapy. *Results*. At baseline, a total number of progenitor hematopoietic cells in both types of cultures varied in wide ranges; LTC-IC of 0- 874 with median 77.71/mL and CFU-GM of 0-531 with median $30{,}58\times10^2$ /mL. Bone marrow involvement, sex, stage of disease or previous therapy had no influence on LTC-IC and CFU-GM counts. Interestingly, we identified an increase in numbers of LTC-IC but not of CFU-GM positively associated with age (p=0.01). In remission (85 pts), the post-treatment numbers of CFU-GM and LTC-IC dropped significantly after R±FND (n=28) and R±COPP (n=16) but not after R±CHOP-like therapy (n=41) compared to baseline value (p<0.01); using paired comparison of samples the differences in LTC-IC only were found to be significant (p=0.019 and p=0.013, respectively). *Conclusions*. Fludarabine as well as procarbazine reduced significantly numbers of LTC-IC in follicular lymphoma patients, which could reflect the impairment of the most immature hematopoietic cells. Even if the both of drugs impair hematopoietic stem cell, regarding its popularity, myelotoxicity of fludarabine should be emphasized. This finding is consistent with clinical observations (poor mobilization and long-term cytopenias) and offers an insight into mechanism of myelotoxicity caused by these drugs in relatively well defined group of FL. We can also hypothetize, the increased number of LTC-IC in elderly patients could be compensatory mechanism of decreasing one-cell function related to aging.

0971

IN MANTLE CELL LYMPHOMA (MCL) THE MCL-INTERNATIONAL PROGNOSTIC INDEX (MIPI) IS A BETTER PREDICTOR OF EVENT-FREE AND OVERALL SURVIVAL THAN INTERNATIONAL PROGNOSTIC INDEX (IPI) IN PATIENTS RECEIVING INTENSIVE 1ST LINE TREATMENT INCLUDING ASCT

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Background. The MIPI has been shown to be a better predictor of survival in MCL following conventional treatment than the IPI.[1] It is not known if MIPI can also predict outcome in patients receiving intensive immunochemotherapy with stem cell support. *Aims*. To validate the MIPI in the Nordic MCL2 protocol, in which 160 patients, after giving informed consent, received intensive 1st-line induction therapy of Rituximab (R) + CHOP (R-CHOP) alternating with R-AraC, followed by consolidation with high-dose chemotherapy with BEAM or BEAC + autologous stem cell support.[2] *Methods*. Data allowing MIPI analysis

were available in 155 patients. We compared IPI, MIPI and simplified MIPI (s-MIPI). Regarding the IPI, since the curves of high-intermediate and high risk groups were identical, these were merged into one group. *Results*. MIPI and s-MIPI predicted survival better (p<0.0001) than the IPI (p<0.002). MIPI and s-MIPI did not identify the same patient subgroups, however (Figure 1). By the MIPI most patients were in the low risk group, whereas by the s-MIPI most patients were in the intermediate risk group. The MIPI discriminated significantly between low and intermediate, and intermediate and high risk groups, respectively (p=0.04 and <0.02 respectively), whereas the s-MIPI could not distinguish significantly between low and intermediate risk groups (p=0.13). Regarding vent-free survival, the IPI did not identify significant differences, (p=0.66) in contrast to MIPI (p=0.01) and s-MIPI (p=0.03). The addition to the MIPI of the % Ki-67-positive cells multiplied by its Cox analysis β value (0.02124), did not add further information as it moved almost half (47%) of the patients to the high risk group. *Conclusions*. We confirm that also in MCL patients receiving intensive immunochemotherapy incl. ASCT, MIPI and s-MIPI predict survival better than IPI. However, MIPI and s-MIPI did not group the patients identically. Although MIPI was more discriminative than s-MIPI, the simplicity of s-MIPI may make it preferable.

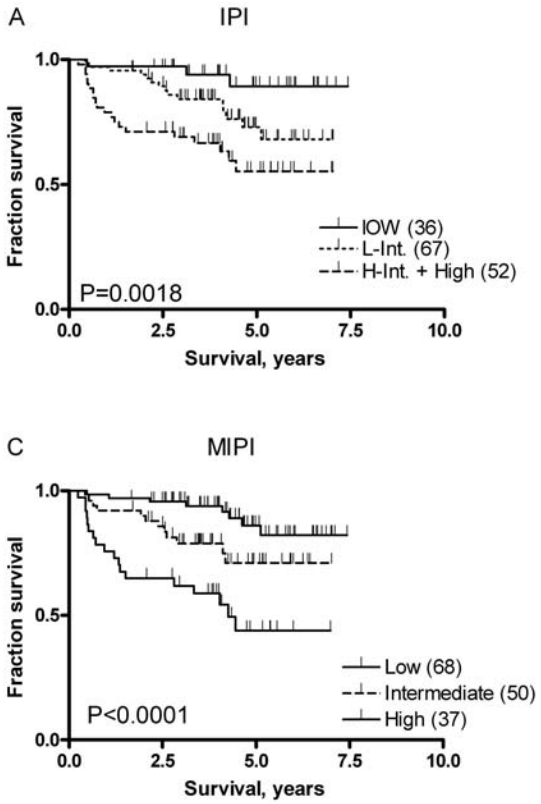

Figure 1. Survival Nodic MCL-2: IPI, MIPI and simplified MIP.

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0972

RETREATMENT WITH RITUXIMAB IN 178 PATIENTS WITH RELAPSED AND REFRACTORY B-CELL LYMPHOMAS: A SINGLE INSTITUTION STUDY

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Background. The role of rituximab retreatment in patients who have responded to a previous course of rituximab is not well known. For patients who relapse after initial rituximab treatment an important clinical issue is the efficacy of retreatment with rituximab at the time of relapse. *Aims*. To investigate the efficacy and toxicity of retreatment with rituximab with or without chemotherapy in patients with relapsed and refractory B-cell lymphomas. *Methods*. This was a single centre retrospective cohort study. We included patients with relapsed and refractory B-cell lymphomas treated in first line and in first progression with rituximab, with or without chemotherapy. We also attempted to put the results into context by conducting a comparison of the rituximab-retreated patients with a group of matched historical control patients who had not received rituximab in first or second-line therapy but had received approximately identical chemotherapy. *Results*. 178 rituximab-retreated patients were included in the study of which 29% had diffuse large B-cell lymphoma and 28% had follicular lymphoma. The remainder of patients had a variety of B-cell lymphomas. The overall response rate for the first treatment was 81% and for the second treatment was 66%. The median progression-free survival from diagnosis (PFS1) was 13 months and from relapse (PFS2) was 18 months (not statistically different). The 5-year overall survival was 57%. No significant toxicities were identified and the yield of stem cell collection was acceptable in this population. 301 historical controls were identified from the same institution who were well matched with the patients for age, histological subtype and proportion treated with high dose therapy (p>0.05). There was a slight excess of males in the control group and more patients in the rituximab-retreated group had a high or high-intermediate second-line International Prognostic Index. Compared to the historical control patients, the rituximab-retreated patients had a shorter PFS1 (90% progressed at 3 years compared to 78% in the control group, p<0.001). There were no differences between the two groups for PFS2. Despite this, the 5-year overall survival for the rituximab-retreated group was superior (57% versus 40%, p<0.02). *Conclusions*. In conclusion, retreatment with rituximab, with or without chemotherapy, in patients having previously received rituximab yields a high overall response rate in patients with relapsed and refractory B-cell lymphomas. The outcome of retreatment, in terms of progression-free survival, is not inferior to that of primary treatment. Relapse occurring after rituximab-containing therapy may be more aggressive than that occurring after chemotherapy alone.

0973

TEMSIROLIMUS FOR THE TREATMENT OF PATIENTS WITH RELAPSED OR REFRACTORY MANTLE CELL LYMPHOMA: SUPPORTIVE EFFICACY ANALYSES FROM THE PHASE 3 STUDY

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Background. Temsirolimus (Torisel®) is a specific inhibitor of the mTOR kinase and is approved in Europe for the first-line treatment of patients with advanced renal cell carcinoma who have at least 3 of 6 poor-prognostic risk factors. Temsirolimus also was evaluated in patients with relapsed or refractory mantle cell lymphoma (MCL). In a phase 3, randomized, open-label study, patients treated with temsirolimus 175 mg weekly 3 times followed by 75 mg weekly (175/75-mg) had significantly longer progression-free survival (PFS) than those treated with investigator's choice therapy (p value temsirolimus: investigator's choice = 0.0009; hazard ratio = 0.44; 97.5% CI = 0.25, 0.78; Hess *et al.* J Clin Oncol. 2008, 26:abs 8513). Patients treated with temsirolimus 175 mg weekly 3 times followed by 25 mg weekly (175/25-mg) showed a trend towards longer PFS than those treated with investigator's choice therapy (p=0.0618; hazard ratio = 0.65; 97.5% CI = 0.39, 1.10). *Aims*. These results were obtained for the intent-to-treat population, which included all ran-

domized patients (n=54 for both temsirolimus groups and the investigator's choice group). We now report the results of sensitivity analyses for the recommended temsirolimus dose, 175/75-mg, compared with those for investigator's choice therapy. *Methods*. The primary endpoint of the study was PFS, the time from the date of randomization to the earlier date of either progressive disease (PD) or death from any cause, if within 4 months of the last valid tumor assessment (per FDA guidance), censored at that assessment. Progression was assessed by independent review of radiographic and clinical data. Progression-free survival was analyzed by Kaplan-Meier estimates and an unstratified Cox proportional hazards model. All patients provided written informed consent. *Results*. Sensitivity analyses for PFS included: 1) evaluable population: those who remained on treatment for at least 8 weeks and did not discontinue early for PD or death, had no major protocol violations, and had at least 1 screening tumor assessment and at least 1 postbaseline independent tumor assessment; 2) all deaths: those patients who had PD or died at any time during the study; 3) all deaths + withdrawal from therapy + initiation of anticancer therapy: those patients who had PD, died, or stopped treatment because of withdrawal from therapy or initiation of other anticancer therapy; and 4) all deaths, excluding patients with blastoid morphology. The latter analysis was performed because 0 patients in the 175/75-mg group and 4 patients in the investigator's choice group had blastoid morphology. The characteristics of PFS for the 4 sensitivity analyses are shown (Table). For each analysis, PFS was significantly longer for the patients treated with temsirolimus 175/75-mg than for those treated with investigator's choice therapy, consistent with the PFS results for the intent-to-treat population. Conclusions: Based on several analyses, patients with relapsed or refractory MCL treated with temsirolimus 175/75-mg showed significant improvement in PFS compared with those treated with investigator's choice therapy. Additional exploratory analyses will be presented.

Table 1. Sensitivity analyses of Temsirolimus 175/75-mg vs. Investigator's Choice Therapy.

PFS Analysis*	Temsirolimus 175/75-mg		Investigator's Choice		p-Value	Hazard Ratio (95% CI)
	n	Median PFS, mo	n	Median PFS, mo		
Evaluable population	29	5.2	26	1.9	0.0002	0.29 (0.15, 0.57)
All deaths	54	5.2	54	2.0	0.0007	0.46 (0.29, 0.72)
All deaths, excluding pts with blastoid morphology	54	5.2	50	2.1	0.0020	0.48 (0.30, 0.77)
All deaths + withdrawal from therapy + initiation of anticancer therapy	54	2.6	54	0.8	<0.0001	0.43 (0.28, 0.65)

*Independent assessment.

0974

HIGH DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL SUPPORT IN FIRST LINE TREATMENT OF AGGRESSIVE NON HODGKIN LYMPHOMA: RESULTS OF AN INDIVIDUAL PATIENT DATA META-ANALYSIS

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Background. Randomised controlled trials (RCTs) reported conflicting results on the impact of high-dose chemotherapy (HDCT) and autologous stem cell transplantation in the first-line treatment of patients with aggressive non-Hodgkin Lymphoma (NHL). *Aims and Methods*. We performed a meta-analysis based on individual patient data (IPD) to assess the efficacy of HDCT compared to conventional chemotherapy in aggressive NHL patients with regard to overall survival (OS) and progression-free survival (PFS). Furthermore, we wanted to determine the efficacy on the intervention in specific subgroups of patients. Particularly we analysed the impact of the age-adjusted International Prognostic Index (aaIPI). We searched the Cochrane Library, MEDLINE and other databases (1/1990 to 12/2007). The RCTs were conducted mainly without Rituximab. Hazard ratios (HR) with 95% confidence intervals (CIs) were calculated using the Cox proportional hazards model stratified by study. The conventional chemotherapy-arm is taken as reference in the analysis. *Results*. Individual patient data were available from 11 RCTs including 2,132 randomised patients. Information on patient characteristics, treatment, events and survival was collected. Overall, there was no evidence for HDCT to improve OS (HR 1.09; 95% CI 0.95-1.24) or PFS (HR 1.04; 95% CI 0.92-1.17) when compared with conventional chemotherapy. In subgroup analysis hazard ratios for OS was 1.34 (95% CI 0.98-1.82) for good risk patients and 1.01 (95% CI 0.87-1.17) for poor risk patients (p value for interaction = 0.10). Subgroup analysis did not indicate differences in terms of PFS between good (HR 1.07, 95% CI 0.84-1.36) and poor risk (HR 0.99, 95% CI 0.87-1.14) patients (p value for interaction = 0.61). *Summary and Conclusions*. Preliminary analyses suggest that there is no evidence for HDCT to improve OS or PFS in NHL patients compared to conventional chemotherapy. There was no evidence for different treatment outcomes in patients with good or poor IPI risk group. Further results will be presented.

0975

PHARMACOKINETICS OF RITUXIMAB AND THE DEVELOPMENT OF ANTIBODIES AGAINST RITUXIMAB IN PATIENTS WITH A B-CELL CD20+ MALIGNANCY

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Background. Limited information exists about the pharmacokinetics of rituximab in patients with CD20+ B-cell malignancies. In an attempt to increase the therapeutic efficacy, several randomised trials were performed varying the number of infusions, intervals between cycles, duration of treatment, and administration of rituximab in combination with several chemotherapeutic compounds. But still, the optimal dosing regimen has to be elucidated. Additionally, the development of human anti-chimeric antibodies (HACAs) could affect rituximab pharmacokinetics. Although HACAs are observed in patients with auto-immune diseases treated with rituximab, it is rare in patients treated with rituximab for CD20+B-cell malignancies. Most obervations were done in patients during induction, but not on maintenance treatment. It is therefore of interest to determine the presence of HACAs, especially with longer duration of treatment. *Aims*. To get more insight into the pharmacokinetics of rituximab for further improvement of rituximab containing therapies. To assess the development of HACAs against rituximab for patients who are treated with rituximab in combination with chemotherapy or are on maintenance therapy. *Methods*. In this pilot study, patients with a CD20+ B-cell malignancy who were treated with rituximab containing regimens were included. The patients were informed in line with the rules of good clinical practice. Induction treatment schedules consisted of a 2,3,4 weekly schedule of 375 mg/m^2 rituximab intravenously in combination with chemotherapy for 4-8 cycles. Maintenance treatment consisted of a two-three monthly dose of 375 mg/m^2 rituximab intravenously for 2 years. On the day of the treatment with rituximab, preinfusion blood samples were taken. After the end of treatment, selected blood samples were taken. Rituximab levels were measured by enzyme-linked immunosorbent assay (ELISA). The minimum quantifiable concentration was 7.8 ng/mL. An assay for determination of HACAs was designed with a lower limit of quantification of 12 units/mL. *Results*. Eight patients were on induction therapy. Figure 1 shows rituximab levels over the time of treatment. No steady state was reached after four cycles. Trough levels after 4 cycles ranged between 55.7 and 135 μg/mL, with a median of 111 μg/mL. Rituximab levels of 1 patient remained very low after the first course. This patient had a CLL with circulating tumour cells. Apart from 1 patient with mantle cell lymphoma, all patients had a complete response. For all patients, concentration of HACAs was below the quantification limit. Five patients were on maintenance therapy. One patient received two-

monthly maintenance treatment. Trough levels of 4 patients on three-monthly schedule remained constant, with a median concentration of 6 μg/mL (range 0.5-11.7μg/mL). After eight cycles on maintenance therapy, no HACAs were observed. *Summary and Conclusions*. Considerable inter-individual variability of rituximab levels was observed. There were no correlations between rituximab levels, therapy regimen, renal function, solid tumour load and response. For patients with circulating tumour cells, a loading dose could be considered. No HACAs were observed in these patients, which strongly supports the previously described antigen mediated clearance of rituximab.

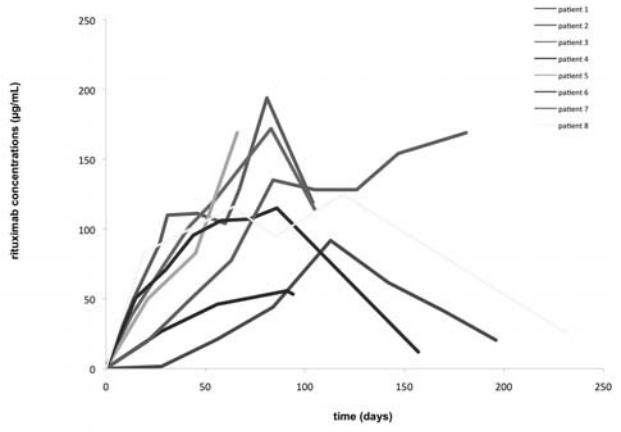

Figure 1. Rituximab levels over the time of treatment

0976

POST-TRANSPLANTATION LYMPHOPROLIFERATIVE DISORDER: REDUCTION OF IMMUNOSUPPRESSION AS INITIAL THERAPY. RETROSPECTIVE ANALYSIS OF CLINICAL CHARACTERISTICS AND PROGNOSTIC VARIABLES

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Background. Post-transplantation lymphoproliferative disorder (PTLD) is a heterogeneous group of lymphoid neoplasms arising in pharmacologically immunosuppressed organ transplant recipients. While it has long been known that reduction of immunosuppression (RI) is an effective therapy, previous reports on RI are of limited value due to small numbers of patients. *Aims*. We sought to estimate outcomes and predictors of outcomes, in patients treated with RI for PTLD. *Methods*. We retrospectively studied 162 organ transplant recipients diagnosed with PTLD at the University of Pennsylvania between 1988 and 2008. Stem-cell transplant recipients were excluded. We analyzed clinical and pathological characteristics, response to therapy, graft outcome and survival of patients treated with RI alone as initial therapy for PTLD in comparison to patients who were treated with other therapies. We used logistic regression to identify predictors of response, and Cox regression to identify predictors of survival, in patients treated with RI. *Results*. 67 patients received RI alone as initial therapy. These patients were similar to those treated with other modalities with respect to demographic parameters, transplanted organ type, immunosuppressive medications, number of previous rejection episodes, pathological subtype, and EBV positivity. 40/67 patients (59.7%) required additional therapy after initial RI; the most common second-line therapies were rituximab (40%), followed by chemotherapy (39%) and radiotherapy (15%). The overall response rate to RI alone was 44% (37% CR, 8% PR), and additional 18% achieved stable disease. Of 23 patients who experienced a CR, only 4 later required therapy for relapsed disease. Median survival was 44 months on RI alone, 24 months on rituximab and 19 months on chemotherapy. We also identified 30 patients who were treated with complete surgical excision of the disease site and received RI as adjuvant therapy. The outcome of these patients was favorable and their median survival was not reached after a mean follow-up of 64 months. In patients treated with RI alone, 41% developed acute allograft rejection. Five of these patients later underwent retransplantation without recurrence of PTLD. Predictors of response to RI: Lack of bulky disease, multiple previous allografting and younger age were associated with better response by univariate analysis. Notably, EBV status, pathological subtype and type of transplanted organ were not associated with significant differences in response to RI. A step-wise multivariate logistic regression analysis confirmed that older age and bulky disease were strong predictors of RI failure. Survival analysis revealed that B symptoms, weight loss, renal failure, dyspnea and liver involvement at diagnosis were associated with worse survival in patients treated with RI alone. *Conclusions*. In our large, single-center series, RI was found to be a powerful tool in controlling PTLD, resulting in responses in 44% of patients, with many patients permanently cured. These findings illustrate once again the role of the immune system in shaping the development of tumors, and support the use of RI, either alone or in combination with other therapies.

Table 1.

Response to RI		
Variable	OR (95% CI)	p-value
>1 previous allograft	0.11 (0.01,0.99)	0.049
Anorexia as initial symptom	3.67 (1.19,11.3)	0.024
Bulky Disease	9.72 (1.15,82.32)	0.037
Survival		
Variable	HR (95% CI)	p-value
Weight loss	2.03 (1.08,3.82)	0.028
B symptoms	2.04 (1.01,4.1)	0.046
Renal failure	2.05 (0.99,4.23)	0.052
Dyspnea	2.68 (1.17,6.13)	0.02
Liver involvement	2.79 (1.26,6.2)	0.012

0977

SERUM B2-MICROGLOBULIN LEVELS IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL): PROGNOSTIC SIGNIFICANCE UNDER TREATMENT WITH RITUXIMAB-CHOP (R-CHOP)

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Background. Elevated serum β2-microglobulin levels have been implicated as a prognostic factor in aggressive non-Hodgkin's lymphomas. However they have not been adopted in current prognostic models, partly due to the high rate of missing data in multicenter studies. Given that the introduction of Rituximab has overcome the adverse significance of several prognostic factors, which had been established in the pre-Rituximab era, the extrapolation of the results of prior studies in the Rituximab era is not scientifically acceptable. Therefore, the prognostic significance of serum β2-microglobulin levels in patients with DLBCL treated with R-CHOP has not been adequately investigated. *Aims*. The determination of serum β2-microglobulin levels and the evaluation of their correlation with other baseline features and the outcome of patients with DLBCL treated with R-CHOP. *Patients and Methods*. We analyzed 344 patients with DLBCL, who were treated with R-CHOP or similar anthracycline-based combinations and had available serum β2-microglobulin levels. Baseline patient characteristics were recorded, 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 (18-88), and 59% were males. The distribution of our patients according to the IPI was as follows: Low Risk (L) 49%, Low-Intermediate (LI) 19%, High-Intermediate (HI) 21% and High Risk (H) 12%. According to the R-IPI, 22% were classified as Low Risk (L), 46% as Intermediate Risk (INT), and 33% as High Risk (H). Elevated serum β2-microglobulin levels (>2.4 mg/L) were recorded in 220 patients (64%), being highly correlated with all baseline patient characteristics studied (individual IPI parameters, B-symptoms, haemoglobin, serum albumin; all p values <0.01) with the exception of gender. The frequency of elevated serum β2-microglobulin levels in patients with L, INT and

H R-IPI was 25%, 64% and 90% (p<0.001). The 5-year FFS was 85% vs 70% in patients with normal and elevated serum β2-microglobulin levels (p=0.006). The corresponding 5-year OS rates were 85% vs 79% (p=0.06). A better prognostic discrimination was achieved at the cutoff of 3.5 mg/L: The 5-year FFS was 83% vs 64% in patients with serum β2-microglobulin levels lower or higher than 3.5 mg/L (p=0.0004), with 5-year OS rates of 85% vs 74% (p=0.01). However the prognostic significance of elevated serum β2-microglobulin levels was not retained in multivariate analysis, where it was overcome by R-IPI or its individual components. Conclusions: Approximately 2/3 of patients with DLBCL had elevated serum β2-microglobulin levels, which were highly correlated with all the other baseline adverse features, including IPI and R-IPI. Irrespectively of the cutoff used, the statistically significant effect of serum β2-microglobulin levels on the outcome of DLBCL treated with R-CHOP, which was demonstrated in univariate analysis, was not retained in multivariate analysis. Thus the combination of Rituximab with CHOP appears to overcome the potential adverse prognostic significance of serum β2-microglobulin levels in DLBCL.

0978

LENALIDOMIDE ORAL MONOTHERAPY IN PATIENTS WITH RELAPSED OR REFRACTORY MANTLE-CELL LYMPHOMA: EFFICACY AND SAFETY RESULTS FROM AN INTERNATIONAL STUDY (NHL-003)

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Background. Mantle cell lymphoma (MCL), which accounts for 5% to 10% of all lymphoid malignancies, is usually characterized by an aggressive course with only short-term remissions after conventional chemotherapy. Historically, the median duration of response ranges between 3 and 4 years indicating a significant unmet therapeutic need to improve patient outcomes. Lenalidomide is an immunomodulatory agent that has demonstrated considerable clinical activity in a variety of hematologic malignancies. A recent sub-analysis of the phase II NHL-002 trial revealed that 15 heavily pretreated patients with advanced MCL achieved an overall response rate (ORR) of 53%, and a median duration of response lasting 13.7 months. A supporting international phase II trial (NHL-003) was initiated to evaluate the clinical utility of single-agent lenalidomide in patients with relapsed or refractory aggressive NHL. Here we present the safety and efficacy results for the MCL subgroup. *Methods*. Key inclusion criteria for patients with relapsed or refractory MCL were ≥1 prior therapy, measurable disease of 2 cm, and an ECOG PS ≤2. The primary endpoint was ORR, and secondary endpoints included duration of response, PFS, and safety. Lenalidomide 25 mg was administered orally once daily on days 1-21 of every 28-day cycle until disease progression or toxicity. Response and progression was measured using the 1999 IWLR Criteria.

Table 1. Response rates with lenalidomide in MCL patients.

Patient sub-group	N	ORR, n (%)	CR/CRu, n (%)	PR, n (%)
MCL	54	**23** (43)	9 (17)	14 (26)
MCL-bortezomib	17	**9** (53)	3 (18)	6 (35)
MCL-stem cell	14	**8** (57)	2 (14)	6 (43)

Results. Fifty-four patients with MCL enrolled on the trial were evaluable for response. Median age for these patients was 69 years (range 33-82), 40 patients (74%) were male, and median time from diagnosis was 3.2 years (range 0.4-10.4). Patients had progressed after a median of 3 prior therapies (range 1-8), which included prior bortezomib for 17 (32%) patients and prior stem cell transplant in 14 (26%) patients. The ORR for the whole MCL cohort was 43%: 9 patients (17%) achieved a complete response (CR) and 14 (26%) achieved a partial response (PR). Responses according to type of prior therapy are summarized in the Table 1. Of the 17 patients who had failed bortezomib, 3 (18%) achieved a CR/CRu and 6 (35%) achieved a PR. Most common grade 3 or 4 adverse events were neutropenia (43%), thrombocytopenia (22%) and anemia (11%). *Conclusions*. Lenalidomide oral monotherapy has demonstrated significant efficacy in treating patients with relapsed or refractory MCL, and has a manageable tolerability profile. These findings are consistent with the previous NHL-002 study, and warrant further investigation of lenalidomide in earlier lines of MCL therapy alone or in combination with other agents.

0979

IMPACT OF TIME-DEPENDENT VARIABLES ON THE OUTCOME OF SPLENIC MARGINAL ZONE LYMPHOMA: A DYNAMIC MODEL ON 84 PATIENTS

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Background. Splenic marginal zone lymphoma (SMZL) is a low grade B-cell lymphoma that represents nearly 2% of lymphoid malignancies. Main manifestations are splenomegaly, lymphocytosis and bone marrow (BM) infiltration. Cytopenias can be present at onset or can develop during the course of disease and are usually a clear indication for treatment. Noteworthy, anemia (defined as hemoglobin level less than 12 g/dL) is one of the 3 parameters included in the SMZL score (Blood 2006). *Aims*. To evaluate the dynamic changes of clinical and laboratory parameters in SMZL and the impact of their modifications on the outcome of disease. *Methods*. From a series of 84 pts with SMZL diagnosed at Division of Hematology of Pavia from 1987 to 2007, we collected clinical and laboratory parameters at onset and at any time during the course of disease. We studied peripheral cytopenias, LDH, β2-microglobulin and serum albumin levels (as categorical respect to normal values and as continuous time-dependent variables), the degree of splenomegaly and BM infiltration (both as continuous variables). Progression-free survival (PFS) was analyzed by univariate and multivariate Cox proportional hazards regression models, with time-dependent covariates. The correlation among clinical and laboratory features was tested with multiple regression model (non-parametric Spearman correlation). *Results*. We studied 84 consecutive pts (38 M, 46 F; median age 62 yrs, range 37-79). Diagnosis of SMZL was established according to WHO classification (2008) and the proposed diagnostic criteria (Leukemia 2008). ECOG PS was 0 in 59%. Twelve pts showed bulky disease. A serum MC was detected in 27 pts (32%). HCV serology was positive in 14 pts (17%). Two pts developed histological shift. 5-years overall survival (OS) was 70% and median PFS was 30 months. In univariate analysis, PFS was influenced at any time during the course of disease by the decrease of hemoglobin (p=0.003) and platelets (p=0.001) and by the increase of LDH levels (p=0.001) and β2-microglobulin levels (p=0.005) (all parameters are predictive as continuous variables). As time-dependent covariates in multivariate Cox model, decrease of platelets (p<0.0001) and raising levels of LDH (p=0.002) are predictive of a shorter PFS. We applied a multiple regression model to evaluate the relationship of cytopenias with other features of SMZL. In this analysis, decrease of platelets is directly related with degree of splenomegaly (p<0.0001), with increase of β2-microglobulin levels (p=0.0001) and reduction of albumin levels (p=0.03). Interestingly, there is no relationship between platelets level and degree of BM infiltration (p=0.1). Levels of hemoglobin and albumin are highly related variables (p=0.0001), but anemia is related neither with splenomegaly (p=0.3) nor with BM infiltration (p=0.1). *Conclusions*. In this dynamic model for SMZL, PFS resulted directly related to time-dependent modifications of lymphoma indexes (LDH, β2-microglobulin) and of hemoglobin and platelets levels. Thrombocytopenia is related to lymphoma mass and its aetiology seems mainly linked to hypersplenism. On the contrary, anemia is not directly caused by hypersplenism or by BM infiltration; its relationship with albumin levels could be explained by a progressive dysregulation of cytokine expression during the course of disease.

0980

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. 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. There is no prospective study result for the treatment of advanced stage MZBCL, mainly because of the rarity of this disorder and some difficulties in the differential diagnosis from other low-grade lymphoma subtypes. This is the first clinical trial for advanced MZBCL ever reported. *Aims*. We conducted this 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. *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 paitents 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 patients (75%) had extranodal sites involvement, 17 (43%) 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. The survival curves will be presented at the conference. *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.

0981

MANTLE CELL INTERNATIONAL PROGNOSTIC INDEX (MIPI) AND BIOLOGICAL-MIPI ARE BETTER PREDICTORS OF THE OUTCOME OF MANTLE CELL LYMPHOMA (MCL) PATIENTS THAN IPI: A RETROSPECTIVE ANALYSIS

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Background. MCL has poor prognosis, with a continuous pattern of relapse and a median Overall Survival (OS) of four years. Clinical MIPI score (including age, performance status, LDH and leukocyte count) seems to be able to give a more reliable estimation of clinical course; cell proliferation (Ki-67) was tested to include a biologic predictive marker in MIPI. *Aims*. To validate MIPI and biological-MIPI on a retrospective group of MCL patients. *Patients and Methods*. Between 1996 and 2008, 102 patients with MCL at diagnosis entered into the study. Clinical characteristics were: median age 62 (37-85) years, 78% stage IV and 17% with blastoid variant. First-line treatments were: high-dose chemoimmunotherapy including Rituximab with autologous stem cell transplantation in 38 patients, Rituximab-Fludarabine based chemotherapy in 18 and Rituximab-CHOP-like in 46. MIPI and biological-MIPI were calculated as published by Hoster 2008. Central pathologist review with Ki-67 evaluation is ongoing. Crude Kaplan-Meier OS and progression-free survival (PFS) curves were estimated both overall and stratified by MIPI, biological-MIPI and IPI score. Differences between curves were tested using the 2-tailed log-rank test. In order to quantify the predictive discrimination of MIPI, biological-MIPI and IPI scores, univariate logistic models (with death and progression event as binary outcomes) were fitted and the area under the receiver operating characteristic (ROC) curves (c-index) was estimated. *Results*. According to MIPI 30 patients (30%) were at low-risk (LR, 0-3), 29 (29%) at intermediate-risk (IR, 4-5), 34 (33%) at high-risk (HR, >5) and 9 missing. Biological-MIPI was calculated on 60 patients with Ki-67 evaluation: 45 patients (75%) were at low-risk (LR, 0-5.699), 6 (10%) at intermediate-risk (IR, 5.7-6.499), 9 (15%) at high-risk (HR, >6.5). According to IPI 29 patients (28%) were at low-risk (LR), 31 (30%) at low-intermediate-risk (LIR), 34 (33%) at intermediate-high and high-risk (IH-HR) and 8 missing. Fifty-two patients achieved a complete-response, 26 a partial-response and 17 did not respond. Failures occurred in 58 patients and 35 of them died, 30 because of lymphoma. With a median follow-up (FU) of 34 months, OS was 69% (95%CI:57%-78%); with a median FU of 28 months, PFS was 61% (95%CI:50%-70%). Thirty-four months OS rates for MIPI were: LR 97%, IR 80%, HR 33% (p<0.00001), for biological-MIPI: LR 89%, IR 75%, HR 26% (p=0.0003) and for IPI: LR 85%, LIR 80%, IH-HR 47% (p=0.0015). Twenty-eight months PFS rates for MIPI were: LR 82%, IR 72%, HR 29% (p<0.00001), for biological-MIPI: LR 82%, IR 28%, HR 22% (p=0.0001) and for IPI: LR 70%, LIR 83%, IH-HR 38% (p=0.0015). MIPI was more predictive than IPI for death event and for failure event: the c-index was 75% and 70% for MIPI, compared to 68% and 65% for IPI respectively. Although assessed on a subsample, biological-MIPI c-index was 71% for death and 67% for failure event. *Conclusions*. in our retrospective series of patients, MIPI and biological-MIPI prognostic scores seem to discriminate among patients with different OS and PFS better than IPI. New therapeutic strategies are warranted to improve the outcome of MCL namely in HR group.

0982

PATIENTS WITH RELAPSED OR REFRACTORY DIFFUSE LARGE-B-CELL LYMPHOMA TREATED WITH ORAL LENALIDOMIDE MONOTHERAPY: RESULTS FROM THE INTERNATIONAL STUDY NHL-003

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Background. Diffuse large-B-cell lymphoma (DLBCL) is the most com-

mon subtype of aggressive lymphoma with considerable biologic and clinical heterogeneity. Despite recent therapeutic advances, up to 50% of patients relapse, and the prognosis remains dismal for those who are not cured with R-CHOP or high-dose chemotherapy with autologous stem cell rescue (SCT). Lenalidomide is an immunomodulatory agent that has demonstrated considerable clinical activity in a variety of hematologic malignancies. In a sub-analysis of a phase II trial (NHL-002) in which 49 patients with relapsed/refractory aggressive NHL received lenalidomide, 26 patients with DLBCL achieved a 19% overall response rate (ORR), and a duration of response lasting a median of 7 months. Here we report on the safety and efficacy results of a subset of patients with DLBCL from the international phase II NHL-003 study, in which patients with relapsed or refractory aggressive NHL who had progressed following ≥1 prior regimen received treatment with single-agent lenalidomide. *Methods*. Oral lenalidomide 25 mg was administered once daily on days 1-21 of every 28-day cycle and continued until disease progression or toxicity. Response and progression was assessed using the 1999 IWLR criteria. *Results*. For the 103 patients with DLBCL who had enrolled and were evaluable for response, the median age was 66 years (21-87), median time from diagnosis was 2 years (0.4-18.6) and 70 patients (68%) of patients were male. Patients had progressed through a median of 3 prior regimens (1-10), and 46 (45%) patients had failed prior SCT. The ORR for the entire patient population was 30% with 7% (7/103) of patients achieving a complete response (CR) and 23% (24/103) of patients a partial response. The ORR was also 30% (14/46) among the 46 patients who had a prior SCT, with 5 patients achieving a CR (11%) and 9 patients a PR (20%). Adverse events were manageable. Most common (>5%) grade 3 or 4 adverse events included neutropenia (34%), thrombocytopenia (18%), asthenia (9%), anemia (8%), leucopenia (7%), back pain (6%) and dyspnea (6%). *Conclusions*. In heavily pretreated patients with relapsed/refractory DLBCL lenalidomide has demonstrated significant clinical activity and a manageable safety profile. Further investigation is warranted to better assess a role for lenalidomide in the treatment of patients with DLBCL.

0983

SURVIVAL IMPACT OF RITUXIMAB COMBINED TO ACVBP (R-ACVBP) IN 209 POOR RISK DLBCL NHL PATIENTS TREATED WITH UPFRONT HIGH-DOSE CONSOLIDATIVE AUTOTRANSPLANTATION (HDC): A GELA PHASE II STUDY

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Rituximab (R) combined with CHOP improves complete remission (CR) rate and overall survival (OS) in DLBCL patients (pts). More intensive regimen followed by auto transplantation have been used in pts < 60y with 2-3 adverse age-adjusted International-Prognostic-Index (aa-IPI) factors, providing a 5y OS of 65% (CI 60-68%), (Haioun LNH 98-3B, ASCO 2007). The objective of the present study was to assess whether or not combining R (375 mg/m^2) to the dose intense ACVBP (Doxorubicin 75 mg/m^2 d1, Cyclophosphamide 1.200 mg/m^2 d1, Vindesine 2 mg/m^2 and Bleomycin 10 mg d1 and d5, prednisone 60 mg/m^2 d1-d5) also translates into a survival benefit. *Methods*. From 01/2004 to 12/2005, 209 DLCBL pts < 60y with DLBCL and aaIPI 2 or 3 received 4 cycles of R-ACVBP every 15 days supported by G-CSF. Responding patients received a consolidative BEAM and peripheral blood stem cell rescue. Median age was 49 years (range: 18-60), 22% of patients presented with aa-IPI 3, 58% with IPI 3-5 (93% with elevated LDH and 54% with extranodal sites >1). Based on International W Criteria, CR + CRu rate after induction was 61%, PR rate 24% leading to an overall response rate of 84% (176 pts). Deaths occurred in 4% of the pts during R-ACVBP procedure and collection failure was observed in 18 pts (10%). 155 pts received auto transplantation, representing 75% of the study population. A case-controlled study was performed by matching the present R-ACVBP population with ACVBP patients selected from the LNH-98-3 trial. Patients were fully matched (1:1) on histology, aa-IPI score, gender, age and follow-up duration. *Results*. With a median follow-up of 27 months, according to the updated IWC 2007, 3y PFS and OS were estimated at 76% (CI 69-81%) and 81% (CI 75-86%), respectively. There was no significant difference between pts in CR or PR before transplant. 3y PFS was higher in R-ACVBP than in ACVBP patients: 75% (CI 67-81%) vs 58% (CI 50-65%), p=0.0003. 3y OS were estimated at 78% (CI 71-84%) vs 67% (CI 58-74%), p=0.05. The gain in 3y OS was significant in patients who received auto transplantation: 89% (CI 81-93%) vs 77% (CI 67-84%), p=0.02. *Conclusions*. These results with R-ACVBP induction and consolidative auto-transplantation suggest a major survival benefit with an increase remission rate after HDC according the criteria used. An impressive PFS of 76% and OS 81% were observed even with 3 adverse factors. Comparison with previous study without R in induction suggests a major improvement which needs confirmatory prospective study.

0984

CEREBROSPINAL FLUID FLOW CYTOMETRY ANALYSIS IN NEWLY DIAGNOSED AGGRESSIVE NON-HODGKIN LYMPHOMAS AT HIGH RISK FOR LEPTOMENINGEAL DISEASE: A MULTICENTRIC PROSPECTIVE ITALIAN STUDY

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Background. Leptomeningeal disease (LD) is an infrequent but nearly always fatal complication occurring in patients with NHL. The diagnostic standard conventional cytologic examination (CC) of cerebrospinal fluid (CSF), is considered with low sensitivity and low specificity. Recently, studies demonstrated that flow cytometry (FCM) assessment of CSF could increase the proportion of positive cases detected with LD in comparison to CC. It's still unknown if detecting occult LD and consequently changeling prophylactic therapy may improve the outcome in these patients. *Aims*. The aim of this study was to compare CC vs FCM in a large cohort of patients with newly diagnosed aggressive NHL at high risk for LD. We also assessed the impact of detecting occult LD with FCM on progression-free survival (PFS). *Methods*. Patients considered at high risk for LD included: diffuse large B-cell lymphoma (DLBCL) with IPI 2-3, elevated LDH along with at least two extranodal sites or with bone marrow or testis or palate or socket involvement; Burkitt lymphoma (BL); blastoid variant of mantle cell lymphoma (B-MCL); B-cell precursor lymphoblastic lymphoma (B-LL); HIV+ patients. All patients, with no evidence or signs of neurological disease, received intrathecal standard prophylactic therapy. Both FCM and CC were performed and the incidence of positive test for occult LD was compared using the McNemar test for paired data. 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. *Results*. From August 2004 to June 2008, 118 patients were enrolled by 10 centres. Clinical characteristics were: 78 males and 40 females, median age 55 years (IQR:43-63); 88 patients (74,6%) with DLBCL, 18 pts (15.2%) with BL, 8 pts (6.7%) with B-MCL and 4 pts (3.4%) with B-LL. Eighteen patients (15.3%) were HIV positive. FCM was able to detect a clonal population in 16 out of 118 patients (13.6%) whereas CC detected abnormal cells only among 7 pts (6%)(p= 0.0002, McNemar's $\chi 2$). Therefore, 9 patients (7.6%) were discordant: FCM positive/CC negative. From date of diagnosis, overall median follow up of survivors was 11 months (IQR:5-18). We observed 22(18.6%) systemic progressions, 5(4.2%) CNS progressions and 19(16.1%) deaths (17 PD, 2 infections). PFS at 6 months was 94% (95%CI:86-97) in pts both negative in FCM and CC, 57% (95%CI:17-84) in pts both positive, 83% (95%CI:27-97) in pts discordant (p=0.0029, log-rank test). *Conclusions*. FCM assessment of CSF in pts with aggressive NHL at risk of LD seems to have an higher sensitivity than CC but, in absence of a gold standard, it's non yet clear what is the clinical relevance of a positive FCM. Our preliminary data suggest that patients both positive for FCM and CC have an higher risk of progression compared with those both negative, whereas discordant cases seem to have an intermediate prognosis. However a longer follow up is needed to further validate these results.

0985

EFFICACY AND SAFETY OF LOW-INTENSITY ORAL AND INTRAVENOUS FLUDARABINE-CYCLOPHOSPHAMYDE WITH AND WITHOUT RITUXIMAB FOR FIRST-LINE TREATMENT OF FOLLICULAR LYMPHOMA

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Background. Fludarabine-Cyclophosphamyde (FC) is a potent regimen for untreated and relapsed follicular lymphoma (FL). However, myelosuppression limits its use at standard doses. Oral F appears as effective as intravenously, lowering hospital expenses and favouring compliance. The association of Rituximab (R) with FC improves ORR/OS and PFS in induction, maintenance and salvage regimens, without adding relevant toxicity. *Aims*. 1) To evaluate efficacy and safety of a lower intensity FC ± R in first-line for FL. 2) To assess whether oral administration improves the toxicity profile while maintaining the efficacy. *Methods*. Follicular lymphoma (WHO histological grades 1-2) patients (pts) aged ≥18, previously untreated, WHO-PS≤≤2, ≤2-fold renal and hepatic function values were eligible. *Outpatient treatment*. Intravenous FC and FCR (iv): Fludarabine (F) 25 mg/m^2/d plus Cyclophosphamyde (C) 250 mg/m^2/d (d 1-3), Rituximab (R) 375 mg/m^2 (d1) x 4 28-day cycles; Oral FCR (po): F 30 mg/m^2/d plus C 175 mg/m^2/d (d 1-3), R 375 mg/m^2/ (d1) x 4-6 28-day cycles; R maintenance (advanced stage achieving CR): R 375 mg/m^2 q/3 months x 8 doses. *Results*. From March'99 to January'09, 81 pts were prospectively included: M age: 56 (32-81), M:F 40:41, Stages III-IV: 85.3%, B-symptoms: 28.4%, FLIPI≥2: 81%. Seven (8.6%) pts received FC iv; 28 (34.5%) FCR iv; 46 (56.8%) FCR po. M cycles: 4 (1-4) FC/FCRiv; 5 (3-6) FCRpo. MFU: 29 m (1-116). Seventy-five pts were evaluable for response: ORR rates FCRiv versus FCRpo: 89% vs 97.6% (p<0001). CR/PR rates FCRiv versus FCRpo: 59.3%/29.6% vs 87.8%/9.8% (p<0001). Regarding toxicity, neutropenia was the most limiting AE: 25 neutropenia 3-4 episodes: 15 (55.5%) and 8 (17.4%) in FCRiv and FCRpo respectively (p<0001); grade 3-4 infection rate: 13 (46.4%) and 7 (15.2%) in FCRiv and FCRpo, respectively (p<0001), causing death in 2/28 FCRiv pts. Grade 3-4 thrombocytopenia occurred in 3 FCRiv and 2 FCRpo pts, anemia 3-4 in 2 FCRiv and 1 FCRpo, and 1 case of liver toxicity (FCRiv). Forty pts received R-Maintenance: FCRiv: 20/27 (74%), FCRpo: 20/46. Withdrawal of R-maintenance due to neutropenia and infections: 9 (45%) FCRiv and 3 (15%) FCRpo. Three pts relapsed with a M TTP: 12 m (9-14); and 3 pts died (2 infections, 1 progression). With a MFU: 29 m (1-116), overall survival (OS) was 94.6% (IC al 95%: 88,5 to 100%), and event-free survival (EFS) was 90%, with no significant differences between oral and iv FCR Transformation to aggressive NHL occurred in 2 pts, no cases of myelodysplasia. *Conclusions*. 1) Low-dose FCR is a potent first-line and safe regimen for FL (including pts> 65 yo), with very high ORR and CR rate. 2) The oral low-dose FCR regimen significantly decreases myelosuppression, while achieving a high ORR and CR rates, equivalent to those of FCR iv.

0986

A NEW SHORT-TERM HIGH INTENSIVE PROTOCOL BL-M-04 FOR ADULT PATIENTS WITH BURKITT LYMPHOMA: EFFICACY AND TOXICITY

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Background. Burkitt lymphoma (BL) is the most aggressive B-cell lymphoid neoplasm, whose growth fraction approximates 100%, with specific chromosomal abnormalities [t(8;14)(q24;q32), rarely - t(2;8)(p12;q32), t(8;22)(q24;q11)]. Despite the rapid proliferative rate, BL is one of the most chemosensitive lymphoid neoplasm.Though it was clear, that high intensive short-term alternating multiagent chemotherapy regimens are most effective in patients with BL, adults have a less favorable outcome than pediatric patients with BL. *Aims*. to evaluate the efficacy and toxicity of the protocol BL-M-04 for adult patients with BL. *Methods*. 37 previously untreated patients with BL were eligible for our study (they had specific translocations involving chromosome 8 [t(8;14)(q24;q32), t(2;8)(p12;q32), and t(8;22)(q24;q11)]. All the patients (23 males and 14 females, mean age 29 years (from 15 to 69 years)) participated in the study performed in the Russian Hematological Research Center between August 2003 and December 2008. The treatment was based on experimental high intensive protocol BL-M-04. BL staging criteria developed by S. B. Murphy were used to stage the patients. Stage I, II, III, IV were diagnosed in 2, 3, 15, 6 patients respectively. B-acute lymphoblastic leukemia (L3) was diagnosed in 11 (30%) patients. B-symptoms (night sweets, fever and weight loss) were present in 30 (81%) patients. Serum lactate dehydrogenase level (LDH) was increased in 30 (81%) patients. Acute renal failure was found in 14 patients: specific renal involvement in 6 patients, ureteral obstruction with postrenal azotemia in 2 patients, and tumor lysis syndrome with uric acid nephropathy in 6 patients. Hemodialysis was used in 5 patients, and urgent nephrostomy in 1 patient. Acute renal failure regressed due to the institution of chemotherapy and intensive care in 8 patients. The main aim of the new treatment regimen was greater efficacy of therapy due to intensification and shorter treatment duration. The new treatment protocol is based on the modified NHL-BFM protocol for high risk patients with a reduced dose of methotrexate from 5000 mg/m^2 to 1500 mg/m^2. As BL, is a chemosensitive tumor that often regresses after 1-2 courses of chemotherapy, we decided to treat patients with BL in 4 courses of chemotherapy (2 induction and 2 consolidation) irrespective of the initial tumor mass. As BL is most sensitive to high dose methotrexate and cytarabine, we used these drugs in the induction phase to achieve to maximize the cytoreductive effect. Courses A and C were used to achieve remission. Doxorubicin was added to course A, and methotrexate to course C. Consolidation courses were similar to induction courses. Hence, we used A and C courses (without course B), intensified with course B drugs (doxorubicin and methotrexate), the interval between the courses being 21 days. *Results*. 34 patients (90%) achieved a complete remission (CR) after 1-2 courses (16 patients - after the 1st course, 18 - after the 2d). 33 are alive in the first CR during 32 months (median 2-62 months). Four patients died. The cause of death in three patietns were chemotherapy related complication. One patient died due to early relapse. The 5-year disease-free survival was 97% with an overall survival of 89%. BL-M-04 therapy resulted in higher CR rates and longer disease-free survival in adult patients with BL and confirmed high efficacy of short-term intensive therapy. Treatment duration was 3-3,5 months. Grade III-IV neutropenia occurred after all courses. Most infectious and hemorrhagic complications occured during the first course (course A), which can be explained by the initial poor condition of the patients. Unfavorable prognostic factors, which increase the number of chemotherapy complications, were stage IV/B-ALL, acute renal failure, and failure of previous treatment (surgery and chemotherapy). *Summary and Conclusions*. BL-M-04 is a highly effective protocol. The 5-year disease-free survival was 97% with 5-year overall survival of 88%. The use of this protocol can achieve rapid tumor regression with a short treatment duration due to chemotherapy intensification and acceptable toxicity. As the majority of relapses occur after 8-12 months of treatment, most of these patients can be considered cured.

0987

RETROSPECTIVE AND PROSPECTIVE ANALYSIS ON THE IMPACT OF HEPATITIS VIRUSES ON CHRONIC LYMPHOPROLIFERATIVE DISORDERS

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Background. Patients with chronic lymphoproliferative disorders (CLD) frequently associate infections with B, C, or D hepatitis viruses, which can also infect and replicate in hematopoietic cells. *Aims*. Analysis of hepatitis viral infection's influence on clinical and biological evolution of CLD - data from patients diagnosed and monitored between December 2007-January 2009; preliminary data of an ongoing multidisciplinary study. *Methods*. Retrospective and prospective study of patients with CLD and hepatitis viral infection. The diagnosis of the CLD was established on lymph node/bone marrow biopsy completed by immunophenotyping analysis, according to WHO classification. All patients had positive serological tests for at least one hepatitis virus; quantitative viremia was determined, by TaqMan PCR method. *Results*. Group of 41 patients: 25 females (60.97%), 16 males (39.02%); median age - 64 years old (min30, max85), with 56.09% between 51-70 years old. B-cell lym-

phoma was found in 28/41 (68.29%) patients: 13/28 (46.42%) - diffuse large B-cell lymphoma, 11/28 (39.28%) - small B-cell lymphoma (marginal/lymphocytic type), 3/28 (10.71%) - follicular lymphoma, 1/28 (3.57%) - lymphoblastic lymphoma. 13/41 patients (31.71%) had: T-cell CLD (2/41), Hodgkin's disease (2/41), CLL (7/41) and Waldenström macroglobulinemia (2/41). Indolent types of CLD were found in 22/41 (53.65%), and aggressive types in 46.35%. HCV was the most frequent virus (24/41-58.53%) and HBV - the second: 14/41 (34.14%); 3 patients presented double/triple viral infection. We observed the association between HBV infection and aggressive histological types (9/14-64.28%), and between HCV and indolent types (15/24-62.50%). Clinical examination revealed adenopathies in 29/41patients (70.73%), hepatomegaly in 38/41 (92.68%) and splenomegaly in 21/41 (51.21%); splenomegaly was found mostly in patients with HCV (12/21-57.14%); 3/41 (7.31%) had diagnostic or therapeutic splenectomy. Extranodal disease was found in 10/41patients (24.39%), especially in patients with indolent CLD and HCV infection. Anemia was present in 9/41 patients (21.95%); autoimmune hemolytic anemia (AIHA) was associated in 4/41 cases (9.75%), all with HCV infection. Thrombocytopenia was revealed in 13/41 (31/70%), mainly associated with HBV infection. The majority of the patients had lymphocytosis (33/41-80.48%), particularly the ones with HCV infection and indolent types of CLD. Serum cryoglobulins were found in 8/41 (19.51%), all with HCV infection. Increased ALT level occurred in 32/41 (78.04%); increased GGT level - 20/41 cases (48.78%); hepatic cytolysis associated predominantly with indolent types of CLD, with HCV infection. Serum LDH level (monitored as prognostic marker), was increased in 17/41 patients (41.46%); of these, 52.94% associated HCV infection. Chemotherapy was given in 32/41 patients with CLD (78%), as monotherapy (5/41 - 13.19%) or combined chemotherapy/chemoimmunotherapy (27/41 - 65.85%); 6/41 patients (14.63%) received interferon and 6/41 (14.63%) - rituximab; antiviral therapy (lamivudin/ribavirin±interferon) was administered in 12/41 patients (29.26%). *Conclusions*. The analysis showed a higher incidence of CLD with hepatitis viral infection in women over 50 years old, with a predominance of HCV infection, which was particularly linked to: indolent CLD subtypes, extranodal disease, splenomegaly, AIHA and cryoglobulinemia, lymphocytosis, liver dysfunction, and high LDH level. The study will further determine how the antiviral and chemotherapy will influence the studied parameters and the clinical and biological outcome of these patients.

Non-Hodgkin lymphoma - Clinical IV

0988

METHOTREXATE RELATED LYMPHOPROLIFERATIVE DISORDER -EXPERIENCE OF A DISTRICT GENERAL HOSPITAL IN THE UK

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Patients with Rheumatoid arthritis are estimated to have 2-20- fold increased risk of lymphoproliferative disorder (LPD) in the absence of Methotrexate (MTX) therapy. It remains debated whether MTX per se increases the risk of LPD development. Aim of this study was to evaluate the patient and disease characteristic of LPD in patients of rheumatoid arthritis (RA) who were treated with MTX. The catchments population of this small district general hospital is 300,000. We observed 6 patients of RA treated with MTX who developed LPD. Only one patient was male and the rest were females as RA is more common in females. Mean age was 63 years (range 51-79 years). Mean duration of RA was 13 years while the mean duration of MTX treatment was 7.5 years. Most common type of LPD was diffuse large B cell lymphoma (3) with one each of marginal zone lymphoma, lymphoplasmacytic lymphoma and follicular lymphoma. 4 patients presented with advanced stage disease (>stage II) while one patient had stage IA and the other was limited extranodal disease. EBV stain was positive only in one patient. MTX was withdrawn in all 6 patients after lymphoma diagnosis. 3 patients put on watch and wait approach with continued complete remission in two. Rest of the patient received chemotherapy ranging from rituximab only to high dose combination chemotherapy. All 6 patients are currently in complete remission with mean duration of 16 months post chemotherapy. Our findings were quite consistent with the literature. MTX related LPD is seen generally in older age group with female preponderance as compared to sporadic cases of LPD. EBV positivity is not a consistent finding. MTX withdrawal is effective in small proportion of patient but these patients should have close follow-up. Interesting findings could emerge if patients of RA and LPD with and without MTX are compared with sporadic cases of LPD.

0989

CHLORAMBUCIL WITH OR WITHOUT RITUXIMAB IS AN EFFECTIVE THERAPY IN NON-GASTRIC MALT LYMPHOMAS

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Background. MALT lymphomas are characterized by an indolent clinical course and prolonged survival. Therapy is not standardized. Chlorambucil and rituximab have been shown to be effective. However data is limited regarding the efficacy of their combination. *Aims.* The objective of this retrospective study was to compare the outcome of patients with non-gastric MALT lymphoma who received first line treatment with chlorambucil or rituximab plus chlorambucil. *Patients and Methods.* Patients with a primary non-gastric MALT site of involvement followed in our Departments between 1985 and 2007 were included in the study. Chlorambucil was given at a dose of 10 mg/d for 10d/mo for a total of 12 mos. Combined therapy included rituximab at a dose of 375mg/m^2 every mo for 8 mos plus chlorambucil at the same dose and duration as in the monotherapy schedule. *Results*. 44 of 78 patients who were registered in the database with a diagnosis of non-gastric MALT lymphoma (15 salivary glands, 10 lung, 7 ocular adnexae, 7 intestine, 3 Waldeyer ring, 1 skin, 1 bladder) were included in the present study. 24 received chlorambucil alone and 20 combined treatment. The main clinical characteristics of the two groups are shown on Table 1. Therapy was well tolerated in both groups. Complete remission was achieved in 77% with an overall response rate of 84%. Rituximab plus chlorambucil treatment was superior to chlorambucil in terms of overall and complete response rates (95% vs 79% and 90% vs 75%), without however this difference to be significant (p=0.28). Moreover, no differences were observed in the 5-year FFS and OS between chlorambucil alone and combined treatment (68% vs. 52% and 90% vs 100% respectively). The median follow up of living patients in the chlorambucil arm was 74 months (9-224) vs 21 months (6-97). *Summary and Conclusions.* In this retrospective study the outcome of non-gastric MALT lymphomas was excellent either fol-

lowing chlorambucil or chlorambucil plus rituximab. However imbalances of patients in favor of chlorambucil alone may have an impact on these results. Further studies are needed in order to evaluate the role of rituximab in the treatment of MALT lymphomas.

Table 1. Characteristics of non-gastric MALT lymphoma pts.

Parameter	Chlorambucil 24 pts # (%)	R-Chlorambucil 20 pts # (%)	*p value*
Age >60 years	6 (25)	10 (50)	0.09
Male gender	7 (29)	12 (60)	0.04
Stage IV	7 (29)	10 (50)	0.16
>1 MALT	4 (17)	7 (35)	NS
BM infiltration	5 (21)	5 (25)	NS
Nodal involvement	4 (17)	2 (10)	NS
IPI 2-5	4 (17)	11(55)	0.01
Hepatitis C	0	0	
B-symptoms	2 (8)	3 (15)	NS
Autoimmune disorders	5 (21)	6 (30)	NS
Paraproteinemia	5 (21)	4 (20)	NS
Elevated LDH	3 (13)	5 (25)	NS

0990

REVIEW OF THE USE OF GDCVP (GEMCITABINE, DACARBAZINE, CYCLOPHOSPHAMIDE, VINCRISTINE AND PREDNISOLONE) COMBINATION CHEMOTHERAPY IN RELAPSED/ REFRACTORY LYMPHOMA IN A LARGE UK HAEMATOLOGY AND BONE MARROW TRANSPLANT CENTRE

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Introduction. Despite advances in the treatment of Hodgkin's (HL) and Non-Hodgkin's lymphoma (NHL), approximately 50% patients relapse or are refractory to first line chemotherapy. Currently no consensus exists regarding optimal salvage drug regimen. Gemcitabine, a novel nucleoside analogue, has *in vitro*, ability to circumvent MDR secondary to increased P glycoprotein and MDR protein 1 over expression. *Methodology*. We aimed to study the effects of GDCVP combination chemotherapy in these patients. Case notes and computerised data base of patients receiving GDCVP from June 2004 to June 2007 were retrospectively analysed. *Results*. 47 patients (Median age 48 years; 66% male; median Karnofsky score 70; median previous relapses 2; progressive disease documented in all) received GDCVP. Indications comprised Transformed Follicular Lymphoma 9% (4/47), Follicular Lymphoma 4% (2/47), HL 51% (24/47), T NHL 4% (2/47), DLBCL 26% (12/47) and Anaplastic 6% (3/47). 35 (74%) patients had Stage 3B and above disease; 25 (53%) had extra nodal involvement and 35 (75%) exhibited B symptoms. GDCVP (total number of cycles 198; median 4) was used as 1st, 2nd, 3rd and 4th line treatment in 32%, 49%, 15% and 4% patients respectively. Percentage of planned dose delivered was 67%, 66%, 67% and 56% for Gemcitabine, Dacarbazine, Cyclophosphamide and Vincristine respectively. 6 (13%) patients underwent previous stem cell autograft; 16 (34%) received Rituximab, 17 (36%) received Platinum whilst 20 (43%) had radiotherapy. Disease nature prior to GDCVP use was chemosensitive 35 (74%); chemoresistant 12 (26%) of which 10 were Primary Progressive. Myelosuppression was moderate (Grade 3 or 4 anaemia, neutropenia and thrombocytopenia was 12, 63.0 and 48% respectively). Other side effects included nausea 60% (grade 2 -39%), vomiting 26% (grade 2 - 25%), stomatitis 17%, diarrhoea17% (grade2-13%), constipation 11%, alopecia 45%(grade2-24%), sensory loss 13% and infection 32% (grade 2,3 and 4 -73%,13%,13% respectively). Table 1 depicts data on required dose reduction. Dosing was delayed in 4 (9%); GI side effects (2) and severe infection (2) and prematurely stopped in 20(43%) after 1, 2 and 3 cycles in 4, 5 and 11 patients respectively due to progressive disease (17/20) and intolerable side effects(3/20). 39 (83%) patients required G-CSF (Neulasta) prophylaxis (9/39 primary, 30/39 secondary). The overall response rate was 51% - complete 4/47 (8.5%), partial 20/47 (42.5%), stable disease 4 /47 (8.5%) and progressive disease 19/47 (40.4%).Stem cell harvesting occurred in 14 (30%) with median CD34 cell count of 2.3×10^6/kg. 9 (19%) underwent stem cell transplant. 22 (47%) patients died by review completion. Median overall survival was 13.3 months (Kaplan-Meir; 95% CI 14.14 - 1.86) and median progression free survival was 12 months (Kaplan-Meir; 95% CI 16.39 - 7.62). *Conclusions*. GDCVP combination chemotherapy is safe, well tolerated and efficacious in relapsed/refractory lymphoma adding to the evidence underlining the efficacy and minimal toxicity of Gemcitabine based regimens. Importantly it does not interfere with stem cell harvesting and may be of use when patients are unsuitable for or have received prior platinum. We recommend detailed prospective studies to further define the role of GDCVP combination chemotherapy in this setting.

Table 1.

Scheduled dose (G-1g/m2,D-500mg/m2,C-750mg/m2,V-1.4mg/m2,IV,P-40mg/m2 PO) Dose reduction	Reason
G – FULL, DCVP – 50%	GI SIDE EFECTS
75% OF GDCVP – 2 PATIENTS	PATIENT COMORBIDITY, GI SIDE EFFECTS, NEUTROPENIC SEPSI
75% OF G, 50% OF DCVP	GI SIDE EFECTS
75% OF G, FULL OF DCVP - 3 PATIENTS	DERANGED RENAL FUCTION, PREVIOUS AUTO
75%GDC, 50% OF VP	PATIENT COMORBIDITY, NEUROPATHY
50% GDCVP - 2 PATIENTS	PATIENT COMORBIDITY
50% V	NEUROPATHY

0991

VALUE OF 18F-FDG PET/CT FOR THE STAGING OF NON-HODGKIN'S LYMPHOMA: SIGNIFICANT IMPROVEMENT IN THE DETECTION OF NODAL AND EXTRANODAL LESIONS BUT MARGINAL IMPACT ON DISEASE STAGE

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Background. An accurate disease staging of patients with non-Hodgkin's lymphoma (NHL) has a fundamental impact on their optimal management and prognosis. 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) combined with computed tomography (CT) is a promising imaging technique that combines metabolic and morphologic data and may markedly enhance detection accuracy in NHL. *Aims*. The aim of this prospective study was to compare PET, CT and PET/CT findings in patients with newly diagnosed untreated NHL, analyze frequency of discrepant images and assess the direct impact of PET/CT on disease stage. *Methods*. 118 patients with biopsy-proven NHL were included in this study (53 DLBCL, 22 FL, 13 T-NHL, 11 MCL, 8 MZL, 3 SLL, 3 LBL, 5 B-cell NHL NS). Whole-body PET/CT was performed in each patient. The PET and CT images were independently evaluated by a nuclear medicine physician and a radiologist who then interpreted the PET/CT images in consensus. A total of 9516 anatomic sites on PET, CT and PET/CT scans were reviewed. For each patient, the true clinical Ann Arbor stage was finally determined by hemato-oncologist with knowledge of histopathologic, laboratory, PET/CT and clinical data. *Results*. Only 1 SLL patient was classified as completely PET-negative (18F-FDG non-avid) case. In 21 patients (18%) PET-negative abnormal lymphadenomegaly according CT criteria was revealed and 15 patients (13%) had PET-positive normal sized lymph node on CT scans. On the other hand, 29 patients (25%) had PET-negative organomegaly and in 22 patients (19%) PET-positive lesion in normal sized extranodal organ was detected. Taken together, PET revealed 87 clinically important changes in 73 patients (62%) in direct comparison with CT. However, PET/CT scans changed Ann Arbor stage only in 13 patients (11%). Using PET/CT results, the disease stage was decreased in 7 patients and increased in 6 patients. *Conclusions*. 18F-FDG PET/CT markedly improves accuracy in initial diagnostic work-up of NHL patients. 18F-FDG PET is superior in the detection of nodal and extranodal lymphoma involvement compared

with CT, but has only limited impact on disease stage. 18F-FDG PET/CT scans may better characterize pathological lesions in patients with NHL.

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0992

FRONT-LINE AUTOLOGOUS PURGED TRANSPLANTATION IN INDOLENT NON-HODGKIN'S LYMPHOMAS: A LONG-TERM FOLLOW-UP STUDY

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Background. Indolent lymphoid neoplasms have a long survival. However, in advanced stages are commonly incurables with conventional chemotherapy. We present our experience with a up-front intensive treatment program which includes chemotherapy until maximum response followed by double-purged autologous hematopoietic stem cell transplantation (HSCT) at a single center. *Methods.* Forty patients with indolent lymphomas were enrolled between 1996 and 2006. The inclusion criteria were (with some minor exception in age): III-IV stages, 18-60 years old, indolent lymphoid neoplasms according to the NCI (PDQ) modification of the WHO classification, adequate hematologic, renal, and hepatic function and informed consent. We followed the principle of maximum response with the induction chemotherapy and the last 14 patients also received rituximab. All patients received an autologous HSCT, 28 cases received a purged product with double immunoselection and 7 cases with only positive immunoselection ($CD34^+$). The conditioning regimen was TBI + an alkylator or BEAM according a predefined procedure. *Results*. Complete response (CR) rate was 30.77% after first line induction chemotherapy, 82.5% before HSCT (after maximum response treatment), and 92.5% after HSCT. Three patients achieved a good partial response (PR) after HSCT, all these patients achieved a CR after a complementary treatment (radiotherapy with or without rituximab), for that the final CR was 100%. With a median followup of 6.5 years the projected overall survival (SV) at 12.5 years was 80% and the projected disease free survival (DFS) at 11 years was 72%. With the univariate analysis (log-rank test) patients with less than 58 years old (p=0.004), follicular lymphoma (vs other indolent lymphomas) (p=0.005), normal range LDH (p=0.045) and those that achieved RC at the time of harvest (p=0.008) or at the time HSCT (p=0.023), showed a significant better DFS. It highlights a projected DFS at 11 years of 88% and an evident plateau after 2 years. Using multivariate Cox regression analysis only the follicular type of lymphoma (OR 9.512; p=0.023) and age less than 58 years old (OR 8.835; p=0.043) remained as independent predictors of superior DFS. At the time of analysis 7 patients have died, 4 (10%) of deaths were transplant related (TRM), of those 1 (2.5%) died before day +100 and 2 (5%) died in the first year. The infections were frequent complications and the cause of TRM. The most frequent infection was herpes zoster. Three secondary malignancies were observed: 1 case of secondary MDS/AML, 1 case of gastric stroma tumor and 1 case of metastatic bladder cancer. *Conclusions.* With this approach a long overall survival and DFS were achieved. A plateau seems showed in both survival curves. This is specially apparent in follicular lymphomas, whereas in other indolent lymphomas it is not so evident.

0993

FRONT-LINE RISK-ADAPTED IMMUNOCHEMOTHERAPY IMPROVES OUTCOME IN INTERMEDIATE/HIGH-RISK PATIENTS WITH FOLLICULAR LYMPHOMA

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Background. The Follicular Lymphoma International Prognostic Index (FLIPI) is a valuable prognostic tool for risk stratification, but its prognostic power was postulated from retrospective data in the pre-rituximab era. Front-line prognostic stratification of treatment intensity may improve outcome, especially of high-risk patients with follicular lymphoma (FL). *Aims*. To assess whether risk-adapted immunochemotherapy overcomes the negative prognostic impact of the FLIPI score in newly diagnosed patients with FL. *Methods*. The prospective study comprised 137 patients with newly diagnosed FL (grades I-IIIa) who fulfilled the GELF criteria. The median age was 53 years (31-84), 84.7% of the patients had advanced (III/IV) clinical stages, 55% had bulky (>7 cm) disease. The FLIPI scores were as follows: low 33.3%, intermediate 31%, high 35.7%. The front-line treatment was stratified according to the commonly used risk factors (FLIPI, β-2-m and s-TK levels, bulky disease) into 3 treatment groups: (1) patients with FLIPI 0-1 with a maximum of 2 additional risk factors treated with (R)-CHOP, (2) patients under 60 (65) years of age with intermediate-risk disease (FLIPI 2 or less with >2 additional risk factors) indicated for an intensive chemotherapy protocol (ProMACE-CytaBOM or Sequential chemotherapy), and (3) patients under 60 (65) years with high-risk disease (FLIPI ≥3) treated with intensive chemotherapy plus autologous stem cell transplantation (BEAM). (1) 62% of patients were treated conventionally, (2) 13% underwent an intensive chemotherapy protocol, and (3) 25% received intensive chemotherapy with ASCT consolidation. Rituximab was added to chemotherapy in 51% of the patients. The treatment response was classified according to the standard Cheson criteria (1999). Patients who achieved a complete response (CR) or unconfirmed complete response (CRu) plus PCR bcl-2/IgH negativity were classified as molecular CR (CRm). *Results*. Generally, CR or CRu was achieved in 88% of patients, 12% had partial remission (PR). CRm was evaluable in 85 patients: 66% of them achieved molecular CR, 17% CR with bcl-2/IgH positivity and 17% PR. After median follow-up of 52 months, 57% of the patients are still in the 1st CR, 26% relapsed or progressed and 17% of the patients died. Overall survival at 5 years (5y OS) reached 85% (95% CI 0.77-0.92); progression-free survival at 5 years (5y PFS) was 61% (95% CI 0.51-0.72). The FLIPI scores do not stratify risk subgroups for OS (log-rank 0.12). For PFS, the population is divided into only 2 groups: low/intermediate (5y PFS 70%; 95% CI 0.56-0.83) and high-risk (5y PFS 55%; 95% CI 0.38-0.71), log-rank 0.026. Cox regression analysis identified only CRm and s-TK level as independent predictive factors (PFS). *Summary*. Front-line risk-adapted immunochemotherapy improves both the quality of treatment response and survival of FL patients. Some of the conventional prognostic factors lose their power in this population. Further investigation will help to develop the role of FLIPI in current treatment decision-making. New biological factors such as molecular remission and s-TK level identify patients at high risk of early relapse.

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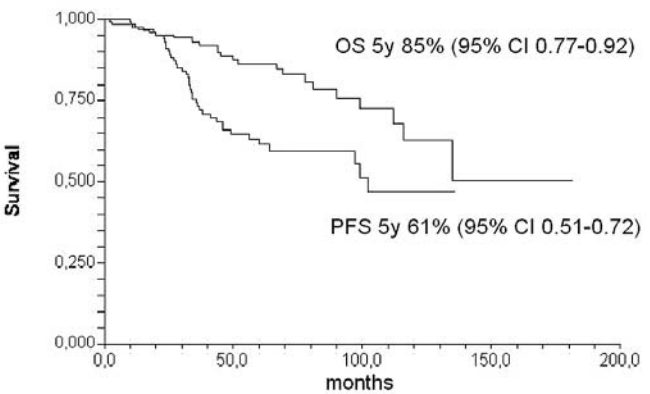

Figure 1.

0994

THE PROGNOSTIC SIGNIFICANCE OF THE NUMBER OF EXTRANODAL INVOLVEMENT SITES IN THE PATIENTS WITH DISSEMINATED DIFFUSE LARGE B-CELL LYMPHOMA TREATED WITH R-CHOP

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Background. The combination of rituximab and CHOP chemotherapy (R-CHOP) has improved survival of patients with diffuse large B-cell lymphoma (DLBCL). Recently, several reports have shown that standard International Prognostic Index (IPI) became less powerful prognostic predictor in patients with DLBCL in the era of R-CHOP. *Aims*. We evaluated the prognostic factors of DLBCL patients treated with R-CHOP. Detailed analysis was planned regarding the number of extranodal sites because of its higher frequency in Korea. *Methods*. Between January

2002 and May 2008, 126 patients with stage III/IV DLBCL treated with R-CHOP were identified. We performed the retrospective analysis of the clinicopathologic factors and verified the predictive power of standard IPI and revised IPI (R-IPI) which was reported by the study group of British Columbia. Various numbers of extranodal sites were analyzed for further stratification and we set E-IPI as the IPI when the number of extranodal sites is stratified in ≤2 vs >2. *Results*. In the univariate analysis, the number of extranodal sites (≤2 vs >2) was a significant prognostic factor for complete response (CR) (p=0.04), event-free survival (EFS) (p=0.01) and overall survival (OS) (p<0.001). Age was also significant for EFS (p=0.03). When the number of extranodal site was stratified differently (0 vs >0, or ≤1 vs >1), these were not associated with CR, EFS and OS. On the multivariate analysis, the number of extranodal sites (≤2 vs >2) remained significant for EFS (p<0.01, HR 2.6) and OS (p<0.01, HR 3.5). The standard IPI identified 3 risk groups with 2-year EFS; 68%, 55%, 56% (p=0.17) and 2-year OS; 85%, 68%, 58% (p=0.04). The R-IPI classified 2 risk groups with 2-year EFS; 65%, 50% (p=0.02) and 2-year OS 76%, 62% (p=0.04). The E-IPI represented 3 risk groups with 2-year EFS; 79%, 56%, 42% (p=0.01) and 2-year OS; 86%, 70%, 39% (p=0.001). The patient group with survival of less than 50% was only recognized by E-IPI. *Conclusions*. The number of extranodal sites (≤2 vs >2) is the most significant prognostic factor of EFS and OS. Although all three indices remain predictive, E-IPI is the best model to identify the prognostic group in this cohort with stage III/IV DLBCL treated with R-CHOP.

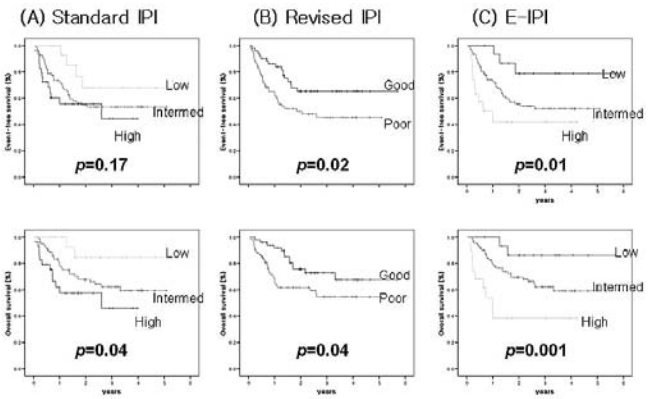

Figure 1. Outcomes according to three prognostic models.

0995

ADDITION OF YTTRIUM-90 IBRITUMOMAB TIUXETAN TO BEAM CONDITIONING PRIOR TO AUTOGRAFTING DOES NOT IMPROVE OUTCOME OF CHEMOREFRACTORY PATIENTS WITH AGGRESSIVE B-CELL NHL

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Background. The outcome of patients with aggressive B-NHL failing standard front-line and salvage chemotherapy is extremely poor even after autografting. Yttrium-90 ibritumomab tiuxetan (Zevalin®) is a radioimmunoconjugate consisting of an anti-CD20 monoclonal antibody coupled to radioactive yttrium-90. Yttrium-90 ibritumomab tiuxetan is active against B-NHL, has mainly hematological toxicity and is not cross-resistant with chemotherapy. We therefore hypothesized that the addition of yttrium-90 ibritumomab tiuxetan to conditioning might improve the outcome of autografting in these patients. *Aims*. We performed this trial to investigate the feasibility and efficacy of the addition of standard-dose yttrium-90 ibritumomab tiuxetan to the standard pretransplant BEAM chemotherapy conditioning regimen consisting of carmustine, etoposide, cytarabine and melfalan (Z-BEAM) in patients with chemorefractory aggressive B-NHL. *Methods*. Patients with diffuse large B-cell (DLBCL) and mantle-cell lymphoma (MCL) were eligible for the trial provided they have failed front-line antracycline-based chemotherapy and the last salvage regimen, were transplant-eligible and did not have bone marrow infiltration with lymphoma. Yttrium-90 ibritumomab tiuxetan was given at a dose of 14.8 MBq/kg (but not more than 1.26 GBq) on day -10. BEAM was administered between days -7 and -2 and stem cells were reinfused on day 0. *Results*. We identified 18 patients with chemorefractory NHL potentially eligible for the trial, 16 with DLBCL and 2 with MCL. Stem cell collection was successful in 11, all with DLBCL. In 4 out of these 11, rapidly progressive disease precluded autografting. Seven patients received Z-BEAM. Three were men and 4 women; median age was 47 years, range 33-62. All were previously exposed to rituximab. Median number of previous treatment lines was 2, range 2-4, and median disease duration 21 months, range 15-23. Two patients died before engraftment, one due to refractory ventricular fibrillation during stem-cell reinfusion, and the other due to pneumonia caused by a multiresistant Pseudomonas strain. Time to platelet engraftment was 12-39 days (median 18) and time to granulocyte engraftment 8-18 days (median 10). One patient died after engraftment due to multiorgan failure. There were no other cases of serious non-hematological, non-infectious toxicity. None of the four patients surviving to discharge responded and all died within 5 months of transplantation due to tumor progression or complications of further treatment. The trial was stopped prematurely because of lack of efficacy and toxicity. *Conclusions*. In this small series of very high-risk patients with chemorefractory aggressive B-NHL the addition of yttrium-90 ibritumomab tiuxetan to BEAM conditioning prior to autografting was toxic and did not improve outcomes.

0996

A POSSIBLE ROLE OF 18F-FDG PET SCANNING IN THE EARLY DETECTION OF RITUXIMAB-INDUCED PNEUMONITIS IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA

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Background. Rituximab is safe and effective in the treatment of patients with non-Hodgkin's lymphoma (NHL). Recently, rituximab-induced pneumonitis (RP) has been reported as side-effect of rituximab. *Aims*. To evaluate the possible role of 18F-FDG PET scanning in the early detection of rituximab-induced pneumonitis in patients with Non-Hodgkin's lymphoma.

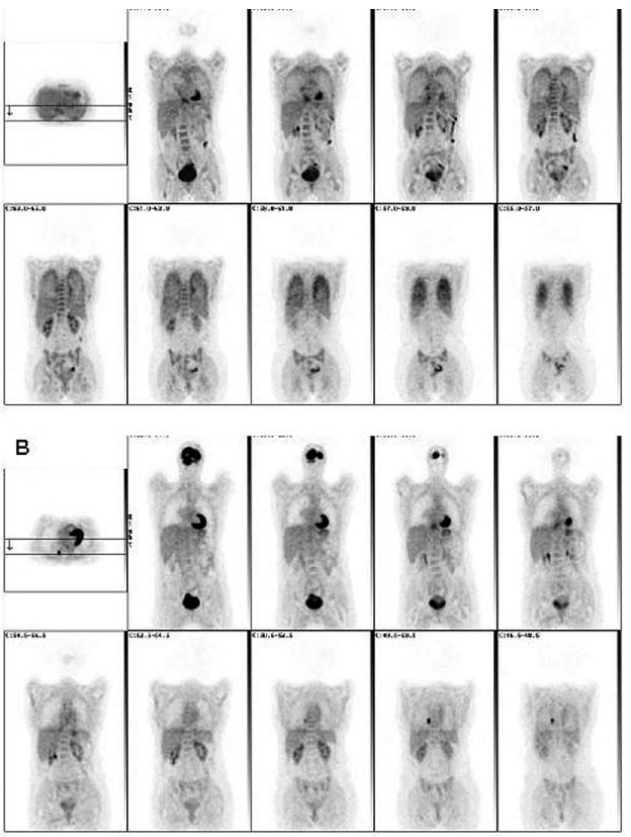

Figure 1. 18F-FDG PET in rituximab induced pneumonitis.

Methods. We performed a single centre, retrospective case-control study of NHL patients treated with C[H]OP-rituximab to investigate

variables associated with RP and to investigate the abnormalities found on 18F-FDG PET. We included patients with a documented 18F-FDG PET before rituximab therapy and in whom a priori the lymphoma response was evaluated by 18F-FDG PET. RP was defined as the presence of characteristic clinical findings and the presence of diffuse unilateral or bilateral pulmonary activity detected by 18F-FDG PET. The diagnosis RP was only made after exclusion of other causes of diffuse lung disease. All subjects were reviewed for treatment schedule, clinical, laboratory, and radiological characteristics. 18F-FDG PET were analysed for activity pattern and maximum standardized uptake values (SUVmax). Patients with RP were considered as cases (case group) and were compared with patients without RP (control group).*Results*. A total of 36 patients were treated with R-C[H]OP, including four patients in which RP was diagnosed. The cases and controls were comparable for age, gender, NHL-classification, Ann Arbor stage and treatment schedule. There were no differences with regard to laboratory parameters, number of administered rituximab doses, interval of rituximab administration and cumulative dose of administered rituximab. All four patients with RP showed bilateral diffuse pulmonary uptake on 18F-FDG PET. The mean SUVmax was 3.5 (range 1.5-7.8). In one patient 18F-FDG PET abnormalities preceeded abnormalities found on HRCT and chest X-ray. The activity on 18F-FDG PET was reversible in all patients. No abnormalities were noted on 18F-FDG PET in the control group. *Conclusions*. In conclusion, we described four patients, in whom 18F-FDG PET imaging proved to be a useful and early diagnostic tool in the detection of RP.

0997

FIRST-LINE TREATMENT WITH RITUXIMAB AND BENDAMUSTINE IN OLD PATIENTS (80 YEARS AND OLDER) WITH AGGRESSIVE B-CELL LYMPHOMAS - AN INTERIM ANALYSIS OF AN ONGOING PHASE II STUDY

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Background. Rituximab plus CHOP (R-CHOP) is widely recommended for the treatment of patients with aggressive B-cell lymphomas. There is a paucity of information from clinical studies regarding the management of old patients. A recently published, retrospective analysis in patients aged >80 years reported a median overall survival of 1.2 years (Thieblemont *et al*. Ann Oncol 2008;19:774-9). Bendamustine is a purine analog/alkylator hybrid with single-agent activity in various lymphomas. Acceptable response rates and good tolerability have been achieved with bendamustine in a phase II study in relapsed/refractory aggressive lymphomas (Weidmann *et al*. Ann Oncol 2002;13:1285-9). *Aims*. We initiated a phase II study of first-line treatment with rituximab and bendamustine in elderly patients (≥80 years) with aggressive B-cell lymphomas who were not eligible for R-CHOP or who denied aggressive treatment. Primary objectives were feasibility and efficacy. *Methods*. Judgement whether a patient qualified for R-CHOP was left to physicians. When a decision against R-CHOP was reached, patients with stage I/II disease received 4 cycles of rituximab (375 mg/m^2, day 1) and bendamustine (120 mg/m^2, days 2 and 3) every 21 days, followed by involved field irradiation; patients with stage III/IV disease received 6 cycles of the above treatment followed by 2 consolidating administrations of rituximab. *Results*. To date, 13 patients (9 male, 4 female) with a median age of 85 years (range 80-89 years) have entered the study. The age-adjusted International Prognostic Index was 0 in 6 patients, 1 in 2 patients, 2 in 4 patients, and 3 in 1 patient. Seven patients had stage I/II disease, and 6 had stage III/IV disease. Eleven patients were diagnosed with diffuse large B-cell lymphomas, 1 with mantle cell lymphoma, and 1 with grade 3 follicular lymphoma. Response: Ten patients were evaluable for response (1 patient refused further treatment after the first cycle, treatment was discontinued after the first cycle in 1 patient because of a psychotic episode, and in 1 patient it is too early for response assessment). Nine of the 10 (90%) patients achieved a response: 6 (60%) had a CR, 3 (30%) had a PR, and 1 (10%) had progressive disease. Survival: In an intention-to-treat analysis of all 13 patients, with a mean observation time of 17.3 months (range 1-49 months), the estimated overall survival at 2 years was 56% (Figure 1); the mean overall survival had not yet been reached. Mean progression-free survival was 14.4 months. Toxicity: Toxicity was generally low. Of 46 evaluable treatment cycles to date, only 1 grade 4 toxicity (neutropenia; 2%) was observed. Grade 3 toxicities included leukopenia (7%), neutropenia (4%), thrombopenia (2%), infections (7%), nausea/vomiting (4%), diarrhea (2%), and renal insufficiency (2%). *Summary and Conclusions*. Bendamustine in combination with rituximab may be an alternative treatment for aggressive lymphomas in elderly patients not eligible for R-CHOP. Furthermore, treatment toxicity was low. These results, however, need to be confirmed in larger numbers of patients.

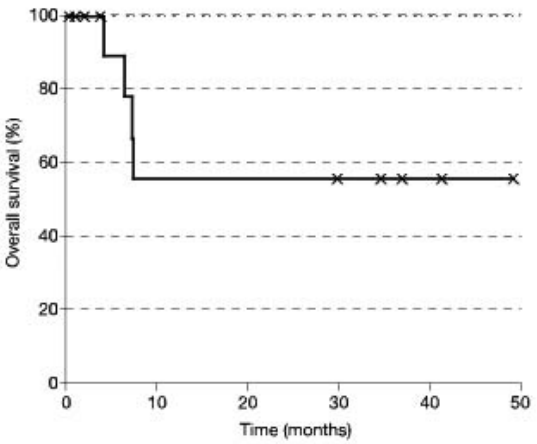

Figure 1.

0998

KI-67 EXPRESSION IS PREDICTIVE OF PROGNOSIS IN PATIENTS OF DIFFUSE LARGE B-CELL LYMPHOMA TREATED WITH RITUXIMAB PLUS CHOP

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Background. Tumor cell proliferation as assessed by the Ki-67 expression has yielded conflicting results in predicting prognosis of diffuse large B-cell lymphoma (DLBCL). Furthermore, introduction of rituximab to the treatment of DLBCL has changed significance of previously known prognostic factors. *Aims*. The objective of this study is to investigate the relationship of Ki-67 expression and prognosis in DLBCL treated with rituximab plus cylophosphamide, doxorubicin hydrochloride, vincristine, prednisolone (CHOP) chemotherapy, which is the mainstay of the disease. *Methods*. Between July 2003 and January 2008, 146 adult DLBCL were treated with R-CHOP at Asan Medical Center in Seoul. We retrospectively analyzed their Ki-67 expression and its relationship with prognosis.

Table 1.

	Ki-67 > 80% (n=46)	Ki-67 ≤ 80% (n=100)	P value
Complete remission (CR)	32 (69.6%)	82 (82.0%)	0.092
Relapse after CR	9 out of 32 (28.1%)	8 out of 82 (9.8%)	0.013
2-year overall survival	66.8%	82.3%	0.017
2-year event-free survival	37.4%	74.3%	0.004

Results. There was no significant difference in achieving complete remission (CR) with R-CHOP by the status of Ki-67 expression. Thirty two patients out of 46 (69.6%) achieved CR in the group of high Ki-67 expression (Ki-67 > 80%) and 82 out of 100 (82.0%) gained CR in the group of low Ki-67 expression (Ki-67 <= 80%)(p=0.092). However, more relapse among patients who gained CR after the initial chemotherapy

of R-CHOP was noted in the group of high Ki-67 expression. Nine patients out of 32 (28.1%) who gained CR relapsed in the group of high Ki-67 expression compared to 8 out of 82 (9.8%) in the group of low Ki-67 expression (p=0.013). The group with high Ki-67 expression had a shorter OS and EFS. The 2-year OS rate was 66.8% in the group of high Ki-67 expression and 82.3% in the group of low Ki-67 expression (p=0.017). The 2-year EFS rate was 37.4% in the group of high Ki-67 expression and 74.3% in the group of low Ki-67 expression (p=0.004). Median OS of both groups were not reached at a median follow-up of 25.2 months (range, 5.2-58.8 months). Median EFS in the group of high Ki-67 expression was 22.3 months and it was not reached in the group of low Ki-67 expression. In the multivariate analysis performed with Ki-67 and variables included in the international prognostic index such as age, performance status, Ann-Arbor stage, LDH and extranodal involvement, Ki-67 expression was still found to be an independent prognostic factor for OS (Hazard ratio (HR) = 3.577; 95% CI 1.631-7.845; p=0.001) and EFS (HR = 3.345; CI 1.754-6.378; p<0.001) in this set of patients. *Conclusions*. High Ki-67 expression was associated with more relapse and inferior survival in patients with DLBCL treated with rituximab plus CHOP.

0999

OCULAR ADNEXA NON HODGKIN LYMPHOMAS: A SINGLE CENTRE RETROSPECTIVE STUDY OF CLINICOPATHOLOGIC FEATURES, TREATMENT AND CLINICAL OUTCOME

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Background. Ocular adnexa lymphomas account for about 1% of non Hodgkin Lymphomas and 5% to 15% of all extranodal sites. They are the most frequent malignant tumor of the eye and ocular adnexa. *Aims*. Aim of this study was to define histological characteristics, treatment, prognostic factors, and outcome of patients affected by non Hodgkin lymphoma of ocular adnexa. *Methods*. Thirty patients (pts) affected by ocular adnexa non Hodgkin lymphomas were examined in our institution between 1991 and 2008. Median age was 65 years (range 40-90) with an equal gender distribution. A pathologic review of all cases was performed according to the REAL/ WHO classification. The most common histological diagnosis was "Extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue" (MALT lymphoma) (11 patients, 37%); 9 (30%) cases were "small lymphocytic lymphomas (SLL), 6 (20%) "follicular lymphoma" (FL), 3 (10%) "Mantle cell lymphoma" (MCL) and 1 (3%) Diffuse large B cell lymphoma (DLBCL). Ophthalmologic sites were: intra-orbital in 25 (83%) cases, lachrymal gland in 5 (17%), with bilateral involvement in 1 (3%) case. In 9 (30%) pts extrinsic ocular muscles were involved. The most common symptoms were: palpebral ptosis, exophthalmus and lachrymation. Fifteen 15 (50%) pts had a primary ocular adnexa lymphoma, 2 (7%) had a stage II, while Stage IV was found in 13 (43%) cases: in all cases bone marrow was involved. Four (13%) pts had other extranodal sites: 2 (%) breast, 1 (3%) pharynx, 1(3%) liver. All patients had low LDH values while 7 (23%) pts had a high β2μ value. No pts had a PS>1 and only 2 (7%) referred B symptoms. Twenty four (80%) pts received chemotherapy (11 (37%) with a CHOP-like regimen, 11(37%) a CVP regimen, 2 (7%) a FND regimen), in 14 (47%) pts chemotherapy was in combination with a monoclonal antibody anti CD20 (rituximab). One (3%) pt was treated with Rituximab alone. Five (17%) pts received radiotherapy. Overall response rate was 80%:17 (57%) Complete Remissions and 7 (23%) Partial Remissions. Four (13%) pts had stable disease and 2 (7%) of them progressed. Six (35%) relapses occurred and in 5 (83%) of them there was extranodal involvement:1(3%) breast, 2 (7%) orbit, 1(3%) lachrymal gland and 1 (3%) bone. *Results*. After a median follow-up of 74 (7-220) months, DFS and OS were 72% and 75%. At multivariate analysis, stage IV and histological type (MCL, SLL) had a negative impact on DFS for the overall population. *Conclusions*. In our experience, in accordance with previous studies, ocular adnexa lymphoma is more frequent in elderly pts; the most frequent histological type is MZL, more than 40% of population have a Stage IV disease, and response rate to the treatment is 80%. Stage IV and histological types MCL and SLL are predictive of poorer prognosis, with frequent extranodal relapses. Further studies are needed to evaluate biological and histopathological features of ocular adnexa lymphomas, in order to identify the best therapeutic strategy.

1000

EFFICACY AND TREATMENT COMPLIANCE OF DOSE-DENSE R-CHOP-14 IMMUNOCHEMOTHERAPY PLUS PEGFILGRASTIM FOR FIRST-LINE TREATMENT OF PATIENTS WITH LOW RISK DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL): AN OPEN-LABEL CLINICAL TRIAL IN SPAIN

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Background. Three-weekly R-CHOP has become the standard treatment for DLBCL. Dose-dense immunochemotherapy could improve the efficacy of treatment; however these regimens are worse tolerated due to mielotoxicity. *Aims*. The aim of this analysis was to evaluate the efficacy and treatment compliance of dose-dense R-CHOP supported by pegfilgrastim in patients with DLBCL and IPI 0-2. *Methods*. This is a prospective clinical trial in patients with DLBCL, CD20 positive disease, ECOG PS 0-2, older than 65 with IPI 0-5 or younger than 65 with IPI 0-2. Patients were required to have normal renal, liver and cardiac function. Treatment: 6 cycles of R-CHOP administered every 14 days followed by pegfilgrastim (6 mg per cycle) on day 2. In this intention to treat subanalysis we have selected patients with low or low-intermediate risk IPI to evaluate response after 2° inmunochemotherapy cycle and after 130-days follow up. *Results*. 68 patients with IPI 0-2 were included in this analysis, median age was 58 years old (range 18-82), 24 (35.3%) were older than 65, 37 (54%) were male. Sixty-for (94%) had ECOG 0-1. Characteristics of the disease at diagnosis were as follows: stage III-IV: 33 (49%), bulky disease: 15 (22%), extra nodal involvement: 42 (62%), B symptoms: 13 (19%), elevated LDH: 26 (38%), elevated β-2-microglobulin: 28 (41%), IPI 0-1 33 (49%). Fifty-nine patients completed 6 cycles of treatment. Febrile neutropenia episodes were reported in 10 (14.7%) patients. Overall, 379 inmunochemotherapy cycles were administered and 26 (6.9%) were delayed (6% in younger patients and 8.2% in patients older than 65). The causes of delay were: neutropenia (3), febrile neutropenia (4), hepatotoxicity (1), fever or infection without neutropenia (13) and other adverse events (5). Doses of myelotoxic drugs were reduced in 9 (2.4%) cycles (4 patients). Most cycles (91.8%) were administered as scheduled and most patients (67.7%) completed their full treatment schedule as planned. After second inmunochemotherapy cycle, 30 (45.5%) patients achieved complete remission, 31 (47%) partial remission, 1 (1.5%) stable disease and 4 (6%) were not evaluable. Ten patients did not complete treatment (5 exitus, 1 drop out due to an adverse event, 1 protocol violation, 3 missings). Among patients who completed treatment, 58 patients were evaluated for efficacy after 130-days follow-up, achieving 54 (93.1%) responses of which 49/54 (90.7%) were complete remissions. Responses in IPI 0-1 and IPI 2 patients were 26/29 (89.6%) and 28/29 (96.5%) respectively. *Conclusions*. High remission rate shows that dose-dense immunochemotherapy with pegfilgrastim support is an efficacious treatment for DLBCL in low risk patients. The regimen is well tolerated, treatment compliance is high and most of cycles were administered as scheduled.

1001

NEW VERTEBRAL FRACTURES IN NON-HODGKIN-LYMPHOMA PATIENTS TREATED WITH CHEMOTHERAPY AND HIGH DOSE STEROIDS ARE RELATED TO THE INITIAL VALUES OF BONE MINERAL DENSITY

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Background. Osteoporosis and bone fractures are a well known adverse events of steroid therapy: the majority of fractures are described within the first year of steroids administration. Plenty of data are available regarding the effects of low doses of steroids chronically administered in autoimmune diseases, but very few data are available as regards patients with lymphoma and treated with very high doses of steroids (HDS) within few months. *Aims*. To prospectively assess the incidence of pathological bone fractures and bone mineral density loss in a cohort of Non-Hodgkin-lymphoma (NHL) patients treated with R-CHOP-like

chemotherapy and thus receiving 3000-4500 mg of steroids within 3-4 months. *Materials and Methods*. From January 2005 to November 2008, 96 patients with newly diagnosed non-Hodgkin-Lymphoma and elegible for R-CHOP like therapy, were enrolled in this study. Thirthy-three were post menopausal females (34.3%), 9 pre-menopausal females (9%) and 54 were males (56.2%). All patients underwent CT scan of the vertebral spine before and within 1 months by the end of chemotherapy. Forty-one patients were also assessed for bone mineral density (BMD), using a dual-energy X-ray absorptiometer (DXA), at the spine and hip, vitD3 and ostase, before and after chemotherapy, while during chemotherapy bone and urine turnover as plasma β cross laps and urine cross-links, were serially measured. The main outcomes were the incidence of vertebral fractures and changes in bone mineral density of the lumbar spine and proximal femur. *Results*. During the 25 months study, 15 patients (15.6%) underwent vertebral fractures during or shortly after chemotherapy. In 10 cases (73.3%) in post-menopausal females, in 5 cases (33.3%) in males. Fourteen patients who were on oral biphosphonate had to discontinue it during chemotherapy because of gastric intolerance. Mean percentage changes in bone mineral density were -2.5% in the lumbar spine and -1,2% in the hip. New vertebral factures were related to the pre-chemotherapy BMD values, but not to BMD loss during chemotherapy. *Conclusions*. Vertebral fractures are a frequent event in the course of chemotherapy with high dose steroids for lymphoma. Because post menopausal females and older men with low BMD are at very high risk of developing vertebral fractures, specific preventive therapy should be given to patients with osteopenia and osteoporosis, who starts chemotherapy with HDS.

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HAEMATOPATHOLOGY DIAGNOSTIC SKILLS ACQUIRED IN A VIRTUAL LABORATORY: PRACTICAL TEACHING IN THE 21ST CENTURY

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Traditionally, acquiring laboratory skills involved donning a white coat and physically carrying out tests at the bench. There is a need in the age of 'Modernising Scientific Careers' with regional cancer-networks and a 'Knowledge & Skills' based workforce to have higher specialist training for clinicians, clinical scientists and other scientists. Higher specialist training in newly emerging specialist disciplines such as Haematopathology is difficult to acquire, it involves cross-disciplinary knowledge and training remains patchy. Short courses are available for some aspects of specialist training but these often involve attending residential courses away from the site of employment. These short courses serve a useful purpose but there are many key skills that are not covered by training courses and individuals wishing to specialise must create their own training program. Distance learning courses are popular and are successful in delivering traditional academic material. However, these courses whilst good at teaching theoretical knowledge fail to teach practical bench skills. Yorkshire and Humberside cancer network have established a model to teach practical skills via distance learning. The Haematological Malignancy Diagnostic Service (HMDS), St James's Institute of Oncology in partnership with the Epidemiology and Genetics Unit (EGU), York University has developed a Masters degree in Haematopathology that is delivered via a virtual learning environment (VLE). A novel aspect is the teaching of practical skills using a virtual laboratory, SimLab®. The course consists of six modules, three academic and three practical laboratory based. During practical modules students enter SimLab® and carry out their laboratory work using a novel program SimTest®. Students are given a series of cases based on original cases seen in HMDS. Cases cover a wide range of haematological malignancies. The structure of the course is unconventional as case time is allocated according to disease incidence i.e. chronic lymphocytic leukaemia cases are assessed frequently. Students will 'screen' the biopsy by assessing clinical information and looking at morphological images or virtual slides of the tissue biopsy. Students can order a range of tests, raw data/primary results will be available electronically for analysis/interpretation. Tests include morphology, multi-colour flow cytometry, immunohistochemistry, molecular cytogenetics (FISH/karyotype images), and PCR/gene sequencing (gel images or raw sequence text file). Where this course differs from others that provide case studies is that the students have the same starting material that is available in the 'real' laboratory. They are free to make mistakes. To mimic real life as much as possible the students have time and sample constraints imposed. Students should acquire the skills needed in an integrated haematopathology service. We believe that virtual laboratories such as SimLab® and SimTest® offer a solution to some of the problems of delivering higher specialist training.

1003

IMMUNOHISTOCHEMICAL DETECTION OF ZAP-70 IN A SERIES OF MARGINAL ZONE LYMPHOMA (MZL) PATIENTS

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Background. ZAP-70 has emerged as a prognostic factor in CLL, since it represents a surrogate marker for the IgVH gene mutational status. ZAP-70 expression in MZL is less well defined. Splenic marginal zone lymphoma (SMZL) display molecular heterogeneity since almost half of the cases carries unmutated IgVH genes, analogous to B-CLL. *Aims*. To evaluate ZAP-70 expression in a series of MZL patients and correlate these findings with other clinical and laboratory features as well as with the mutation status in SMZL patients. *Methods*. Using an immunohistochemical method we evaluated the expression of ZAP-70 in 68 MZL patients diagnosed and followed in our Departments between 1985 and 2008. The study population included 39 SMZL, 21 non-gastric MALT and 8 nodal MZL (NMZL) cases. CD3 staining was also performed in order to exclude normal reactive T-cells being present among the lymphomatous population. Cases demonstrating nuclear staining in greater than 20% of tumor cells were considered positive. For the detection of IgVH somatic hypermutation DNA was extracted from mononuclear cells of spleen tissues or bone marrow in 24 SMZL cases. DNA was amplified using a mixture of six 5' leader region specific primers for each one of the six VH families (VH1-VH6) and a 3'J consensus primer. Amplified products were next ligated and cloned and plasmid DNA was isolated from overnight cultures of randomly selected colonies. At least five plasmids per case were sequenced for both orientations. Clinical, laboratory and outcome data of all patients were recorded and evaluated in relation to ZAP-70 expression. Furthermore in SMZL cases, ZAP-70 expression was evaluated in relation to mutational status of IgVH genes. *Results*. Of the 68 patients studied for ZAP-70 expression only six cases (9%) were found to be positive. Thus, ZAP-70 positivity was detected in three of the 39 (8%) SMZL, in one of the 21 (5%) MALT lymphomas and in two of the 8 (25%) NMZL patients. 24 SMZL cases were simultaneously studied for the detection of their IgVH somatic hypermutation status. 14 (58%) of them were found to be mutated and 10 (42%) unmutated. Of the 3 ZAP-70 positive SMZL cases, all three belonged to the unmutated IgVH group while no positive ZAP-70 cases was detected in the mutated group. Due to the small number of ZAP-70 positive cases in the individual groups studied, it was not possible to perform a meaningful analysis in relation to the clinical and laboratory features as well as survival data of our patients. *Summary and Conclusions*. ZAP-70 is expressed in MZL, although in significantly lower rate than that of CLL. In the present study only 9% of MZL patients were positive for this protein. NMZL presented the higher rate of expression. In SMZL no correlation was found between mutational status and ZAP-70 expression, a finding that clearly shows that ZAP-70 cannot be used as an alternative marker for the mutational status of IgVH genes in SMZL.

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OUTCOME OF AGGRESSIVE ATL-HTLV1 PATIENTS AFTER CHOP21 AS FIRST LINE TREATMENT - BUCHAREST EXPERIENCE

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Background. Adult T-cell Leukemia/lymphoma (ATL) is a peripheral T-cell malignancy caused by a retrovirus, HTLV-1. ATL has the most unfavorable prognosis compared to B-cell non-Hodgkin Lymphoma and

Peripheral T-Cell Lymphoma. Large clinical trails for ATL were conducted in Japan by the Japan Clinical Oncology Group (JCOG). In Europe, the majority of newly diagnosed patients with ATL are treated with CHOP21 as first line therapy, although a large trial to asses the efficiency of this approach in aggressive ATL subtypes does not exist, mainly because the specific geographical distribution of ATL. *Aims*. Evaluating the efficiency of CHOP21 as first line treatment on aggressive ATL-HTLV1 patients. *Methods*. We have retrospectively evaluated 34patients diagnosed with ATL-HTLV1 positive, between January 1998 and January 2007 in Hematology Clinics of the University of Medicine and Pharmacy Carol Davila Bucharest. All patients previously untreated received CHOP21 as first line treatment. We have analyzed the rate of complete response (CR), partial response (PR), rate of response (RR), median time until relapse, median survival time (MST) and 2 years overall survival (OS). Patients' baseline characteristics: median age was 44.5 years (range 19-68), and 20patients (58.8%) were male. Among the 34patients, 19patients (55.9%) were diagnosed with acute ATL, 12patients (35.3%) with lymphoma subtype, and 3patients (8.8%) with chronic ATL subtype. B symptoms were present at 18patients (52.9%). Central nervous system involvement at onset was detected in 4patients (12.5%). 30patients (88.2%) of ATL-HTLV1 positive presented the classic T-helper phenotype ($CD4^+$ $CD8^-$), 3patients (8.8%) presented T-suppressor ($CD4^-$ $CD8^+$) and 1patient (2.9%) had an atypical T phenotype $CD4^+$ $CD8^+$. Hypercalcemia was present at 17patients (50%) and increased serum LDH (over 3times normal value) in 14 patients (41.2%). *Results*. Complete response to CHOP21 was observed in 8patients (23.5%), partial response in 6patients (17.6%), adding up to a 41.1% RR. Regarding the persistence of the response, the median time until relapse was 18 months, range 2.0-60.0. MST was 5months and the 2 years OS was 20.5%. We analyzed the response to CHOP21 in each ATL subtype. In acute ATL, 1patient (5.2%) had CR, and 2patients (10.5%) had PR, with a median time of response of 4months, range 2.0-24.0. In lymphoma subtype, 5patients (41.6%) had CR, 2patients (16.6%) had PR, with a 58.2% RR; median time until relapse was 11months, range 3.0-36.0. In chronic ATL subtype, 2patients (66.6%) had CR, and 1patient had PR, with a median time to response of 48months, range 6.0-60.0. *Conclusions.* CHOP21 is not a satisfactory therapy for aggressive ATL; this approach showed a modest benefit. A literature search revealed that the best results (81% RR, 35% CR) are obtained with JCOG LSG15 (VCAP/AMP/VECP) a multidrug regimen supported by G-CSF which included drugs that are not affected by P-glycoprotein expression. This regimen should be as well a standard therapy for aggressive ATL in Europe. Several reports have demonstrated the clinical benefit of allo-HSCT for ATL patients, the only approach that can cure. Other potential therapeutic compounds are studied.

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INTENSIFIED CHOP (ICHOP) ± RITUXIMAB IN PRIMARY MEDIASTINAL DIFFUSE LARGE B CELL LYMPHOMA (PMBCL): THE ROLE OF DOXORUBICIN/ CYCLOPHOSPHAMIDE DOSE-INTENSITY

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Background. PMBCL is a clinical/biological distinct entity, sharing some characteristics with both classical DLBCL and Hodgkin's lymphoma. MACOP B is considered the treatment of choice. *Aims*. To evaluate the role of doxorubicin/cyclophosphamide dose intensity in this setting of patients.Methods Starting from 1997, we treated PMBCL with an ICHOP regimen including cyclophosphamide 1750 mg/mq with MESNA uroprotection, doxorubicin 75 mg/mq, vincristine 1.4 mg/mq with 2 mg cap, and prednisone 100 mg d 1-5 of each 14-day courses, GCSF from day 7 to day 12. Rituximab (R) 375mg/mq/course was added to ICHOP (R-ICHOP) from 2002. Treatment plan included five courses of ICHOP±R. Cases with unfavourable prognosis according to age-adjusted International Prognostic Index (aaIPI2-3) were submitted to high dose chemotherapy (HDT) and peripheral stem cell rescue. Radiotherapy on involved sites was then delivered to all patients if at least partial remission (PR) was reached. Clinical response was evaluated through CT +/- Gallium scan (14 pts) up to 2002, and thorough CT + PET scan (16 pts) thereafter, according to Cheson criteria. *Results*. up to 2006, 30 pts were treated, with the following characteristics: M/F 10/20, median age 34 years (range 22-53), Ann Arbor stage I: 4, II -IIE:19, III: 1, IV: 6; bulky disease: 29; B symptoms: 14; aa IPI 0-1: 24, 2-3: 6; RICHOP / ICHOP 21/9. After ICHOP±R 15 patients achieved complete (CR) or unconfirmed complete remission (CR-U), 14 PR, 1 stable disease. At the end of the whole program 29/30 pts reached CR and one progressed. Seven pts received HDT, six following ICHOP±R and one after II line chemotherapy for refractory disease. After a median observation time of 60 months 1 patient progressed and 1 patient relapsed, respectively. Both died of lymphoma. One patient with stage IIE IPI 0 relapsed 18 months after completion of ICHOP and RT and died after further 5 treatment lines including alloBMT. The other patient with stage II EB IPI 1, progressed shortly after R-ICHOP and RT and died five months later. Five-yr failure free survival and overall survival are 93.2 and 92.8, respectively. ICHOP±R was well tolerated, with neither toxic death or life-threatening toxicity. No patient interrupted the planned treatment because of toxicity. Hospitalization was required in seven cases due to febrile neutropenia (6), hemorrhagic cystitis (3 cases), and pneumonia (1). Five episodes of grade III-IV mucositis were observed in 4 patients. Of 147 delivered cycles, 25 were delayed (13 pts). *Conclusions*. in PMBCL, the results obtained with the ICHOP protocol are better than standard CHOP and comparable to MACOP-B, emphasizing the role of doxorubicin and cyclophosphamide dose-intensity. In this limited series, the impact of adding rituximab is not clear.

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SUBCLINICAL ATHEROSCLEROSIS IN YOUNG B-THALASSEMIA MAJOR PATIENTS

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Background. Although clinical atherosclerosis usually occurs in late age, recent studies have shown that it begins in childhood in the presence of risk factors. Endothelial dysfunction which is an important precursor of atherosclerosis has been demonstrated in β thalassemia major (B-TM) patients, who are subjected to peroxidative tissue injury because of continuous blood transfusions. *Aims*. To assess subclinical atherosclerosis in young β-TM by measurement of carotid intima thickness (CIMT) and the associated risk factors including dyslipidemia in comparison to young type 1 diabetics as a high risk group for premature atherosclerosis. *Study design*. This study is a cross sectional study including 90 children and adolescents divided into 3 groups: 30 β-TM patients of mean age 18.4±6.18 years(Group I); 30 type 1 diabetic patients of mean age 19.23±4.25 years(Group II); and 30 healthy subjects age and sex matched (control group). The followings were done for all studied subjects: history,clinical examination and investigations including: fasting lipid profile assessing serum total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG) and lipoprotein (a), Apoprotein A, Apoprotein B; as well as hemoglobin electrophoresis and serum ferritin for thalassemics; random blood sugar and glycosylated hemoglobin for diabetics. High resolution ultrasound for measurement of carotid intima thickness (CIMT)was done for patients and controls. *Results*. There was a significant increase in serum TG and TC levels in thalasemia and type 1 diabetics compared to controls (p<0.001 and p=0.003, respectively). Type 1 diabetics showed the highest level of LDL-cholesterol compared to thalassemic patients and controls (p<0.0001). Patients with TM and type I diabetics had significantly lower HDL-C compared to controls (p<0.0001). Apo A level was significantly higher in thalassemic and diabetic patients compared to controls (p<0.001). Apo B level was significantly lower in thalassemics and diabetics compared to controls (p<0.001). A significant increase in CIMT was found in thalassemics and diabetic patients compared to controls (p<0.001). In TM patients, CIMT was positively correlated with age (r=0.39, p=0.05), HbF (r=0.5, p=0.004), serum ferritin (r=0.73, p=0.001) and serum cholesterol (r = 0.59; p=0.001). In diabetic patients CIMT was positively correlated with age (r=0.52, p=0.003), random blood sugar (RBS) (r=0.43, p=0.02), hemoglobin A1c (HbA1c) (r=0.59, p=0.001) and serum TC (r=0.45, p=0.01). *Conclusions*. Atherogenic lipid profile was prevalent in young β-TM with increased carotid intima thickness (CIMT) suggesting increased risk of premature atherosclerosis. CIMT assessment can be used as a prognostic factor for vascular risk stratification and planning for preventive measures.

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SILICA CLOTTING TIME IN LABORATORY DIAGNOSIS OF LUPUS ANTICOAGULANT (LA). AN USEFUL TOOL FOR DIAGNOSIS OF ANTIPHOSPHOLIPID SYNDROME (APS) IN PATIENTS ON ANTICOAGULANT TREATMENT

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Background. LA autoantibodies can interfere with phospholipid-dependent clotting tests *in vitro*, and inhibit both the common and intrinsic pathways of coagulation. The activated partial thromboplastin time (APTT), and the dilute Rusell's viper venom time (dRVVT) are currently the most frequently utilized assays. Silica clotting time (SCT) is not affected by oral anticoagulants and could be a very sensitive/ specific test for detecting LA. *Aims*. To evaluate the sensitivity and specificity of the SCT for detecting LA in patients with clinical criteria of APS, with/without associated connective tissue disease or prolonged APTT compared to the results obtained by APTT and dRVVT assays. *Patients and Methods*. Along a period of two months, 220 patients underwent evaluation for suspected hypercoagulability status. In cases with clinical criteria of APS according to the Sapporo International Consensus Statement, the clinical files (demographic, clinical and inherited and acquired thrombophilic risk factors) of patients, were reviewed. PTT-LA, dRVVT and SCT were performed using an ACL TOP-3G automatized coagulometer (Instrumentation Laboratory). *Results*. 220 patients were evaluated for hyperocoagulable status (including LA). Positive result for LA was detected in 43 patients (19.5%) male 18 (41.8%) and female 25 (58.1%) with a median age of 50 years (range: 5-82). Clinical events were deep vein thrombosis/pulmonary embolism 14 (32.5%); cerebrovascular disease 9 (20.9%); arterial ischaemia 5 (11.6%); venous and arterial thrombosis 2 (4.6%); obstetric complications 1 (2.3%); connective tissue disease 8 patients (18.6%) and 4 (9.3%) prolonged APTT. 14 of 43 (32.5%) were under oral anticoagulant treatment (OAT). 17 patients were diagnosed of APS on the basis of clinical and biological data. dRVVT were positive in 40 (93.02%) and 18 patients (41.8%) for SCT. Sensitivity and specificity: SCT relative to dRVVT 50% and 66.6%. PTT-LA relative to dRVVT 42.5% and 66.6%. In the 14 receiving OAT sensitivity and specificity values were: SCT relative to dRVVT 46.1% and 100% respectively. In 17 APS patients sensitivity and specificity values were: SCT relative to dRVVT 70.6% and 100%. In patients with APS receiving AOT sensitivity and specificity values increased: SCT relative to dRVVT 85.7% and 100% respectively. The presence of vascular thrombosis in patients diagnosed of APS was compared to the dRVVT and SCT: 9 patients (53%) showed deep vein thrombosis/pulmonary embolism, 2 (11.7%) showed cerebrovascular disease, 2 (11.7%) showed obstetric complications and 4 (23.5%) showed arterial ischaemia. In 12 patients (70.5%) dRVVT and SCT were both positive, in the remaining 5 patients (29,41%) SCT was negative and dRVVT positive, altough 2 of these patients showed a post-surgical thrombosis. *Conclusions*. 1) In our serie Sensitivity of SCT relative to dRVVT is similar to previously reported, but specificity values are lower. 2) Higher values concerning sensitivity and specificity of SCT relative to dRVVT were obtained in APS patients receiving OAT, according with previously reported data. 3) In our experience SCT seems to be an useful tool in laboratory diagnosis of LA specially in patients on OAT.

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POTENTIAL FUNCTION OF EGF1 DOMAIN PEPTIDE IN ANTICOAGULATION THERAPY: BINDING TO TISSUE FACTOR WITHOUT THE ABILITY OF COAGULATION

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Background. Tissue factor (TF) is exposed to blood upon vascular damage and binds to activated factor VII (FVIIa) to initiate blood coagulation cascade *in vivo*. The first epidermal growth factor-like (EGF1) domain is reported to be important for activation of FVII/TF and we presume that the clone and expression of EGF1 domain peptide would be helpful in studying the anticoagulation drugs based on FVIIa/TF interaction. *Aims*. To explore the potential function of EGF1 domain peptide in anticoagulation therapy.

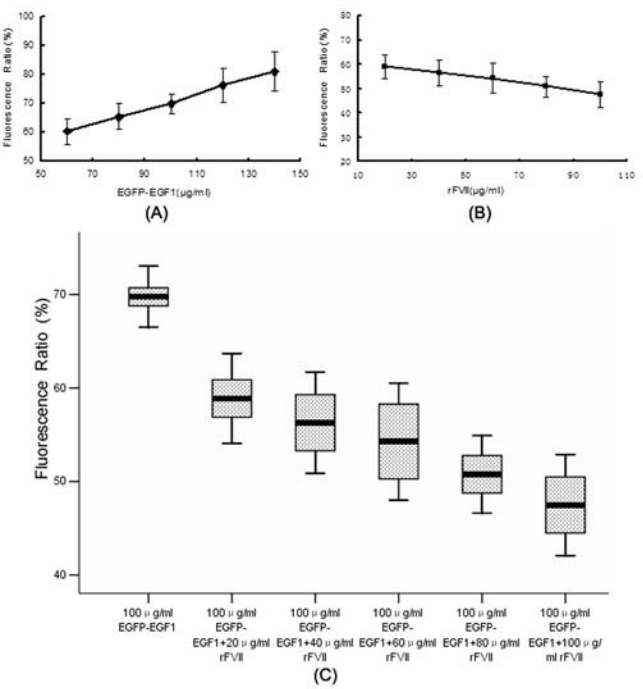

Figure 1. Competition assay of EGFP-EGF1 protein by FASCS.

Methods. We amplified the EGF1 domain from a rat liver and the EGFP-EGF1 fusion proteins were expressed in E.coli BL21. The affinity of rat soluble TF (sTF) was quantitated by surface plasmon resonance (SPR). Subsequently, the model of rat TF (rTF) expression *in vitro* was established by lipopolysaccharide induction. The affinity of TF expressed cells was analyzed by confocal microscope and flow cytometry. Blood clotting activity was tested by prothrombin time assay using FVII depleted human plasma. *Results*. The SPR results indicated the association constant ka of EGFP-EGF1 proteins was higher when compared with rat FVII (rFVII) (8.29±1.39 VS 3.75±0.32, $p<0.01$). The rFVII could definitely depress the integration of EGFP-EGF1 with rTF (69.76±3.29% VS 58.88%±4.81%, $p<0.01$). However, the EGFP-EGF1 proteins lost the activity of coagulation when compared with that of blood plasma of normal SD rats (56.8±3.2s VS 17.8±3.4s, $p<0.01$). *Conclusions*. The rat EGF1 domain peptide could specifically bind to TF without the ability of coagulation, which might be a molecular target for anticoagulation therapy.

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RELATIONSHIP BETWEEN INR MEASURED WITH DIFFERENT THROMBOPLASTINS, COAGULATION FACTOR LEVELS AND THROMBIN GENERATION IN PATIENTS RECEIVING ORAL VITAMIN K ANTAGONISTS

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Background. The International Normalised Ratio (INR) is the standard method for monitoring oral anticoagulant therapy with vitamin K antagonists. In clinical practice we observe variation in INR measured with different thromboplastins. In contrast to the prothrombin time (PT) thrombin generation (TG) assays offer a more global assessment of coagulation. *Aims*. We evaluated whether differences in INR could be explained by sensitivity of thromboplastins to coagulation factor levels. We studied also the association between INR and parameters of TG. *Methods*. INR was measured with 6 thromboplastins (Coaguchek S®-Roche, Thromborel S®- Siemens, Innovin®- Siemens, Thrombotest®- Axis-Shield, Neoplastine CI Plus®- Stago, Neoplastin R®- Stago) on 83 plasmas of anticoagulated patients (in a range of INR-Coaguchek between 0.9 and 4.7). Innovin and Neoplastin R are recombinant thromboplastins, while all others are not. FII, FVII, FX and FV levels were determined by one stage PT-based clotting assay triggered with Innovin and Neoplastin CI plus. TG was measured by Calibrated automated Thrombinography (CAT) triggered with Innovin. *Results*. Passing and Bablok regression comparing the point of care method (Coaguchek) and the INR by Innovin, Thrombotest and Neoplastin R yielded a slope of 1.077 (95% confidence interval, CI, 1-1.2), 0.967 (95% CI: 0.875-1), 1.008 (95%CI: 0.885-1.118), respectively, and an intercept of -0.096 (95%CI: -0.4 to 0.1), -0.133 (95%CI: -0.2-0.131), -0.108 (95% CI: -0.362-0.229), respectively. Comparison of Coaguchek with Thromborel S and Neoplastin R yielded CI of slope and intercept indicating a difference between methods. Paired Wilcoxon test comparing the FII, FVII and FX PT-based clotting assays triggered with Innovin and Neoplastin CI plus showed significant different levels ($p<0.0001$). Correlation between all INR values and mentioned coagulation factor levels was good, with the highest Pearson correlation coefficient (r) for INR-Thromborel S and factor assays performed with Innovin. A progressive decrease of endogenous thrombin potential (ETP) and peak height (PH) was observed as the INR increases. ETP and PH correlated well with INR values obtained with all thromboplastins (r for ETP ranged from -0.601 to -0.684 and for PH from -0.576 to -0.678). Correlation between coagulation factor levels and ETP and PH was good. FII PT-based clotting level triggered with Neoplastine CI Plus correlated best with ETP (r= 0.873) and PH (r=0.838). No correlation was found with the lag time. 33 samples out of the 83 showed a difference of INR-Coaguchek and INR-Innovin of more than 10% (-22.22 to 24.0%). In this subpopulation coagulation factor levels were comparable with those in the total population (Man Whitney test p= 0.1920-0.8256). ETP correlated well with INR values obtained with all thromboplastins (r from -0.500 to-0.621). *Conclusions*. Our results show that TG and INR are closely correlated. There is no difference in correlation between recombinant and non-recombinant thromboplastins. Despite the efforts of standardisation by expressing results of PT in INR in anticoagulated patients, differences in PT-INR are observed depending on the thromboplastins used. The differences in INR measured with different thromboplastins can not be explained by sensitivity of thromboplastins to coagulation factor levels.

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ENDOTHELIAL KLF4 LINKS PRO-INFLAMMATORY LEVELS TO THE EXPRESSION OF PAI-1 AND VWF GENE

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Background. The vascular endothelium plays a critical role in vascular homeostasis and maintains blood fluidity. Both biomechanical and biochemical stimuli can affect endothelial gene expression. *Aims*. Identification of factors that mediate the effects of these stimuli on endothelial function is of considerable interest. Pro-inflammatory cytokines can affect prothrombotic properties and regulate blood fluidity. Some studies indicated that endothelial cells can express Krüppel-like factor 4(KLF4), which may have an anti-inflammatory effect. The role for KLF4 in endothelial thrombotic function has not been elucidated. *Methods.* The human umbilical vein endothelial cells(HUVECs), which has been transfected by recombinant adenoviral vector carrying KLF4 antisence RNA, were treated with human IL-1β, TNF-α at final concentrations of 2.5 ng/mL and 10 ng/mL, respectively, for 4 hours. The mRNA expression and the protein diversity of plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF) were detected by virtue of Real Time-PCR and Western blot, meanwhile, the expression and location of KLF4 were observed by confocal laser microscope. *Results*. Our study observed that pro-inflammatory mediators significantly up-regulated endothelial KLF4, PAI-1 and vWF expression. Comparing with the control infected cells, in the experimental cells, down-regulated of KLF4 leads to increase of TNF -α and IL-1β induced endothelial cell PAI-1 and vWF expression. Conclusions KLF4 can suppress the expression of PAI-1and vWF under basal and cytokine-stimulated conditions. These observations implicate that, in response to pro-inflammatory stimuli, KLF4 is an anti-thrombotic factors.

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THE ANTITHROMBOTIC AND BLEEDING EFFECTS OF DABIGATRAN, A DIRECT THROMBIN INHIBITOR, AS COMPARED TO ENOXAPARIN, HEPARIN AND WARFARIN IN RATS

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Dabigatran etexilate is an orally available direct thrombin inhibitor approved in Europe for the prevention of DVT following orthopedic surgery. Chronic treatment for stroke prevention in patients with atrial fibrillation and VTE treatment Phase III studies are ongoing. Comparators in these studies are not only heparins, but also warfarin. *Objective*. To determine the antithrombotic efficacy of dabigatran as compared to clinically used agents in an arterial venous (AV) shunt model in the rat and compare this to the bleeding potential of these compounds in the same species. *Methods*. The antithrombotic doses of dabigatran, heparin, enoxaparin and warfarin were tested in a rat AV shunt model (determined as ED50). All compounds were given as an iv infusion over 1 hr, except warfarin, which was dosed orally for 4 days. Thrombus formation was measured over 15min within the shunt. Bleeding was measured in the rat tail model using a similar dosing scheme. Standardized cuts were made in the tail of the anesthetized rat following increasing doses of the coagulation inhibitors. The time until bleeding stopped was recorded. Blood sampling for plasma levels and clotting tests were also performed. *Results*. A dose-dependent antithrombotic effect was obtained with all compounds. The dose that resulted in 50% thrombus inhibition (ED50) was 0.07 µmol/kg/hr for dabigatran, 0.02 µmol/kg/hr heparin (~50 U/kg), and 0.07 µmol/kg/hr (~0.3 mg/kg) enoxaparin and 0.58 µmol/kg for warfarin (~0.18 mg/kg oral daily dose). All compounds also had increased bleeding with increased doses. Doses of dabigatran up to 15-fold the ED50 were required before significant bleeding was observed in this model, similar to both heparin and enoxaparin. Doses of warfarin, however, only 2-3-fold the ED50 resulted in significant bleeding. *Conclusions*. Direct inhibition of thrombin by dabigatran is as effective and potent as clinically used antithrombotic agents. Its safety profile is also similar to heparin and enoxaparin, the latter being confirmed in Phase III clinical trials after orthopedic surgery. However, the safety profile in this model was improved vs warfarin, which may be a further benefit for chronic antithrombotic therapy with dabigatran.

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MIRNA EFFECT ON THE SEPTIC PHENOTYPE

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Background. MicroRNAs are small non-coding regulatory RNAs that control the expression of more than one third of the human genes. Although some studies have explained the role of miRNAs in immunity and inflammation, no information is yet available about their role in sepsis. *Aims*. to identify which miRNA species are involved in regulating the function of the endothelial cells in a state of sepsis and whether activated protein C (APC)- a common treatment in severe sepsis- can affect the expression of these miRNAs. *Methods*. endothelia cells (line EA.hy926) were treated in duplicates as follows: baseline (no treatment), 20 μg/mL LPS, 100nM APC and a combination of 20 μg/mL LPS and 100 nM APC. miRNAs were isolated from the cells using mirVanaTM miRNA Isolation Kit (Ambion). Reverse transcription to cDNA and quantitative real time PCR (qRT-PCR) was performed in an array to show the expression profile of a wide range of miRNAs. *Results*. The following miRNAs showed a fold change of ≥1.5 in a simulated sepsis state (miR-142-5p, miR-142, miR-30d, miR-191, miR-17, miR-130a, let-7i, miR-93, miR-106b, miR-150, miR-186, miR-222, let-7b, miR-27a, let-7f, miR-22, miR-122, miR-302a, miR-7, miR374b, miR-196b, miR-302b, miR-28-3p and miR-302c). The expression of most miRNAs was reduced by APC. Five miRNAs showed dramatic reduction in expression by APC (miR-374b, miR-302c, let-7f, miR-106b and miR-27a). *Conclusions*. several miRNAs are affected by the mimicked sepsis phenotype and they might play a role in controlling endothelial function during sepsis. APC treatment abrogates the effect of LPS on miRNA expression. We propose that these changes will have a functional effect on endothelial cells and this will be our next project.

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GENETIC VARIABILITY OF FIBRINOGEN A-CHAIN GENE DEFINES FIBRINOGEN LEVELS BETWEEN HEALTHY INDIVIDUALS AND PATIENTS WITH DOCUMENTED ATHEROSCLEROSIS: EFFECTS ON PROTHROMBOTIC PROFILE

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Background. Fibrinogen plays an key role in atherogenesis. A genetic polymorphism on fibrinogen a-chain, the G58A, has been associated with fibrinogen levels in healthy individuals, but its effect on patients with coronary artery disease (CAD) is still unknown. In the present study we examined the impact of this polymorphism on fibrinogen levels and its relation to the prothrombotic profile. *Methods*. The study population consisted of 230 subjects, 179 of which with angiographically documented CAD and the rest with angiographically documented absence of any significant coronary stenoses. The G58A polymorphism was detected by Polymerase Chain Reaction (PCR) and appropriate restriction enzymes. Fibrinogen levels were measured by a nephelometric method, while other factors of thrombosis such as plasma levels of D-dimers, factors V, X, plasminogen were measured by standard coagulometry techniques. *Results*. The genotype distribution was GG: 39.6%, AG: 40.2%, AA: 20.2% and GG: 37.3%, AG: 49.0%, AA: 13.7% for CAD patients and healthy individuals respectively. Among the three genotypes there was no significant difference in fibrinogen levels of CAD patients (128.6±32.4 vs 115.8±29.5 vs 127.8±33.4 mg/dL, p=NS for all). Patients with CAD had significantly higher levels of fibrinogen than healthy individuals regarding to the G58A polymorphism (456.3±131.2 vs 385.3±102.0 mg/dL, $p<0.001$). In addition, there were significant differences fibrinogen levels between the same genotypes of the two populations (CAD vs healthy, AA: 477.5±123.1 vs 386.8±62.7, GG: 452.4±146.3 vs. 374.8±114.0, AG: 449.1±119.3 vs 393.6±104.0 mg/dL $p<0.05$ for all). Similarly, d-dimers levels were significantly higher in the CAD than healthy subjects regarding to the G58A polymorphism (555.8±628.7 vs 360.3±336.7 mg/L). On the contrary, levels of thrombotic markers did not differ significantly between CAD and healthy individuals: fV (124.0±31.8 vs 115.0±25.1%), fX (94.2±23.2 vs 90.3±18.7%), plasminogen (109.5±19.0 vs 107.7±14.7 u/mL) p=NS for all. *Conclusions*. Genetic polymorphism G58A on fibrinogen a-chain gene fails to affect the prothrombotic profile as healthy subjects and CAD patients presented with no differences on specific markers. On the contrary, it turns to be effective on fibrinogen levels implying a potential mechanism which promotes atherosclerosis.

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THE IMPACT OF THE GENETIC POLYMORPHISM G455A OF FIBRINOGEN B-CHAIN GENE ON FIBRINOGEN LEVELS, LOW-GRADE INFLAMMATORY PROCESS AND ENDOTHELIAL FUNCTION IN PATIENTS WITH ADVANCED ATHEROSCLEROSIS

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Background. The genetic polymorphism G455A on fibrinogen b-chain gene has been associated with fibrinogen levels in healthy individuals, but its effect on fibrinogen levels, low-grade inflammation and endothelial function in patients with advanced atherosclerosis remains unknown. Therefore, in the present study we examined the impact of this polymorphism on fibrinogen levels and other pro-thrombotic molecules, low-grade inflammation and endothelial function in patients with coronary artery disease (CAD). *Methods*. The study population consisted of 293 patients undergoing cardiac catheterization with stable or no CAD. The G455A polymorphism was estimated by PCR and suitable restriction enzymes. Serum levels of C-reactive protein (CRP) and fibrinogen were measured by nephelometric methods. In addition, levels of sCD40-ligand (cd40L) were determined by enzyme-linked immunosorbent assay (Elisa). Endothelial function was measured by estimating flow-mediated dilation of the brachial artery (FMD), with ultrasound. *Results*. The genotype distribution was GG: 51.8%, GA: 37% and AA: 11.2%. There were significant differences among fibrinogen levels across the genotypes of the studied population (AA: 517.4±145.0, GA: 431.5±130.0, GG: 434.2±127.3 mg/dL, $p<0.01$ for AA vs GA and AA vs GG respectively, p=NS for GA vs GG). Among patients with CAD AA patients had significantly higher levels of fibrinogen only than GA patients (AA: 517.5±144.0, GA: 434.0±132.2, GG: 443.0±121.0 mg/dL $p<0.05$ for AA vs GA). C-reactive protein and sCD40L levels did not differ among the study genotypes GG (2.8±4.3 mg/L and 2.4±1.1 μg/mL), GA (3.1±4.5 mg/L and 2.9±2.1 μg/mL) and GG (3.5±3.5mg/L and 2.9±1.3 μg/mL) p=NS for all. Similarly, in patients with established CAD there was no significant difference in CRP or sCD40L levels between GG (2.9±5.2mg/L and 2.5±1.2 μg/mL), GA (2.5±2.5 mg/L and 3.0±2.1μg/mL) and AA (3.1±2.6 and 2.7±1.2mg/L) p=NS for all. Despite the fact that AA patients with CAD had lower FMD than GG and GA this difference did not reach any statistical significance AA (3.3±3.7%) compared to AG(4.7±3.0%) and GG(4.3±4.0%) p=NS for all. *Conclusions*. Our findings suggest that there is a significant correlation between specific genotypes of G455A polymorphism and fibrinogen levels in patients with CAD. On the contrary, this genetic polymorphism fails to affect thrombotic mechanisms, endothelial function and low-grade inflammation in these patients. These findings indicate that the genetic polymorphism G455A on fibrinogen b-chain gene affects fibrinogen levels independently of inflammatory process and endothelial function in patients with CAD.

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A NORMAL D-DIMER VALUE, MEASURED BY A HIGH SENSITIVITY ASSAY, RULE OUT A SUSPECTED VENOUS THROMBOEMBOLISM EPISODE INDEPENDENTLY OF PRETEST CLINICAL PROBABILITY

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Background. In only 13 to 25% of patients with clinical Venous Thromboembolism (VTE) suspicion, the disease is confirmed by an image study. Academically is recommended, before practice any exploration, to asses the clinical pre-test probability, following explicit rules (ei. Wells criteria); so, when a patient has a low or moderate pretest clinical probability, a normal D-Dimer, measured by a non-necessarily high sensitivity method but previously evaluated, rule-out a VTE episode. However, in general clinical practice, out of clinical trials, these explicit rules are rarely employed. Aims. To prove that the diagnostic strategy to systematically measure D-Dimer levels, whatever the explicit criteria are to all suspicions of VTE, and avoiding the image determination to the patients with normal levels, is safe for them and cost-beneficial for the Institu-

tion. *Methods*. Observational survey and management study, carry out between 12/2006 to 09/2008, in a University Hospital. From all daily D-Dimers orders and image explorations practiced, we register all suspicions of VTE consecutively raised. Deep Vein Thrombosis (DVT) was diagnosed by ultrasounds, while Pulmonary Embolism (PE) was diagnosed by CT or Lung Scan. Blood levels of D-Dimer were measured by a quantitative ELISA by fluorescency rapid assay (D-Dimer Exclusion, VIDAS, BioMerieux, Lyon, France). A cut-off of 500 ngr/mL was chosen. All patients with a normal D-Dimer value were followed by phone calls, for three months, asking for safety to avoid anticoagulants. From these data, a cost-benefit analysis was practiced. *Results*. A total of 3051 suspected VTE episodes were detected. D-Dimer was measured in 2240 cases. From 529 suspicions with a normal D-Dimer value, only one episode of VTE was found (a relapsed DVT) (Table). Similar prevalence in VTE was found, in the suspicion group with high levels of D-Dimer measured (25.07%) that in non-measured group (23.10%). [Chi-square = 1.43, p=0.23, odds ratio 1,13 (CI 95=0.92-1.37)].That's why other criteria, than pre-test clinical probabilities, were used for obviate D-Dimer ordering. D-dimer measurement to all suspicions of VTE is cost-benefit if the prevalence of patients with normal D-Dimer levels is more than 19.7% for DVT or 17,5% in PE suspicions. Increasing age had a lesser probability to found a normal D-Dimer, with values of 29%, 19% and 16% in segments of 60-69, 70-79 and 80-84 years old respectively. Cancer was found in 583 patients in D-Dimer measured group, evidencing a normal D-Dimer value in 138 cases (23.6%). With the strategy to measure D-Dimer to all suspicions of VTE, we compute a saving cost for each VTE episode of 72,6 € for DVT founded, 160,7 € for PE diagnosed by lung scan and 372,2 € for each detected PE when CT is employed. *Conclusions*. D-Dimer determination by this method to all patients with suspicion of VTE, independently of the pretest clinical evaluation, could reject one out of every four patients with safety, providing that the patients in anticoagulant treatment or relapsing episodes are excluded, and also with an important cost reduction in image explorations, if very older patients are excluded.

Table 1.

	Venous Thromboembolism				
		Present	Absent	Total	PPV / NPV (CI 95 %)
D-Dimer	Increased > 500 ngr/ml	**429**	**1282**	1711	PPV **25,07 %** (23,03 – 27,13)
	Normal < 500 ngr/ml	**1**	**528**	529	NPV **99,81 %** (99,79 – 99,83)
	Total	430	1810	2240	
	Sensitivity **99,77 %** CI 95%(98,71 -99,99 %)		Specificity **29,17 %** CI 95%(27,32-31,56 %)		Prevalence **19,2 %**
	Likelihood Ratio Negative **0,008** CI 95%(0,001 – 0,05)		Likelihood Ratio Positive **1,409** CI 95%(1,37 – 1,45)		**NNT = 4,2**

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ELEVATED LEVELS AND DIFFERENT SUBSETS OF PROCOAGULANT MICROPARTICLES IN BREAST CANCER PATIENTS USING HORMONAL THERAPY

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Background. Circulating microparticles (MP) are known to be elevated in cancer and thromboembolic disease. Since breast cancer patients using hormonal therapy have an increased risk for thrombosis, we hypothesized a role for MP in this hypercoagulable state. *Methods*. Plasma samples were collected from 20 breast cancer patients without metastatic disease using adjuvant hormonal therapy, 20 patients with metastatic breast cancer using hormonal therapy and from 20 female controls. The hormonal therapy used was either an anti-estrogen or an aromatase inhibitor. Levels and cellular origin of MP were determined by flowcytometric analysis. In addition, the MP-induced thrombin generation (TG) was determined using the calibrated automated thrombogram method. *Results*. Breast cancer patients had higher levels of MP expressing Annexin V (median 8200 vs 6540×10^6/L, p=0.03) P-selectin (CD62P, median 330 vs 200×10^6/L, p=0.01), Tissue Factor (median 33 vs 15×10^6/L, p=0.001), and of MP derived from epithelial cells (CD227, MUC1) (p=0.005), leucocytes (CD45) (p=0.005) and endothelium (CD144) (p=0.002). MP- associated thrombin generation showed a higher peak height (79 vs 69 nM, p=0.04) and area under the curve (AUC) (1130 vs 1029 nM.min, p=0.006) in breast cancer patients. Both for MP levels and TG parameters no differences were observed between the metastatic and adjuvant group, nor between the anti-estrogens and aromatase inhibitors. *Conclusions*. Altered MP subset characteristics in breast cancer patients using hormonal therapy, especially the higher number of Annexin V and P-selectin positive MP, may induce heightened procoagulant activity. Presence or absence of metastatic disease seemed to have smaller or no impact on these parameters, probably reflecting the inactive disease state of breast cancer patients on endocrine treatment.

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ROLE OF JAK2 MUTATION IN PATIENTS WITH SPLANCHNIC VEIN THROMBOSIS: AIIMS EXPERIENCE

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Background. Splanchnic veins thromboses which include portal vein thrombosis and thrombosis of the hepatic veins causing Budd Chiari syndrome are frequent presenting complication of an undiagnosed myeloproliferative disorder (MPD). The recently identified JAK2 V617F somatic mutation that occurs in MPD patients is a risk factor for portal, hepatic and mesenteric venous thrombosis, independently of the presence of overt MPDs. Screening of JAK2 mutation may be useful in identifying patients who should be carefully observed for the subsequent development of overt MPDs. In view of this, we studied JAK2 V617F mutation in patients with splanchnic vein thrombosis. *Materials and Methods*. A total of 26 patients with splanchnic vein thrombosis and without cirrhosis of liver were screened by ARMS technique for JAK2 mutation. These patients presented to hematology and gastroenterology Department, All India Institute of Medical Sciences, New Delhi, India. The distribution of cases was as follows; 11 portal vein thrombosis (PVT), 13 Budd Chiari syndrome (BCS) and two cases having both PVT and mesenteric venous thrombosis (MVT). *Results*. The JAK2 V617F mutation was identified in 3/13 cases with BCS (23%) and 2/11 patients with PVT (18%). None of the patients with MVT were positive for JAK2 mutation. Overall 5/26 patients (19%) with splanchnic vein thrombosis were positive for JAK2 V617F mutation. Patients with JAK2 V617F mutation had higher median TLC as compared to those who were negative for JAK2 mutation (11.2×10^9/L vs. 6.3×10^9/L). The median platelet count in patients with JAK2 positivity was 207×10^9/L and 105×10^9/L in patients who were JAK2 negative. *Conclusions*. This study shows presence of JAK2 mutation in splanchnic vein thrombosis and its association with higher leukocyte count and platelet count. This observation needs to be confirmed in a larger number of patients. Hence screening for the JAK2 V617F mutation may be useful to recognize patients who should be carefully observed for the subsequent development of overt MPD.

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A TWO YEAR OBSERVATIONAL STUDY OF PATIENTS ATTENDING A DEEP VEIN THROMBOSIS CLINIC-DEMOGRAPHICS, RISK FACTORS AND ANATOMICAL SITE OF THROMBOSIS

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Background. Deep venous thrombosis (DVT) is a common clinical problem. There have been marked advances in the diagnostic and therapeutic strategies for management of DVT. The advent of low molecular heparin (LMWH) has led to outpatient therapy for patients presenting with DVT. However our understanding of the epidemiology of DVT is based on studies of mainly hospital based patients. Similarly there is little information on the anatomical distribution of DVT in the outpatient population. It may be important to identify subgroups such as patient with ileofemoral thrombus for consideration of more intensive management. *Aims*. Outpatient management of DVT has been normal practice in our hospital since October 2000. We wished to investigate the demographics and risk factors of the population presenting with DVT to our clinic and to establish the anatomical distribution of thrombi reported. *Methods*. The sex, age and any risk factors of patients present-

ing to the DVT clinic between October 2006 and October 2008 were obtained from the specially designed DVT record sheet. The radiology results (venogram and Doppler ultrasound) were obtained from the PACS system (patient archiving computer system). *Results*. A total of 3600 patients attended as outpatients for investigation of possible DVT, of these there were 366 (10%) who had DVT. There were slightly more men than women 54% as compared to 46%. The age range was wide from 24-86 in the men and 19-91 in women. The majority of patients were over 50 yrs (73%) with only 2% being less than 30 years. Many patients had significant risk factors for DVT; past history was the most common risk factor (23%). Over 21% had a history of surgery within the last 3 months and 18% had a diagnosis of malignancy. Travel was also a common risk factor with 18% having travelled. In the women 5% were taking either the oral contraceptive pill or hormone replacement therapy. The majority of DVTs were above knee -54%, below knee DVTS -30%, ileofemoral.- 5%, axillary vein- 1% and there were 10% of DVTS that were described as extensive. *Conclusions*. Although the majority of patients are elderly with known risk factors for DVTS they can be managed on an outpatient basis. There were at least 15 cases of ileofemoral DVT that may have been suitable for more aggressive management to reduce morbidity. The number of ileofemoral DVTS may have been underestimated as some radiological reports were ambiguous stating extensive DVT. We recommend all radiological reports clearly state the proximal extent of the thrombus.

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THROMBOEMBOLISM PREDICTIVE FACTORS IN POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA

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Background. Polycythemia vera (PV) and Essential Thrombocythemia (ET) are myeloproliferative chronic diseases characterized by the frequent expression of JAK2V617F mutation (90% and 60% respectively). Thromboembolism is a complication that highly impacts on overall survival. A relationship between JAK2+ and Thrombotic events has been suggested. *Aims*. A retrospective study of a group of patients affected by PV and ET, tested for JAK2V617F mutation and 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 retrospective analysed the incidence of both arterial and venous thrombosis and their relationship with clinical variables. *Results*. We considered 85 patients: 46 female and 39 male. Among 21 patients with PV 16 (76%) were JAK2V617F$^+$ and 5 (24%) JAK2V617F-. Among 64 patients with ET 37 (58%) were JAK2V617F$^+$ and 27 (42%) JAK2V617F-. A thrombotic event occurred in 16 patients during the course of their disease: 5 (24%) within the PV group (3 venous and 2 arterial) and only 11 (17%) within the ET group (7 venous and 4 arterial). The mean and median time between diagnosis and the occurrence of the thrombotic event were 325 and 198 days respectively. The mean and median time between the start of any treatment for the mieloproliferative disorder and the occurrence of the thrombotic event were 220 and 161 days respectively. Platelets (plt), leukocytes (wbc) and haematocrit (ht) at diagnosis were not statistical different between patients who had a thrombotic event and those who did not (Table 1).

Table 1.

Diagnosis	Time	Plt x 10^9	Wbc x 10^9	Ht %
PV without thrombosis	Diagnosis	394	10,8	55,8
PV with thrombosis	Diagnosis	369	10,1	57,9
PV with thrombosis	Event	479	14	51,9
ETwithout thrombosis	Diagnosis	785	9,2	42,1
ET with thrombosis	Diagnosis	1027,2	11,2	42,4
ET with thrombosis	Event	973,2	11,4	46,4

Moreover blood count variables were not different between the diagnosis and the time of the thrombotic event (table 1). JAK2 positivity did not influence the occurrence of a thrombotic event: 15,6% in JAK2- versus 20,8% in JAK2$^+$ patients. At the event time all the 16 patients with thrombosis were treated with ASA, and 12 of them received also cytostatics. Only advanced age showed a significant correlation with the occurrence of a thrombotic event (p=0,004). *Conclusions*. We confirmed the high incidence rate of thromboembolic complications in patients affected by PV and ET. Both JAK2$^+$ and leucocitosis have 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 analysed. Further cooperative prospective studies on large group of patients are needed in order to better identify risk factors, mainly the role of leukocyte and platelets interaction.

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JAK2 MUTATIONAL STATUS, HEMOSTATIC RISK FACTORS AND THROMBOPHILIC FACTORS IN ET PATIENTS

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Background. The recent discovered of tyrosine-kinase activating JAK 2 V617F point mutation has been found in 50-60% of Essential Thrombocythemia (ET) patients. Increased risk of thrombosis in patients with this mutation has been reported. Other factors that increase the risk of thrombosis arising from ET itself include hereditary thrombophilic factors but also increase or decrease level of coagulation factors. *Aims*. In this study we explored if indeed JAK2 V617F mutation in ET patients coexisted thrombophilic factors that would contribute to thrombotic complication and a higher risk of thrombosis. *Methods*. We have examined 32 patients with ET (24 females and 8 males, mean age 56.0±14.2). This group included: 10 untreated patients, 22 treated with anagrelide, and hydroxyurea. The control group (CG) consisted of 20 healthy persons: 6 males and 14 females (mean age 41.4±8.3). We search for pathogenetic JAK2 V617F mutation and prothrombotic: Factor V Leiden, prothrombin gene and MTHFR gene mutations. We evaluated also plasma levels of: Factors: I,VIII,XII; AT, protein C and S. Urokinase concentration was assessed in plasma and in platelet lysates. Platelet activation was studied using flow cytometry to detect CD61/CD42b and CD61/CD 62P expression on the platelet surface. *Results*. Mean platelet count was 785±32G/l and 250±54G/l for ET and the CG, respectively, p<0.001. WBC count was higher in ET patients than in CG: 8.3±3.7G/l and 5.4±1.4G /l, p<0.001. Concentration of uPA was higher in patient plasma as compared to the CG(0.635±0.232ng/mL versus 0.447±0.115ng/mL, p<0.05). Mean uPA concentration measured in platelet lysates was similar in both group (ET 0.317±0.135ng/109 platelets, CG 0.290±0.065 ng/109 platelets). In 11 patients from ET group thrombotic complications occurred and in 7 ET patients bleeding episodes were noticed. Patients with thrombotic complications as compared to those with bleeding episodes had higher fibrinogen and factor VIII level (420 mg/dL versus 302mg/dL p<0.05, and 115% versus 88% p<0.05 respectively). In patients with thrombotic complications compare to patients without this complications lower CD61/42b expression was detected (1.74% versus 10.00% p<0.05). The JAK 2 V617F point mutation was detected in 18(60%)ET patients. In all cases heterozygous genotypes were determined. In 10(37%) ET patients the MTHFR gene mutation was detected, in 6 patients simultaneously with JAK2 mutation. In 13(65%) persons from CG the MTHFR gene mutation was discovered. No prothrombin G20210A mutation was found in ET patient and in CG. In ET patients with JAK2 point mutation the higher level of red blood cells was found (4.38±0.57T /l and 3.86±0.55T /l, p<0.05 respectively). The activity of factor XII in JAK2 positive patients was lower than in negative ones (83.2±25.5% and 109.0±20.4% respectively p<0.05). In ET patients carrying MTHFR gene mutation (compared to wild type for MTHFR) the higher level of plasma urokinase was observed (0.747±0.3ng/l and 0.547±0.2ng/mL p<0.05). *Conclusions*. Summing up these results in the group of patients with heterozygous JAK2 point mutation we have not observed increase frequencies of thrombotic complications. To evaluate the risk of thrombosis it is necessary to assess not only JAK2 mutational status but also additional risk factors of as well thrombosis as bleeding

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ENDOTHELIAL CELLS AND ENDOTHELIAL PRECURSOR CELLS KINETICS IN HAEMATOLOGICAL PATIENTS UNDERGOING CHEMOTHERAPY OR AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Circulating endothelial cells (EC) and endothelial precursor cells (EPC) may reflect tumour load, vessel injury and endothelial healing capacity. Their kinetics during treatment of cancer patients is unknown. *Aims*. To study the kinetics of circulating endothelial cells (EC) and endothelial precursor cells (EPC) in haematological patients during chemotherapy and autologous stem cell transplant (ASCT). *Methods*. 18 newly diagnosed patients and 17 patients undergoing ASCT were studied and compared to healthy controls. ECs were evaluated as $CD146^+CD31^+Lin^-$ cells, while EPCs were evaluated as $CD34^+CD133^+Lin^-$ or $CD34^+VEGFR2^+Lin^-$ cells or CFU-En colony forming units. Numbers of these cells were evaluated before and after treatment, and, in patients treated with ASCT, during mobilization of haematopoietic progenitors. *Results*. Both newly diagnosed patients and patients before ASCT had significantly higher number of $CD146^+CD31^+Lin^-$ cells and significantly lower number of CFU-En colonies than healthy controls. These parameters did not return to normal for at least 3 months after chemotherapy or ASCT. Numbers of CFU-En did not correlated neither with numbers of $CD34^+CD133^+Lin^-$ cells nor with numbers of $CD34^+VEGFR2^+Lin^-$ cells, but they correlated with numbers of $CD4^+$ lymphocytes and NK cells. *Summary and Conclusions*. Haematological patients have higher number of EC and lower numbers of CFU-En than healthy controls. These parameters do not return to normal after treatment, probably as a result of endothelial damage caused by chemotherapy and of ongoing immunological dysfunction.

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THE IMPACT OF THE GENETIC POLYMORPHISM G455A ON THE B-CHAIN FIBRINOGEN GENE ON THROMBOTIC PROCESS IN PATIENTS WITH CORONARY ARTERY DISEASE

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Background. Evidence suggests that fibrinogen plays a critical role in atherosclerosis. A genetic polymorphism on fibrinogen chain B, the G455A, has been associated with fibrinogen levels in healthy individuals, but its effect on thrombotic process in patients with coronary artery disease (CAD) is unclear. In the present study we examined the effect of this polymorphism on prothrombotic profile of patients with CAD. *Methods*. The study population consisted of 243 individuals, 191 of which with angiographically documented CAD and the rest with angiographically documented absence of any significant coronary stenoses. The G455A polymorphism was detected by PCR and suitable restriction enzymes. Fibrinogen levels were measured by immunonephelometry, while other factors of thrombosis such as plasma levels of d-dimers, factors V and X, plasminogen and thrombin time were measured by standard coagulometry techniques. *Results*. The genotype distribution was GG: 48.2%, AG: 40.3%, AA: 11.5% and GG: 50.0%, AG: 34.6%, AA: 15.3% for CAD patients and healthy individuals respectively. Among CAD patients AA patients had significantly higher levels of fibrinogen than GA patients. There was no difference among other genotypes (AA: 517.5±144.0, AG: 434.0±132.2, GG: 443.0±121.0 mg/dL $p<0.05$ for AAvsGA, p=NS for AAvsGG, GAvsGG). Plasma plasminogen levels did not differ across the three genotypes GG (106.3±19.1 u/mL) compared to GA (112.5±20.0 u/mL) and AA (115.8±11.5 u/mL), p=NS for all. Moreover, there was no significant difference in plasma levels of factor X (AA: 94.1±20.3 vs GA: 91.6±26.1 vs GG: 101.2±23.2%, p=NS for all), factor V (AA: 124.9±28.4 vs AG: 125.7±34.4 vs. GG: 122.0±32.2%, p=NS for all), thrombin time (AA: 19.5±3.2 vs. GG: 20.8±18.4 vs GA: 19.0±1.7 sec, p=NS) and D-dimers (AA: 551.7±321.5 vs GA: 511.4±526.7 vs GG: 616.3±817.4 mg/L p=NS). *Conclusions*. Genetic polymorphism G455A on fibrinogen b-chain gene affects fibrinogen levels, but has no effect on other thrombotic markers. These findings indicate that this polymorphism may play important role in the process of atherothrombosis by affecting only fibrinogen levels, but not other thrombotic parameters.

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ASYMPTOMATIC BRAIN MAGNETIC RESONANCE IMAGING ABNORMALITIES IN ADULT PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. A hypercoagulable state in the thalassemia syndromes has been established. Thromboembolic events are more frequently observed in thalassemia intermedia (TI) patients who are splenectomized and not maintained on regular transfusion regimens. Brain involvement has been poorly studied. Although the incidence of clinically overt stroke in TI is low, silent strokes have been observed in 37% of patients by one study. *Aims*. To evaluate the frequency and risk factors for silent brain abnormality in adult patients with TI who are splenectomized and not maintained on regular transfusion regimens. *Methods*. This was a cross-sectional study on a sample of TI patients attending to the Chronic Care Center, Hazmieh, Lebanon. Patients who are above 18 years of age, splenectomized, not on regular transfusion regimens, and on no antiplatelet or anticoagulant medication underwent brain magnetic resonance imaging (MRI). Patients were initially screened to confirm absence of neurological signs or symptoms and other associated stroke related disease such as diabetes, hypertension, carotid stenosis, cardiac disease, or prothrombotic mutations. Patient charts were reviewed for any previous transfusion history and duration since splenectomy. Blood samples were obtained for assessment of total and fetal hemoglobin, nucleated RBC, platelet, and serum ferritin levels. Written informed consent was provided by all patients. *Results*. A total of 30 patients were included in this study. The mean age was 31.9±11 years (range: 18-54 years) and the male to female ratio was 13:17. The mean duration since splenectomy was 17±9.9 years (range: 2-36 years). Eighteen patients (60%) never received blood transfusions while 12 (40%) had occasional transfusions for transient anemia. A total of 18 patients (60%) had abnormalities on brain MRI. All patients with abnormal MRI had evidence of white matter lesions predominantly involving the frontal and/or parietal subcortical white matter (100%), with less frequent involvement of the external capsule (27.8%), occipital white matter (16.7%), temporal white matter and internal capsule (5.6% each). Four patients (22.2%) had single white matter lesions while 14 (77.8%) had evidence of multiple lesions (mean 5±10 lesions, range: 2 to more than 40 lesions). Most patients had small lesions (<0.5 cm) although 7 patients (38.9%) had lesions ranging in size between 0.5-1.5 cm and 1 patient (5.6%) had lesions greater than 1.5 cm. In addition, 11 patients (61.1%) had evidence of concomitant mild cerebral atrophy. Younger age (p=0.015) and occasional transfusion history (p=0.015) were significantly associated with a lower incidence of MRI abnormality. Moreover, occasional transfusion history (p<0.0001) and a shorter duration since splenectomy (p=0.045) were significantly associated with single, rather than multiple, lesions. Gender, total and fetal hemoglobin, nucleated RBC, platelet and serum ferritin levels had no significant correlations with presence of abnormality or number of lesions detected. *Summary and conclusions.* Silent white matter lesions on brain MRI is a common finding in adult, splenectomized and non-regularly transfused TI patients. Increasing age and a longer duration since splenectomy are associated with a higher incidence and multiplicity of abnormality, respectively; while occasional transfusion history seems to be protective for both.

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LOW LEVELS OF PROTEIN Z ARE ASSOCIATED WITH HELLP SYNDROME AND ITS SEVERITY

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Background. Microtrombus formation in the materno-placental circulation and infarcted areas on placenta caused by defects in coagulation cascade have been speculated for the etiology of HELLP syndrome. Protein Z, a cofactor of a vitamin K-dependent protease inhibitor, play a role in the inhibition of factor Xa. Protein Z has been found to be associated with pregnancy complications. However, there is not any data implying an association between HELLP and changes in maternal plasma levels of protein Z. *Aims*. To investigate if HELLP syndrome is associated with maternal plasma concentrations of protein Z. *Methods*. Protein Z levels in 29 women with HELLP syndrome were compared with 29 healthy-nulliparous and 25 pregnant women. Definition and classification of

HELLP syndrome was made according to Tennessee classification. Blood samples were obtained just after delivery in all pregnants. Concentrations of protein Z were determined by a sensitive and specific commercial immunoassays which utilizes sandwich enzyme immunoassay technique (Asserachrom Protein Z®, Diagnostica Stago and Serbio, Asnieres-sur-Saine, France). *Results*. All women were in same ethnicity. The median age was 29 (22-40), 29 (21.40) and 28 (22-47) for healthy, pregnant and HELLP groups, respectively (p=0.893). Median parity, gravidity and mean gestational age in patients with HELLP were 2 (0-9), 3 (1-10), 32.62±3.95 years, respectively. The median protein Z levels in HELLP syndrome were found significantly lower than those of pregnant women [864 (338-2220) ng/mL vs 1732 (726-4000) ng/mL, p=0.0001]. However, no significant difference was found between HELLP and healthy groups (864 (338-2220) ng/mL vs 958 (396-4000) ng/mL, p=0.597). On the other hand, when comparing, protein Z levels were obtained to be higher in pregnant women than those in healthy (1732 (726-4000) ng/mL vs 958 (396-4000) ng/mL, p=0.0001). (Figure 1). Despite protein Z levels in patients with HELLP were comparable with those in healthy women, protein Z levels were observed to be correlated with disease severity in HELLP syndrome. While a positive correlation exists between protein Z levels and platelet counts (rs=0.498, p=0.006), protein Z levels correlated negatively with LDH (rs=-0.458, p=0.012) and AST levels (rs=-0.374, p=0.046). Besides, median protein Z level was higher in partial HELLP than in complete HELLP (74 vs 39.85, p=0.038) (Figure 1). *Conclusions*. Protein Z levels are low in HELLP syndrome and related with its severity. Despite it is not clear whether low levels of protein Z causes HELLP syndrome or its levels lower during disease process, it should be kept in mind that it may play a role in the pathogenesis of HELLP syndrome beside suggested etiological factors like prothrombin 20210A and factor Leiden V mutations.

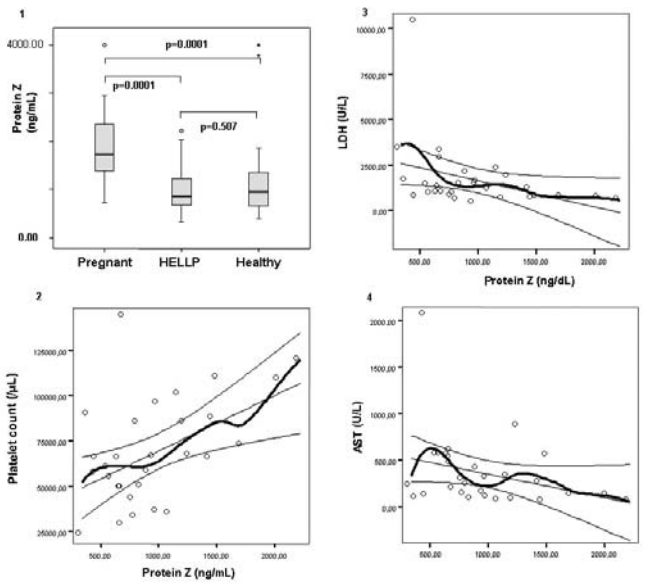

Figure 1. Protein Z levels in groups and its correlations

Infectious diseases, supportive care II

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MOLECULAR TYPING OF P. AERUGINOSA ISOLATES ALLOWS TO TRACE WATER AS THE SOURCE OF AN OUTBREAK OF MULTIRESISTANT PSEUDOMONAS BLOODSTREAM INFECTIONS

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Background. The effectiveness of systematic surveillance culture is still a matter of debate. Epidemiological surveillance at a Haematology Unit allows to detect changing patterns among isolated pathogens and to discover potential external sources, in order to adopt adequate strategies of environmental control and to better plan empiric antibiotic therapy. Aims. To trace potential environmental sources responsible for outbreaks of bloodstream infections (BSIs) occurring at our haematology unit. Methods. We prospectively analyzed all bacteremic episodes occurring among hematological patients admitted at our Institution over 57 months of observation; to evaluate epidemiological changes occurring over time, we compared the frequency of different species among the pathogens isolated during two consecutive periods (A and B, respectively) of 28.5 months each. When an outbreak (defined as occurrence of an infection at a greater frequency than expected) was observed, pertinent environmental screening were performed, including monitoring of water sources. The genetic correlation between isolates was evaluated by molecular typing by Amplified Fragment-Length Polymorphism (AFLP) on strains isolated from environment and patients. *Results*. During the entire period of observation, 349 BSIs were recorded. Gram-positive (G+) bacteria were responsible for bactaeremias in 134 cases (38.4%), Gram-negative (G-) bacteria in 216 (61.9%) and fungi (all Candida spp) in 10 (2.9%). In 16 cases a mixed BSI was detected. Frequency of G+ and G- BSIs did not significantly differ during the two periods, although, in period B, viridans streptococci (10.5% vs 3.1%, p=0.011) and P. aeruginosa bactaeremias (20.5% vs 9.4%, p=0.0046) significantly increased. Viridans streptococci BSIs were not considered an outbreak as increasing in their frequency was slow and continuous over time. Of the 54 P. aeruginosa observed during the entire period, 18 (33.3%) were multiresistant (MR Pseud) and occurred only during the second period of observation. Eleven out of 18 MR Pseud were observed during the last six months of the study (August 2008 - January 2009), which was considered an outbreak. Therefore, cultures from water of the patients' bathrooms were performed on September 2008 and two P. aeruginosa clusters were detected based on antibiotic resistance pattern, one of which consisting of MR Pseud. AFLP analysis, performed on both water clusters and 6 out of 11 MR Pseud from patients, revealed >85% similarity within patient isolates and the environmental strains belonging to the MR Pseud cluster. Overall a fatal outcome was observed in 33/349 BSIs (9.4%). Mortality increased in the second period (12.6% vs 5.7%, p=0.04). While viridans streptococci BSIs were not associated to a bad prognosis, MR Pseud BSIs significantly correlated with a higher risk of death (p<0.0001), as 10/18 MR Pseud BSIs were associated to death. *Summary and Conclusions*. Genetic analysis of strains isolated from environment and patients revealed that the outbreak of MR Pseud BSIs had a clonal origin and was a consequence of water contamination. Our study confirms the importance of epidemiological observation in order to early detect outbreak and to proceed to specific environmental studies.

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CARDIAC BIOMARKERS AND ASSESSMENT OF CARDIAC TOXICITY DURING HEMATOPOIETIC CELL TRANSPLANTATION FOR HEMATOLOGICAL MALIGNANCIES

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Background. Cardiac toxicity is a potentially serious complication of hematooncology treatment. Preparative regimen (PR) followed by hematopoietic cell transplantation (HCT) represents a high risk for devel-

opment of cardiotoxicity. Various methods including cardiac biomarkers have been recommended for monitoring of cardiotoxicity. Experience with new cardiac biomarkers in this context is very limited. *Aims*. Assessment of cardiac toxicity during HCT with multiple biomarkers of cardiac injury - myoglobin, creatine kinase MB (CK-MB mass), cardiac troponin I (cTnI), heart-type fatty acid binding protein (H-FABP), glycogen phosphorylase BB (GPBB). *Methods*. A total of 53 patients (mean age 49.9±12.3 years, median 54 years, 33 males) transplanted for hematological malignancies were studied. The diagnoses were as follows: AML 27, MM 12, NHL 5, HL 4, ALL 3, CML 1, MDS 1. Thirty transplants were autologous, 23 allogeneic. Cardiac biomarkers were measured on Randox Evidence analyzer the day after completion of PR (after PR) and the day after infusion of hematopoietic cell graft (after HCT). Values above the reference range recommended by the manufacturer were considered elevated. *Results*. The cut-off values of all biomarkers and the number of patients with elevated values are shown in the Table 1. We found significant elevations in GPBB (above 7.30 µg/L) in 8 (15.1%) patients after PR and in 9 (17.0%) after HCT. H-FABP increased slightly above the cut-off (4.50 µg/L) after HCT in 1 (1.9%) patient. Other cardiac biomarkers (myoglobin, CK-MB mass, cTnI) remained within the reference range in all patients. *Conclusions*. Our results suggest that administration of PR followed by HCT could be associated with myocardial injury manifested by increased release of GPBB from cardiomyocytes. These findings could be considered a sign of acute subclinical cardiotoxicity. Whether these acute changes will have predictive value for development of treatment-related cardiomyopathy in the future is not clear and should be evaluated during a prospective follow-up. Further studies in a larger number of patients will be needed to confirm our preliminary results and define the potential role of new biomarkers in this context.

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Table 1. Elevated biomarkers of cardiac injury in the peritransplant period (n=53).

cardiac biomarkers	1 day after PR	1 day after HCT
myoglobin above 76.0 µg/L	0	0
CK-MB mass above 4.80 µg/L	0	0
cTnI above 0.40 µg/L	0	0
H-FABP above 4.50 µg/L	0	1 (1.9 %)
GPBB above 7.30 µg/L	8 (15.1 %)	9 (17.0 %)

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SERIOUS EBV ENCEPHALITIS IN PATIENTS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-SCT) - SERIES OF 3 CASES

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Background. Epstein-Barr virus (EBV) infection in alloSCT patients causes various disorders ranging from asymptomatic increase of PCR detected DNA copies in body fluids to highly malignant post transplant lymphoproliferative disease. We show 3 cases of serious encephalitis - a clinical syndrome rarely described in literature. *Aims*. In 3 well-documented cases we describe a clear correlation of neurological status with EBV DNA quantity in cerebrospinal fluid (CSF) as well as prompt improvement of consciousness after receipt of rituximab. *Methods*. In a tertiary haemato-oncological centre we have observed among 344 alloSCT recipients in a period of 6 years (2003-2008) 3 cases of post transplant EBV reactivation with deeply decreased level of consciousness. The three patients recovered their neurological status to normal soon after treatment with rituximab. *Results.* CASE1: 58 years old woman developed abulia, tremor, bradypsychia and nausea on day + 60 of mismatched related alloSCT for high grade myelodysplastic syndrome (MDS) after myeloablative conditioning including anti-thymocyte immunoglobulin (ATG). No gross abnormality was seen on CT scan of her brain. High load of EBV DNA in peripheral blood, bone marrow and CSF (11300 copies/mL) were detected. Shortly after single dose of rituximab the patient improved dramatically. CASE 2: 39 years old woman developed sopor on day +50 of mismatched unrelated alloSCT after myeloablative conditioning (including ATG) for high risk acute myeloid leukemia (AML). Later she developed bilateral fluidothorax. High load of EBV DNA in fluidothorax and peripheral blood and CSF investigations suggesting viral encephalitis lead to therapy by foscarnet and rituximab with prompt recovery of the neurological status and disapearance of fluidothorax. CASE 3: 35 years old man after mismatched sibling SCT for high grade MDS after myeloablative conditioning (including ATG) was readmitted to hospital on day +58 because of fever and vomiting. Mild aGVHD was diagnosed from gastric biopsy. The neurological status of the patient deteriorated rapidly to sopor (without any pathology visible on MRI scan). Lumbar puncture revealed lymphocytosis in CSF and high levels of EBV DNA were detected in both blood and CSF (31200/mL). After first dose of rituximab the neurological status returned to normal within days. *Conclusions*. Our experience suggests that serious encephalitis with sopor without gross morphological changes on CT or MRI scans characterized by high EBV DNA load in cerebrospinal fluid and prompt recovery after rituximab treatment is a distinct clinical syndrome occuring in patients shortly after alloHSCT.

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MOLECULAR DIAGNOSIS APPROACH OF BLOOD STREAM INFECTIONS OF THE NEUTROPENIC PATIENT WITH HAEMATOLOGICAL MALIGNANCES

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Background. Life-threatning Infections are a relative common complication in febrile neutropenic patients (pts) with haematological malignancies, mainly due to depth and duration of neutropenia. Therefore, the evidecence of infectious origin during neutropenic fever is the main point in the management of neutropenic patients and for the antibiotic treatment strategy. The current gold standard for the detection of bacterial pathogens in blood is blood culture. Howeverthe blood culture suffers from several limitations, such as lack of rapidity and low sensitivity, especially if the patient has been already treated by empiric antibiotics. *Aims*. We evaluated a novel molecular tool for the diagnosis of bacteriaemia directly from blood samples, comparing its features to those of an automated continuous-monitoring blood culture system. *Methods*. We analyzed 166 febrile episodes in severe neutropenic patients (neutrophil count $<0.5\times10^9$ L^{-1}) admitted to the Haematology Unit. 71 pts with haematological neoplasm were analysed: 21 pts with ANLL, 30 pts with lymphoproliferative disordrers and 20 pts who underwent hemopoietic stem cell transplantation. At the same time a blood culture was requested and five ml of blood was also sampled for the molecular assay (LightCycler SeptiFast Test, Roche Diagnostics). *Results.* 45/166 (27.1%) bacteriemias were documented. In 20/45 (44%), both the methods arranged for systemic infections by Gram-positive or Gram-negative bacteria or fungi. 23/45 (51%) febrile episodes were identified only by the molecular assay (particularly S. aureus, A. baumanni, K. pneumoniae, P. aeruginosa, S. maltophilia, A. fumigatus, C. albicans and C. krusei). 2/45 (4.4%) febrile events were identified only by the blood culture (S. hominis and Burkholderia cepacia), this last not anticipated as target to molecular method. *Conclusions*. Our data confirm the negative and positive predictive value of the molecular assay in monitoring febrile neutropenia. The new molecular tools allow to elaborate new diagnostic algorithms to underline the microbes in the blood stream of the neutropenic patient with haematological malignances. Indeed, the molecular method allowed a faster identification of the pathogens and a more rapid and correct antibiotic treatment.

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THE ROLE OF PROPHYLACTIC ANTIMICROBIALS DURING AUTOLOGOUS STEM CELL TRANSPLANTATION: A SINGLE CENTER EXPERIENCE

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Background. Although prophylactic antimicrobials had been used frequently for ASCT, the benefits of antimicrobial prophylaxis in ASCT was controversial, partly because ASCT has a relatively short period of neutropenia compared with allogeneic SCT. However, antibacterial chemoprophylaxis with fluoroquinolones in neutropenic patients has reduced the incidence of Gram negative bacteremia in many literatures. *Aims*. The aim of this retrospective study was to investigate the efficacy of fluoroquinolone based antimicrobial prophylaxis during autologous peripheral stem cell transplantation (ASCT) in patients with multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL). *Methods*. We searched Asan Medical Center Registry for NHL and MM. Total 114 cases received fluoroquinolone based antimicrobial prophylaxis consist-

ed of ciprofloxacin (500 mg twice daily p.o.), fluconazole (100 mg twice daily p.o.) and acyclovir (400 mg every 8hr p.o.). The prophylactic antimicrobials was started 1 day before high dose therapy (HDT), and continued until neutrophil engraftment or switching to intravenous broad spectrum antibiotics at the occurrence of signs of infection. Meanwhile, total 118 cases did not receive antimicrobial prophylaxis during ASCT. *Results.* In prophylaxis group, 80 of 114 (70.2%) patients had experienced febrile episodes at median day +6 after transplantation, which showed statistical difference compared with no-prophylaxis group (p<0.001). In no-prophylaxis group, 111 of 118 (94.1%) patients had experienced at median day +5 after transplantation. Documented infection occurred in 14 of 114 (12.3%) patients in prophylaxis group, and 16 of 118 (13.6%) patients in no-prophylaxis group (p=0.846). In these patients, the positive blood culture was seen in 12 (10.5%) of 114 patients in prophylaxis group, and 12 (10.7%) of 118 patients in no-prophylaxis group (p=1.000). Documented viral infection or reactivation was not observed in prophylaxis group, but observed in 4 patients of no-prophylaxis group. Both groups showed no invasive fungal infection or serious adverse event during ASCT. The day of infection resolved was a median day +15 (range, 3-29) in prophylaxis group and day +14 (range, 2-70) in no-prophylaxis group (p=0.945). The duration of antimicrobial treatment was median 10 days both in prophylaxis group and in no-prophylaxis group (p=0.565). The duration of hospitalization was median 20 days and 21 days, respectively. Three patients had expired during ASCT. In prophylaxis group, one patient had died of veno-occlusive disease followed by hepatorenal syndrome, and 1 patient died of MRSA sepsis. In no-prophylaxis group, 1 patient had died of complications of cardiac amyloidosis. Overall, serious adverse events or infection-related mortality rate was associated with infection was extremely rare at both groups (1.8% vs. 0.8%, respectively). *Conclusions*. In our experience, the antimicrobial prophylaxis seems to decrease the incidence of febrile episodes during ASCT. There was statistical significant lower incidence of febrile neutropenia with unknown origin in prophylaxis group. However, antimicrobial prophylaxis seems to have no beneficial effect on reducing infectious complications. The antimicrobial prophylaxis of our study did not show the difference in the detecting rate of causative organism as an infective agent, duration of antimicrobial therapy and hospitalization between two groups.

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EFFECTIVENESS AND SECURITY OF ITRACONAZOL FOR INVASIVE FUNGAL INFECTION (IFI) PRIMARY PROPHYLAXIS IN PATIENTS WITH ACUTE LEUKEMIA

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Background. Invasive fungal infection is one of the main causes of morbidity and mortality in neutropenic and acute leukemia patients, therefore an effective pharmacological primary prophylaxis with a low toxicity profile is necessary. *Aims*.To analyze the effectiveness and security of itraconazol in primary prophylaxis in patients with acute myeloid leukaemia (AML) or myelodisplastic syndrome (MDS) in our clinic. *Material and Methods*. We performed a retrospective study of consecutive patients diagnosed of acute myeloid leukaemia, who received itraconazol for antifungal primary prophylaxis, between June 2004 and April 2007. We study each neutropenic episode (NE) individually. Data were collected for the state of the disease, chemotherapy (induction, intensification), previous NE, steroids administration, HEPA filter room, comorbidities (cardiac insufficiency, bronchopathy, diabetes, hepatopathy, chronic renal failure), duration of neutropenia, liver function tests (LFT), duration of prophylaxis, reasons for withdrawn, and others. The statistical analysis consisted on comparison of variables using Kruskal Wallis and Chi2 or exact of Fisher. *Results.* Seventy-nine NE were analyzed. Median age of patients was 51 years (20-79). 67% were men and the rest female. In seventy-six patients the diagnosis was AML, 2 MDS and 1 blast crisis from a chronic myeloid leukemia. On 36 (45.6%) patients the NE coincided with the diagnosis of the disease. Thirty-five (44.3%) of the NE happened during induction chemotherapy. Only 6 had significant comorbidities previous to the NE. Forty-five patients (57%) were at HEPA filter room. All patients received itraconazol prophilaxis, in 49.4 of cases it was administer orally and the rest intravenously. The incidence of probable and proved IFI (by EORTC criteria) was 3.8% (3 patients). No statistically significant relationship was found between IFI and state of disease (p=0.763), chemotherapy (p=0.290) or way of administration of itraconazol (p=0.591). Effectiveness of itraconazol for antifungal prophylaxis was 83.5%. Sixteen patients presented different adverse effects (20.3%), in 10 of them (12.7%) hepatic toxicity attributable to the drug was observed, and digestive intolerance in 6 (7.6%), but withdrawn of prophylaxis because of adverse effects occurred in only 8 cases (10.1%). We observed a tendency for statistically association between bilirrubin high levels at the begining of prophylaxis and posterior hepatic toxicity (p=0.074). 3 patients (3.8%) died during prophylaxis period; no case was attibutable to IFI nor to drug toxicity. *Conclusions*. Itraconazol has demonstrated in our cohort to be an effective drug for antifungal primary prophylaxis in neutropenic patients with acute leukemia, without a significantive adverse effect rate.

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PROGNOSTIC FACTORS AND OUTCOME OF PATIENTS ADMITTED TO THE INTENSIVE CARE UNIT (ICU) WITH HEMATOLOGICAL MALIGNANCIES

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Background. Patients with haematological malignancies present complications that sometimes require the admission to the ICU. Traditionally, in these cases the prognosis and outcome is poor, so the identification of factors that allow physicians to decide the best management for these patients would be of much interest. *Aims*. 1. To analyze survival of patients with haematological malignancies admitted to the ICU in our University Hospital. 2. To identify prognostic factors at the moment of admission to the ICU, and through the stay to the time of discharge. *Material and Methods.* We performed a retrospective analysis of patients admitted to the ICU with diagnosis of hematological malignancy between January 2000 and December 2007. Data were colleceted for disease, cause of admission, previous hospitalization, blood tests, SOFA and SAPSII scales punctuation, mortality at hospital after 30 days and survival after 1 year after admission. *Results.* 169 episodes of admissions to the ICU were included. Median age was 57 (11-88) years, 95 were men (56.2%) and 74 women (43.8%). The diagnosis were acute leukaemia in 36.7%, lymphoproliferative disorders in 34.3% and multiple myeloma, myeloproliferative and myelodisplastic syndrome in 29%. One hundred one (65.6%) received chemotherapy in the previous month and 34 (20%) underwent haematopoietic stem cell transplantation (HSCT) before admission, 21 of them were allogeneic HSCT. The most frequent cause of admission was major acute respiratory failure (35.5%), followed by sepsis (20.7%) and primary heart failure. 72 (42.6%) received respiratory support with non-invasive ventilation (NIV), in 62 (37.6%) orothraqueal intubation was necessary and in 15 patients (8.9%) occurred the nonintubation order. Median stay at the ICU was 6 days (1-72). Overall mortality was 48.5%. Haematological disease was statistically related to mortality at hospital, being greater for the acute leukemia group (p=0.009). Mortality was also higher in patients with longer previous stay at hospital, ventilatory support required, central venous catheter carriers, need of parenteral nutrition and those with higher SOFA and SAPSII punctuation, as well as greater bilirrubin levels and deeper thrombopenia. *Conclusions*. 1.The most frequent cause of admission to the ICU was the respiratory failure followed by sepsis. 2. Mortality at hospital of these patients in our center was similar to the referred previously in the literature. 3. SOFA and SAPSII scales seem to be useful instruments to predict mortality in these patients.

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COMPLIANCE TO IDSA-GUIDELINES FOR THE TREATMENT OF INVASIVE ASPERGILLOSIS IN ACUTE MYELOID LEUKEMIA

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Background. In February 2008 revised guidelines of Infectious Disease Society of America (IDSA) for the treatment of invasive aspergillosis were introduced. *Aims*. To analyze physician's compliance with IDSA current guidelines and to evaluate guidelines' feasibility and utility in improving patients care. *Methods*. A multicenter registry was conduct-

ed. Patients with acute myeloid leukaemia and proven/probable invasive aspergillosis were evaluated for adherence to the current IDSA-guidelines in terms of choice, dosage and duration of antifungal target therapy. Two groups of patients (IDSA and non-IDSA) were thus defined and compared. *Results.* 136 cases were collected. Treatment choices adhered to IDSA guidelines in 55% of patients only. This cohort of pts was almost identical to that of pts whose treatment deviated from guidelines in terms of the principal epidemiological and clinical carachteristics. Survival analysis at 120th day did not show any difference between the 2 groups, while in terms of 1st line therapy efficacy 24% of patients failed in the IDSA- group versus 41% in remaining patients (p 0.03). *Summary and Conclusions.* Frontline therapy choice remains a crucial point, influencing patients' outcome. Guidelines design cathegories of patients with homogeneous carachteristics and suggest optimal diagnostic and terapeutic options for them. Unfortunatly there are frequent reasons to deviate from these general advises. In this context, our report has a double value: it bears witness of frequent guidelines' inapplicability in clinical daily practice, but it also supports their recommendations, even in a non-selected series.

Table 1. First line antifugal target therapy in the two cohorts of patients (IDSA versus non-IDSA).

	Drug	N° patients	N° failures	*p-value*	N°exitus (AMR)	*p-value*
IDSA	➢Voriconazole ➢L-AmB	38 37	6 (8%) 12 (32%)	0.09	7 (18%) 9 (24%)	0.53
Total IDSA		**75**	**18 (24%)***		**16 (21%)#**	
Non IDSA	➢d-AmB ➢LC-AmB ➢Caspofungin ➢Itraconazole ➢Posaconazole ➢Combined	6 2 28 1 2 22	3 (50%) 2 (100%) 11 (39%) 1 (100%) 1 (50%) 7 (32%)	0.37	1 (17%) 2 (100%) 9 (32%) 1 (100%) 0 5 (23%)	0.1
Total non-IDSA		**61**	**25 (41%)***		**18 (30%)#**	

Legend: d-AmB: deoxycolate amphotericin B; L-AmB: liposomal amphotericin B; LC-AmB: lipid complex amphotericin B

* total IDSA vs total non-IDSA: **p-value 0.03** # total IDSA vs total non-IDSA: p-value 0.56

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EFFICACY AND SAFETY OF MICAFUNGIN AS AN EMPIRICAL ANTIFUNGAL AGENT FOR FEBRILE NEUTROPENIC PATIENTS

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Background. Invasive or possible fungal infection is often fatal and increasing the incidence in febrile neutropenic patients with hematologic malignancies. Empirical antifungal agent should be carefully selected. *Aims*. The study was conducted as a prospective multicenter trial to document the efficacy and safety of micafungin (Micamin), a class of echinocandin, in febrile neutropenic patients with hematologic disorders as an empirical antifungal agent. *Methods*. Micafungin was administrated at a dose of 100 mg/day intravenously for sustained fever (>38.4C) at 3-5 days after starting sufficient empirical antibiotic therapy. Overall success rate according to the composite score (no breakthrough fungal infection, survived for seven days beyond the end of therapy, did not discontinue therapy prematurely, had resolution of fever during the period of neutropenia, and successfully treated a documented base-line fungal infection) was evaluated and so was side effects, too. *Results.* AML (18/30), ALL (4/30), MDS (4/30) and lymphomas (4/30) patients were enrolled. The overall success rate according to the composite score was 56.7% (17/30). 93% (28/30) of the patients did not show breakthrough fungal infection, 93% (28/30) survived for seven days beyond the end of therapy, 67% (20/30) did not discontinue therapy prematurely, 67% (20/30) had resolution of fever during the period of neutropenia, and only 2 patients was documented base-line fungal infection and did not respond to micafungin. Two patients (7%) experienced grade 3 anemia and 13% (4/30) showed grade 3 or 4 of elevated transaminase. 23% (7/30) showed grade 3 or 4 hyperbilirubinemia, and four of them resolved. One patient died of hepatotoxicity. The response of ALL patients were superior to those of AML or MDS patients significantly (p=0.002). *Conclusions*. Micafungin documented an excellent efficacy (56.7%) and safety profile when used as an empirical antifungal agent to treat febrile neutropenic patients with hematologic disorders.

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MOLECULAR DIAGNOSIS BY LIGHTCYCLER? SEPTIFAST IN THE PREEMPTIVE TREATMENT OF INVASIVE FUNGAL INFECTIONS: A 5 CASES REPORT

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Background. Invasive fungal infections (IFI) remain a leading complications with high morbidity in haematological patients undergoing intensive treatment. Despite new drugs active against yeasts and moulds, when the antifungal treatment is started at time of established clinical IFI, the organ damage is leading to severe impairment with significant rate of mortality. An early suspect of incipient IFI is an ideal condition for starting antifungal treatment, thus potentially translating in higher rate of success in infection control. Molecular diagnosis of infections is a new tool for early diagnosis of pathogens circulating in patients with sepsis and neutropenic fever (Mancini, J. Med. Microbiol. 2008). *Aims*. We tested the sensibility and the clinical impact of LightCycler® SeptiFast (Roche Molecular Systems) for molecular diagnosis of invasive fungal infection in a subset of patients at high risk of invasive fungal infections. *Methods*. From January 2007 in cohort of 105 febrile neutropenic patients affected by haematological malignancies each time a blood culture was requested, a small aliquot of blood (1.5 ml) was also sampled for the molecular assay by LightCycler® SeptiFast performed by the Microbiology Laboratory. SeptiFast is a real-time PCR-based assay capable to detect a wide panel of bacterial and mycotic pathogens, and in particular Aspergillus Fumigatus. The time of the PCR-assay is approximately 6 hours. We collected a total of 227 samples and we decided antimicrobial therapy on the LightCycler® SeptiFast result. *Results.* In 5 febrile neutropenic patients (2 AML, 1 ALL, 1 IMF, 1 HD) the SeptiFast was positive for Aspergillus Fumigatus, while the standard blood culture sampled at the same time point was negative in all the patients. The pulmonary CT scan was suggestive for fungal infection in all the 5 patients, while blood sample for detection of the galattomannan antigen was positive only in 2/5 patients. The BAL was performed only in 2 patients, and in 2/2 patients the culture was positive for Aspergillus Fumigatus. In 5/5 patients the SeptiFast result positive for Aspergillus Fumigatus determined a timely switch in the antifungal therapy. We observed in 3 patients the complete resolution of the fungal infection, while in 2 patients the fungal infection became better but they died in the following weeks for progression of the haematological malignancies. *Conclusions*. Molecular diagnosis by LightCycler® SeptiFast is an attractive tool for triggering early pre-emptive therapy for invasive fungal infections, A prospective study with systematic use of SeptiFast in combination with the established galattomannan blood levels and early radiological findings will be run.

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MOLECULAR DIAGNOSIS OF BACTERIEMIA IN NEUTROPENIC FEBRILE ONCOHEMATOLOGICAL PATIENTS. PRELIMINARY RESULTS

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Background. The rapid diagnosis of bacteriemia in the course of fever of unknown origin plays an important role for the appropriate therapeutic treatment in neutropenic patients. In daily practice, blood culture helps identify bacterial and fungal bacteriemia. However, the efficacy of culture methods can be limited due to its low sensitivity when the patient is receiving antibiotic therapy and to the long period of time required to get a definitive identification of the pathogen (24-72 hours). Septifast® test (Roche Molecular Systems) is a new molecular technique for the detection of 25 most important bacterial and fungal species reported to cause bloodstream infections. *Aims*. The aim of the study was to compare the real-time PCR assay (Septifast®) with conventional blood cultures from neutropenic febrile oncohematological patients in a single institution. *Methods*. We prospectively tested 45 blood samples from 19 patients (pts), during a period of 5 months. All of them presented fever (>37.8°C) with a neutrophil count <0.5×10^9/L. Seven pts

were diagnosed with acute leukemia, 2pts with chronic lymphocytic leukemia, 5pts with aggressive lymphoma, 1pts with agranulocytosis, 1pts with medullar aplasia, 1pts with multiple myeloma, 1pts with pancreatic neoplasm and 1pts with grade 4 chondrosarcoma. At the moment of the study the patients were receiving antibiotic therapy with piperaciline-tazobactam or imipenem (19pts), vancomicine (7pts), amikacine (3pts), aciclovir (2pts) and antifungal therapy (9pts). Septifast® test (a real-time PCR assay) was applied in wholeblood samples of neutropenic febrile patients, providing results in only 6 hours. At the same time, two blood culture vials for aerobic and anaerobic cultures were drawn. *Results.* Thirty-two percent of patients presented a positive result. Eight of the 45 (17.7%) blood samples studied were positive by PCR assay (2 for Staphylococcus aureus; 3 for Pseudomonas aeruginosa; 1 for Esterichia coli; 2 Coagulase-negative staphyilococci). Two blood samples were positive for Pseudomona aeruginosa and Coagulase-negative staphyilococci by both Septifast® test and conventional blood culture. The entire negative PCR test results correlated with negative blood culture samples. The negative predictive value was 100% with a sensitivity of 100% and a specificity of 86%. (Table 1). *Conclusions*. Septifast® test is an easy and sensitive method for detecting pathogens in severely immunocompromised patients, allowing results in a shorter time than conventional blood cultures, having also higher sensitivity.

Table 1. Comparision results from Septifast® test and conventional blood culture system.

SEPTIFAST®	BLOOD CULTURE		TOTAL SAMPLES
	Positive Samples (%)	*Negative Samples (%)*	
Positive Samples (%)	*2 (4.4)*	*6 (13.3)*	*8 (17.7)*
Negative Samples (%)	0 (0)	37 (82.2)	37 (82.2)
TOTAL (%)	2 (4.4)	43 (95.5)	45 (100)

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RESPIRATORY SYNCYTIAL VIRUS INFECTION OUTBREAK IN CHILDREN WITH HEMATO-ONCOLOGICAL DISEASES

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Respiratory syncytial virus (RSV) has been reported to cause mortality among adult cancer patients receiving chemotherapy with or without hematopoetic stem cell transplantation (HSCT), but there have been few reports describing the outcome of RSV infection in children with hemato-oncological diseases. Two RSV infection outbreaks developed in hospitalized pediatric patients for various hemato-oncological diseases with or without HSCT between February 20th and April 15th, 2006, and between January 7th and February 25th, 2009. Thirteen of 30 patients developed rhinorrhea, progressive cough and/or dyspnea in these two time periods. A survey of respiratory viruses was done by using direct immunofluorescent antibody assay from nasopharyngeal washing aspirate. RSV antigen was detected in six patients (one high-risk ALL receiving induction, and one standart risk ALL on consolidation chemotherapy (CT); one relapsed ALL, one stage IV neuroblastoma, and one relapsed Hodgkin lymphoma receiving intensive CT; one with post-BMT under treatment for chronic GvHD). In 2009, RSV RNA was assessed in 14 patients with or without symptoms, also. RSV RNA was positive in 9 of 14 patients, two of these nine patients were asymptomatic, and RSV antigen was positive in 3/9 patients with symptoms. The remaining patients with respiratory symptoms in the two different outbreak periods were followed-up for RSV infection, but remained negative during all surveys. Five of six patients with RSV antigen (+), and the sister (RSV antigen was negative) of the boy with hyper-IgM syndrome and RSV antigen (+), who was also transplanted for hyper-IgM syndrome and under treatment for chronic GvHD and pneumonia were all treated with 0.2 g/kg intravenous immune globulin (IVIG) and oral ribavirin. Five patients recovered fully, although two were retreated due to recurrent (+) RSV antigen and respiratory symptoms within two weeks. One of six patients is on ribavirin therapy, now. We did not give oral ribavirin to one patient with (+) RSV antigen due to mild symptoms. All patients are alive. In conclusion, we did not observe any oral ribavirin-related adverse event or mortality in our patients even in post-BMT period. It is possible to give the scheduled anti-neoplastic therapy for not being delayed. However, our study population is too small, and, new studies are needed for cancer patients with RSV infection.

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CLOSTRIDIUM DIFFICILE ASSOCIATED DIARRHEA IN HEMATOLOGY-ONCOLOGY PATIENTS

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Background. Clostridium difficile-associated diarrhea (CDAD) in the cancer patient receiving chemotherapy is a frequent cause of morbidity and prolonged hospitalization. *Objective*. To determine the incidence density and identify risk factors associated with C difficile infection in hematology oncology patients. *Methods*. During a 7 month period (April -October 2007) all patients that were admitted and remained for more 48 hours in a haematology-oncology department in Athens, Greece, were studied. *Results.* The incidence rate of CDAD was 10.8%. The incidence density of C difficile associated diarrhea was 5.0 per 1.000 patient-days (14.2 per 1.000 patient-days at risk. Patients who developed CDAD had significantly longer stay than patients without CDAD (Median 30 vs 17 days, $p<0.001$). The median duration of neutropenia was statistically longer for patients with CDAD compared with patients without CDAD (8 vs 1, $p<0.001$).There was no significant difference in mortality between patients with CDAD and those without CDAD (9,1% vs. 7.7%., $p=0.875$). We found that chemotherapy before admission ($p=0.001$) and number of antibiotics ($p=0.001$) were independently predictive of CDAD by multivariate analysis. *Conclusions*. Special attention should be given to specific practical measures targeted at the practical prevention and control of Clostridium difficile infection.

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IN VITRO ANTIBIOTIC SYNERGY ON MULTIDRUG-RESISTANT STRAINS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM ONCOHAEMATOLOGICAL PATIENTS

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Background. Pseudomonas aeruginosa is a pathogen of great importance in the context of infections in patients with oncohaematologic desease; it is responsible for high morbidity and mortality of these patients. The strains selected from the broad use of antibiotics are often multidrug-resistant (MDR) or pan-resistant; thus the management of infections caused by P. aeruginosa in these patients is very difficult. *Aims*. Our aim was to evaluate *in vitro* synergy arising from the three different antibiotics pairs association on strains of P. aeruginosa pan-resistant isolates from oncohaematological patients, for all the strains an antimicrobial susceptibility testing had been previously performed by conventional methods in use.

Table 1. Frequencies of the categories for antibiotic association.

ANTIBIOTIC ASSOCIATION	CATEGORY		
	Indifference	Synergy	Antagonism
(CAZ) – (TM)	27/33 (81,82%)	6/33 (18,18%)	0/33 (0,00%)
(MEM) – (TM)	33/33 (100%)	0/33 (0,00%)	0/33 (0,00%)
(CAZ) – (TEC)	21/33 (63,64%)	12/33 (36,36%)	0/33 (0,00%)

Methods. 296 strains of P. aeruginosa isolated from oncohaematological patients, were identified through oxidase tests, catalase, VITEK system 2/GN cards and gallery API-20 NE (bioMérieux, Marcy-l'Etoile, France), 33 (11.15%) of which were pan-resistant, 3 with a mucoid morphotype and 30 non-mucoid. For each strain an antimicrobial susceptibility testing (VITEK system 2/card AST-N022 and Kirby-Bauer) was

performed according to the current CLSI guidelines. Through disc-diffusion in agar (susceptibility disks, Bio-Rad, Marnes la Coquette, France) for the 33 pan-resistant the following antibiotics associations were tested: ceftazidime (CAZ) 30 μg - tobramycin (TM) 10 μg, meropenem (MEM) 10 μg - tobramycin (TM) 10 μg, ceftazidime (CAZ) 30 μg - teicoplanin (TEC) 30 μg. *Results.* The 33 strains included in the assessment of synergy showed a pattern of antibiotic category (resistant 'R' for all antibiotics tested) perfectly superimposable by both methods used. The antibiotic pair ceftazidime - tobramycin showed synergy in 18.18% of cases while no synergy was found for meropenem - tobramycin association. The ceftazidime-teicoplanin association gave surprising results with 36.36% of cases of synergy (Table 1). *Summary and Conclusions*.These observations offer new and interesting therapeutic options for the treatment of this pan-resistant pathogen especially in the severe infections that affect oncohaematological patients.

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IFN-GAMMA RELEASE ASSAY RESPONSES IN PATIENTS WITH CLINICAL SUSPICION FOR PULMONARY TUBERCULOSIS, AND IN HEALTH-CARE WORKERS WITH POTENTIAL FOR EXPOSURE TO M. TUBERCULOSIS

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Background. WHO defines tuberculosis as a major cause of illness and death worldwide. Renewed effort to accelerate progress in global TB control in line with the expectations of the Global Plan, supported by intensified resource mobilization from domestic and donor sources, is needed. In many high-income countries with low rates of tuberculosis, serial testing for latent TB infection (LTBI) is recommended for persons at increased risk of TB exposure. In this context, the development of more specific, *in vitro* assays for LTBI, interferon gamma (IFNγ) release assays (IGRAs), is a welcome development. These assays are highly specific, especially in bacillus Calmette Guerin (BCG)-vaccinated populations. *Aims*. To evaluate the tuberculin skin test (TST) and IGRAs responses in patients with clinical suspicion for pulmonary tuberculosis, and in health-care workers who served these patients. All subjects were immunocompetent and HIV negative. *Methods.* We studied TST responses and blood from 29 patients with clinical suspicion for pulmonary tuberculosis, and from 150 health-care workers who had had contacts with patients. The TST was administered by the Mantoux method (5 IU/mL of PPD). T-Spot TB assay was performed on the blood from subjects in study. The assay included two antigen (M. tuberculosis-specific ESAT-6, Early Secretory Antigen Target 6, and CFP10, Culture Filtrate Protein 10). *Results.* 10/29 patients were TST and IGRA positive. Two patients had pulmonary tuberculosis and IGRA prevailing response was directed against CFP10. Eight patients had clinical and/or radiological findings for remote infection and IGRA prevailing response was directed against ESAT-6. 25/69 health-care workers TST and IGRA negative have been vaccinated in past with bacille Calmette-Guérin. 18/50 health-care workers vaccinated were TST positive (induration > or = 10 mm) and IGRA negative and 2/50 were also IGRA positive. Besides, 15/50 TST positive health-care workers were non vaccinated; only three of these subjects were IGRA negative and 22 IGRA positive with prevailing response against ESAT-6 in 21 subjects and one with prevailing response against CFP10 (history positive for pulmonary tuberculosis). *Summary and Conclusions*.The risk of development of active TB has been established in several cohort studies with the TST. Also, from controlled clinical trials, we know that treatment of TST positive persons reduces the risk of active disease. Unfortunately, there are no equivalent data for IGRAs. The association between strong IFNγ response to ESAT-6 and subsequent progression to active TB among household contacts of index cases and the predictive positive value of a positive IGRA result are yet to be determined. Little is known about the response to CFP10. We find a stronger response to CFP10 in cases of pulmonary tuberculosis; moreover, IGRA is a useful diagnostic tool for the surveillance of exposure of HCWs vaccinated with bacille Calmette-Guérin to M. Tuberculosis.

SIMULTANEOUS SESSION III

Indolent non-Hodgkin lymphoma - Clinical

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HAIRY CELL LEUKEMIAS WITH UNMUTATED IGHV GENES DEFINE THE MINOR SUBSET REFRACTORY TO SINGLE AGENT CLADRIBINE AND WITH MORE AGGRESSIVE BEHAVIOR

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Background. Hairy cell leukemia (HCL) is generally responsive to single-agent Cladribine (2CdA) and only a minority of patients are refractory and with poor prognosis. HCL generally express mutated (M) and, in a minority, unmutated (UM) immunoglobulin heavy chain variable region genes (IGHV). *Aims*. i) to investigate the clinical and molecular parameters predicting response and ii) to investigate the immunogenetic and molecular profile of the refractory patients. *Design and Methods*. In a multicenter clinical trial in newly diagnosed HCL, we prospectively investigated clinical and molecular parameters predicting response and event-free survival (EFS) after single-agent 2CdA. Of 58 HCL, 6 expressed UM-IGHV (UM-HCL) and 52 M-IGHV (M-HCL). Beneficial responses were obtained in 53/58 patients (91%), while treatment failures were observed in 5/58 (9%) patients. Responses correlated significantly with UM-IGHV (5/6 failures, p=0.000001), leucocytosis (3/6, p=0.005), and bulky spleen (4/8, p=0.0007). UM-HCL characteristically had bulky spleen (4/6, p=.002), leukocytosis (3/6, p=.01), TP53 defects (2/6, 33%, p=.03) as documented by mutational analysis and genome wide DNA profile (250K SNP Affymetrix array), and progressed rapidly after first treatment (median EFS 7.5 months, p=0.00000000000004). *Conclusions*. Our data suggest that UM-HCL identify the minor subgroup refractory to 2CdA and with more aggressive disease presentation. High incidence of TP53 disfunction indicates a potential mechanism of resistance to 2CdA in the UM-HCL group. Overall, our data provide new molecular elements for treatment concerns in HCL.

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BRIEF CHEMOIMMUNOTHERAPY RITUXIMAB (R)-FND +/- R MAINTENANCE IS EFFECTIVE AND SAFE IN NEWLY DIAGNOSED FOLLICULAR LYMPHOMA ELDERLY PATIENTS: AN INTERGRUPPO ITALIANO LINFOMI (IIL) RANDOMIZED TRIAL

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Background. In order to maintain efficacy and reduce toxicity of the treatment in elderly follicular lymphoma (FL) patients, we designed a study with a short chemo-immunotherapy. *Aims*. Primary endpoint was

to compare the efficacy of Rituximab maintenance versus observation (control) in elderly FL patients responsive to a short chemo-immunotherapy R-FND plus Rituximab consolidation. *Design and methods*. From January 2004 to December 2007, 241 patients (age 60-75) with untreated advanced stage FL were enrolled into 33 IIL centres and 234 patients were eligible. Treatment plan was: 4 courses of R-FND (Rituximab 375 mg/m2 day 0, Fludarabine 25 mg/m^2 dd 1-3, Mitoxantrone 10 mg/m^2 day 1, Dexamethasone 10 mg dd 1-3) every 28 days followed by 4 weekly Rituximab (375 mg/m^2); responding (CR+CRu+PR) patients were randomized to receive Rituximab maintenance every 2 months for 4 doses or observation. Qualitative and quantitative PCR monitoring for IgH/Bcl-2 rearrangement was performed on bone marrow (BM) at diagnosis, after R-FND and R consolidation and during maintenance/observation. *Results*. Median age was 66 (60-75); advanced stage II 14%, stage III 21% and stage IV 65%; 55% had BM involvement and 18% B symptoms; FLIPI score was: Low 11%, Intermediate 34%, High 55%. One or 2 co-morbidities were present in 36% and 23% of the patients respectively. Qualitative PCR analysis was performed in 222 patients at diagnosis: 51% were Bcl-2 positive. Two hundred and two patients were randomized between maintenance or observation. Thirty-two were not randomized because of: stable/progressive disease (15), adverse events (11) or other causes (6). Overall response at the end of treatment was 86% with 69% CR and 17% PR; PCR negativity at the end of treatment was 74% with PCR negativity associated with CR in 67% of the patients. Rituximab consolidation after R-FND was able to induce CR in 36/88 (41%) PRs and to increase PCR negativity from 61% to 74%. With a median follow-up of 18 months, two-years OS and PFS were: 92% and 73% and shown in the Figure.

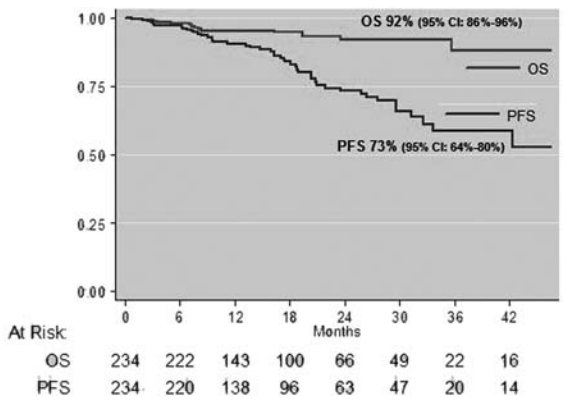

Figure.

Two-years PFS rates according to FLIPI score were: Low/Intermediate risk 85% vs High 65% (p 0.0002). A total of 1119 courses of chemo-immunotherapy were delivered. The most frequent CTC grade 3-4 toxicity was neutropenia in 25% of the courses, with only 13 serious infections. Pulmonary and cardiac toxicities were <1%. Two toxic deaths occurred: 1 HBV reactivation and 1 Steven Johnson Syndrome. Any grade Rituximab related reactions were seen in 6% of the courses with Rituximab discontinuation in only 2 patients. *Conclusions*. The results of this trial, specifically designed for elderly patients, show that a brief chemo-immunotherapy R-FND + Rituximab consolidation is safe and effective with a good 2-yr PFS rate also in patients at high risk FLIPI score. PCR negativity was achieved in the majority of the BCL2-rearranged patients. Further follow-up is needed to validate these observations. The final results of the study will provide insights on the role of Rituximab maintenance after R-chemotherapy.

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90YTTRIUM IBRITUMOMAB TIUXETAN (ZEVALIN) COMBINED WITH BEAM (Z -BEAM) CONDITIONING REGIMEN PLUS AUTOLOGOUS STEM CELL TRANSPLANTATION IN RELAPSED OR REFRACTORY FOLLICULAR LYMPHOMA. A GELA PHASE II PROSPECTIVE STUDY

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Background. Autologous stem cell transplantation (ASCT) proceeded by conditioning with intensive high-dose chemotherapy is a standard treatment for patients with low-grade B-cell lymphoma, who have experienced disease relapse or who are refractory to treatment. The long-term outcome of ASCT is better if complete remission (CR) has been achieved at the time of transplant and the duration of response longer than in the last previous chemotherapy treatment. Conditioning regimens therefore include both chemotherapy and total body irradiation to increase the likelihood of CR., 90Y-ibritumomab tiuxetan, has demonstrated pronounced anti-lymphoma activity when given as monotherapy in relapsed/refractory low-grade lymphoma. Consequently, incorporating the CD20-specific antibody as an alternative source of irradiation during conditioning, has generated much interest. *Aims*. The aims of this study were to evaluate the safety and efficacy of conventional dose of 90Yttrium ibritumomab tiuxetan with BEAM conditioning regimen pre autologous stem cell transplantation (ASCT) in chemo-sensitive relapsed or refractory low-grade B-cell lymphomas. *Design and Methods*. From 3/2005 to 8/2006, a phase II study was undertaken, in which 77 patients with relapsed/refractory low-grade B-cell lymphoma were given conventional-dose 90Y-ibritumomab tiuxetan 1 week before starting a standard BEAM regimen (Z-BEAM) as a pre-ASCT conditioning regimen. *Results*. Patients characteristics at last salvage chemotherapy inclusion: 68 follicular lymphoma, 6 marginal zone and 1 mantle cell plus 2 transformed histology after pathological review; median age 53 yr. (31-64), FLIPI low risk 32, intermediate risk 20, high risk 20. First relapse 39; 2nd relapse 10 partial response 21, stable disease 4 and progressive disease 3 after first line treatment. Response rate before ASCT were CR and CRu 77%, PR 22%, stable 1%. Overall, among the 77 pts last salvage chemotherapy regimen included rituximab in 74 pts and ASCT was performed in 75 patients. The haematological reconstitution after Z-BEAM followed by ASCT in 75 pts was : time to neutrophils > 1G/L 12 days (9-35), time to platelets >20 G/L 12 days (3-42). Three months after transplant 88% of the patients were in CR, CRu .With a median follow-up of 28 months (range: 8-38 months), the 2-year EFS and OS were 63% and 97%, respectively. There was a significant difference in EFS post ASCT for patients in favour of 1st relapse 74% versus second relapse 40% (p= 0.005). Using patients as their own control for the duration of last qualifying phase, the 2 years EFS post ASCT, was in favour of ASCT 62% vs 37% (p=0.007). At week 12 and 42 after ASCT, median haemoglobin level were 11.6 g/dl and 11.3 g/dl, median platelets counts was 111G/L and 148 G/L, leucocytes counts 3.48 G/L and 4.80 G/L respectively. There was no evidence of impaired engraftment. Overall toxicities were comparable to standard ASCT conditioning regimens and no toxicity-related mortality was reported. *Conclusion*.This study demonstrated that 90Y-ibritumomab tiuxetan combined with standard BEAM treatment provides a safe, highly active conditioning regimen and need to be evaluated in further randomized study.

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RESULTS OF A PROSPECTIVE RANDOMISED TRIAL COMPARING CHLORAMBUCIL, MITOXANTRONE AND DEXAMETHASONE (CMD) VERSUS FLUDARABINE, MITOXANTRONE AND DEXAMETHASONE AS PRIMARY THERAPY FOR ADVANCED STAGE FOLLICULAR LYMPHOMA (REAL GRADES I-III, STAGE III/IV). A REPORT FROM THE UK CENTRAL AND SOUTHERN LYMPH

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Background. The choice of the most appropriate primary therapy for the de novo presentation of advanced stage Follicular lymphoma (FL) is controversial. The purine analogue Fludarabine has proven activity in FL either as a monotherapy or within combination regimens such as Fludarabine, Mitoxantrone and Dexamethasone (FMD). In this multicentre study 400 patients presenting with de novo advanced stage FL between May 2000 and April 2006 were randomised between FMD (Fludarabine 25 mg/m^2 iv D1-3, Mitoxantrone 12mg/m^2 iv D1 and Dexamethasone 20 mg po D1-5) and CMD (Chlorambucil 10 mg po D1-10 with M and D as per FMD) on physicians' decision to treat for the presence of B symptoms, bone marrow failure, bulk disease or compressive symptoms. Responding patients received a maximum of 8 cycles of therapy. At the time of the study Rituximab (R) was not available for inclusion within primary therapy. Baseline peripheral blood (PB) and bone marrow (BM) samples were provided for PCR analysis to determine the presence of an informative bcl-2/IgH rearrangement and informative patients had further PCR analyses performed at fixed time points during follow-up. *Aims*. The primary end points for comparison of the two regimens were progression-free survival (PFS) and overall survival (OS). Secondary end points were complete (C) and partial (P) remission rates (RR) together with molecular response rates and the impact of molecular response on PFS and OS. *Design and Methods*. 200 patients were randomised to each arm, central histopatholgical review confirming a diagnosis of FL in all cases. The two arms were well-matched for median age, Stage (III vs IV) and IPI / FLIPI.

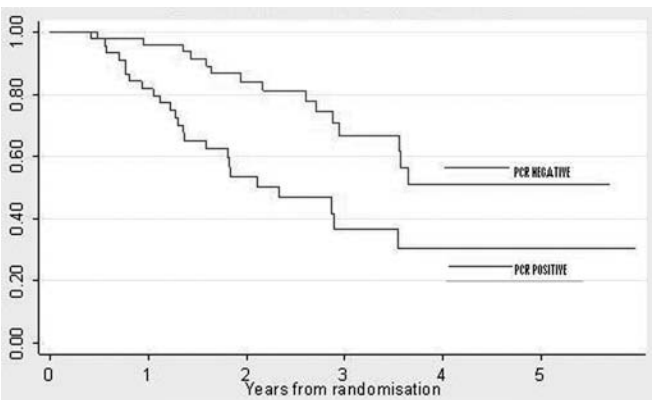

Figure 1. PFS according to PCR result at end of therapy.

Results. At the end of therapy for CMD versus FMD the CRR was 44% and 44% respectively with PRR of 46 versus 47%. Adverse haematological and non-haematological effects were not statistically different between the two arms. 81% of patients allocated CMD received 5 or more cycles of therapy compared with 76% of those allocated FMD. Hazard ratios at 2 years were in favour of CMD for OS at 1.48 (95% CI 0.91-2.31, p=0.11) and for PFS at 1.44 (95% CI 1.08-1.93, p=0.013). Median PFS was 43 months for CMD versus 33 months for FMD. A PCR amplifiable bcl-2/IgH rearrangement was identified in 134 of 267 evaluable patients (50.2%). In a subset analysis of 92 PCR-informative patients 55% of patients receiving CMD and 49% of patients receiving FMD became PCR negative at the end of therapy (p=ns). The achievement of PCR negativity was strongly associated with an advantage in PFS (p=0.03 - see attached figure) but not in OS. The difference in PFS based on molecular response demonstrated utilising a sensitive nested PCR technique could be replicated using a less sensitive single round PCR. *Summary*. In this prospective randomised trial a significant benefit in median PFS was demonstrated for CMD over FMD. Both regimes are deliverable with acceptable toxicity profiles. The PFS demonstrated with CMD compares favourably with other anthracycline-containing regimens without R and with R-CVP. Patients achieving PCR negativity at the end of treatment have a significant PFS advantage.

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SINGLE AGENT BORTEZOMIB IS ACTIVE IN RELAPSED/REFRACTORY MALT LYMPHOMAS. FINAL RESULTS OF AN INTERNATIONAL EXTRANODAL LYMPHOMA STUDY GROUP (IELSG) PHASE II STUDY

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Background. Bortezomib has shown antitumor activity in different subtypes of non-Hodgkin lymphomas, including both indolent and aggressive diseases. The suggested key role of constitutive activation of NF-KB in MALT lymphoma pathogenesis supported the evaluation of bortezomib in this clinical setting. *Aims*. The IELSG has coordinated a phase II multicenter study aimed to assess the antitumor activity and safety of bortezomib in extranodal marginal zone B-cell lymphoma of MALT-type, relapsed or refractory after prior systemic therapy. *Design and Methods*. Bortezomib 1.3 mg/m^2 has been administered on days 1, 4, 8, and 11 of a 21-day cycle, for up to 6 cycles. Activity has been measured after 6 and 18 weeks of treatment and defined according to the revised NCI response criteria. Toxicity was evaluated according to NCI-CTC (version 3). *Results*. On February 2009, 32 patients (pts) have been enrolled and the pt accrual has been closed. Among the 30 pts in whom the clinical data are available, 16 (53%) pts were male. At time of enrollment median age was 61 years (range, 37-82). Median time from first diagnosis was 3.6 years (range, 0.8-15). Median number of prior systemic therapies was 2 (range, 1-4), in most cases immuno-chemotherapy including rituximab. The Ann Arbor stage distributions were the following: stage I= 7 pts (23%), stage II= 7 pts (23%), stage IV= 16 pts (53%). A primary gastric localization was present in 14 pts, primary cutaneous/subcutaneous in 10, primary lung lymphoma in 3; in the remaining 3 pts lymphoma was primarily localized in the orbit, pharynx, and genital tract. Multiple extranodal sites were present in 8 of these pts (27%). All pts had ECOG PS<2; in 3 cases (10%) elevated serum LDH was reported. Twenty-five pts were assessed for response at the end of treatment plan: the overall response rate was 48% (95%CI: 28%-69%), with 6 complete and 6 partial responses. Seven patients experienced stable disease after study conclusion and 6 progressive disease during therapy; 3 other were not evaluable for response, and in 4 pts, with ongoing therapy, no response data is available yet. All responses were durable lasting from 3+ to 34+ months. At a median follow-up of 2 years, three disease progressions were observed among 2 complete responders and in a single pt in partial response. Five deaths were reported, in 2 cases due to disease progression. No toxicity related deaths were observed. The safety profile of bortezomib is similar to that observed in multiple myeloma and other non-Hodgkin lymphoma entities. The most relevant grade 3 adverse events were fatigue, thrombocytopenia, neutropenia and peripheral neuropathy, which was always reversible, but represented the cause of early treatment stop in 5 patients. *Conclusions*. Bortezomib seems to have relevant clinical activity in patients with MALT lymphomas, with tolerable toxicity. Final results will be presented

Myeloproliferative disorders - Biology

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A GERMLINE JAK2 SNP IS ASSOCIATED WITH PREDISPOSITION TO THE DEVELOPMENT OF JAK2 V617F-POSITIVE MYELOPROLIFERATIVE NEOPLASMS

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Background. Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are myeloproliferative neoplasms (MPN) characterized by clonal proliferation of one or more myeloid lineages. The presence of the identical somatic activating mutation in JAK2 (JAK2V617F) in over 90% of PV and 50-60% of ET/PMF suggests that there are additional inherited or acquired genetic events that contribute to the pathogenesis of these phenotypically distinct disorders. Moreover, family members of MPN patients are at higher risk for the development of MPN, consistent with the existence of one or more MPN predisposition loci. *Aims*. We hypothesized that germline variation contributes to MPN phenotypic pleiotropy and to MPN predisposition. *Design and Methods*. We carried out a genome-wide analysis of 250000 single nucleotide polymorphisms (SNPs) in 217 MPN samples, and used a series of 324 germline DNA samples from MPN cases to further analyze our findings. *Results*. Analysis of 250,000 SNPs in MPN samples identified an allele in the JAK2 locus (rs10974944) that is enriched in JAK2V617F-positive MPN compared to JAK2V617F-negative MPN or to control populations, suggesting this haplotype predisposes to the development of JAK2V617F-positive MPN. Analysis of germline DNA PV, ET, and PMF patients found that that the GG/CG genotypes at rs10974944 were more common in MPN cases compared to Wellcome Trust Case-Control Consortium (WTCCC) controls (OR=3.1, p=4.1x10-20), consistent with the G allele functioning as a dominant MPN predisposition allele. Germline allelic variation at rs10974944 was strongly associated with JAK2V617F-positive MPN (OR=4.0, p=7.7x10-22) and much less strongly associated with JAK2V617F-negative MPN (OR=1.6, p=0.06). In patients heterozygous (CG) for rs10974944, we showed that JAK2V617F is preferentially acquired in cis with the predisposition allele (G). These data indicate germline JAK2 variation more strongly influences MPN predisposition than MPN phenotype. We identified three additional germline loci that are enriched in PV or in ET, establishing these loci as candidate modifier loci that influence MPN phenotype. *Conclusions*. These data suggest that germline variation is an important contributor to MPN phenotype and predisposition.

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CHROMATIN MODIFICATION- A NUCLEAR ROLE FOR JAK2

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Background. Activation of JAK2 by chromosomal translocations or point mutations is a frequent event in haematological malignancies. JAK2 is a non-receptor tyrosine kinase that regulates a number of cellular processes by inducing cytoplasmic signalling cascades including MAPK, PI3K-AKT and the STAT family. Thus far, the focus for understanding the molecular mechanisms of JAK2 mediated oncogenesis have centred around the analysis of these downstream cytoplasmic signalling pathways. *Design and Methods*. Mammalian cell lines and/or primary haematopoietic cells were studied using confocal immunofluorescence (IF), biochemical subcellular fractionation, the generation and development of a histone modification specific antibodies, western blot and immunoprecipitation assays, expression of GST-tagged recombinant mammalian proteins, *in vitro* binding assays, genome-wide expression analysis and chromatin immunoprecipitation (ChIP). *Results*. Using IF and subcellular fractionation our results identify for the first time that JAK2 is present in the nucleus of haematopoietic cells. *In vitro* kinase assays demonstrated that JAK2 phosphorylates histone H3. Modification-specific antibodies were then generated, characterised and used to demonstrate that JAK2 signals directly to chromatin by phosphorylating tyrosine-41 on histone H3 (H3Y41). H3Y41 is conserved throughout phylogeny and has been demonstrated to be an essential amino acid necessary for cell viability. Using a combination of cell fractionation and *in vitro* binding assays we show that this region surrounding H3Y41 is bound by heterochromatin protein 1α (HP1α), an essential component of transcriptionally silent heterochromatin. We present a mechanistic insight into the biological function of JAK2 mediated H3Y41-phosphorylation by demonstrating that this modification prevents the binding of HP1α and thus is likely to modulate multiple aspects of chromatin structure. Finally, the combination of genome-wide expression analysis and ChIP was used to identify the LMO2 oncogene as a previously unrecognised target of JAK2 signalling that is independent of STAT5 binding. JAK2 signalling was associated with increased LMO2 transcription together with increased H3Y41 phosphorylation and reduced HP1α occupancy at the LMO2 promoter. *Conclusions*. These results identify a previously unrecognised nuclear role for JAK2 and reveal the first characterised tyrosine phosphorylation of any core histone. Our data demonstrates that phosphorylation of H3Y41 by JAK2 modulates the structure and function of chromatin by regulating the binding of HP1α, and reveals a previously unrecognised direct link between JAK2 and the LMO2 locus. Taken together these results demonstrate a novel signalling pathway by which JAK2 directly regulates chromatin structure in mammalian cells.

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TET2 MUTATION PREVALENCE AND CLINICAL CORRELATES IN A SPECTRUM OF MYELOID NEOPLASMS: A SINGLE INSTITUTION STUDY OF 337 PATIENTS

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Background. Recently acquired loss of heterozygosity (LOH) and somatic deletions at chromosome 4q24 has been described in patients with myeloproliferative neoplasms (MPN). Furthermore, TET2 (TET oncogene family member 2), a putative tumor suppressor gene located in the minimal LOH region of chromosome 4q24 has been shown to harbor loss of function mutations that coexist with JAK2V617F and may predate its acquisition, in patients with myeloid malignancies. *Aims*. To describe the TET2 mutation prevalence and its clinical correlates in a spectrum of myeloid malignancies. *Design and Methods*. High-throughput DNA sequence analysis was used to screen for TET2 mutations in bone marrow-derived DNA from 337 patients with myeloid neoplasms including 239 with BCR-ABL-negative myeloproliferative neoplasms (MPN), 48 with systemic mastocytosis (SM), and 50 with myeloid malignancies other than MPN. *Results*. Among the 239 patients with BCR-ABL-negative MPN, 32 TET2 mutations were identified for an overall mutational frequency of ~13%; specific diagnoses included polycythemia vera (PV; n=89), essential thrombocythemia (ET; n=57), primary myelofibrosis (PMF; n=60), post-PV MF (n=14), post-ET MF (n=7) and blast phase PV/ET/MF (n=12); the corresponding mutational frequencies were approximately 16%, 5%, 17%, 14%, 14% and 17% (p=0.50). Mutant TET2 was detected in ~17% and ~7% of JAK2V617F positive and negative cases, respectively (p=0.04). However, this apparent clustering of the two mutations was accounted for by an independent association between mutant TET2 and advanced age; mutational frequency was ~23% in patients ≤60 years old versus ~4% in younger patients (p<0.0001). Presence of mutant TET2 did not affect survival, leukemic transformation or thrombosis in either PV or PMF. Among the 48 patients with SM, 42 met WHO diagnostic criteria for SM and 6 displayed FIP1L1-PDGFRA; 12 (29%) SM, but no FIP1L1-PDGFRA patients had TET2 mutations. KITD816V was detected by PCR sequencing in 50% or 20% of patients with or without TET2 mutation (p=0.05), respectively. In SM, multivariable analysis showed a significant association between the presence of TET2 mutation and monocytosis (p=0.0003). The remaining 50 patients included 15 with chronic myelomonocytic leukemia (CMML), 16 with myelodysplastic syndrome (MDS), 7 with secondary acute myeloid leukemia (AML), 5 with de novo AML, 3 with MDS/MPN and 4 with MPN, unclassifiable (MPN-

U); the corresponding TET2 mutational frequencies were 20%, 6%, 43%, 20%, 33%, and 50%. A TET2 mutation co-existed with MPLW515L in one MPN-U patient and with PML-RARA in one AML-M3 patient. *Conclusions*. We conclude that TET2 mutations are ubiquitous among myeloid malignancies, are more prevalent in older patients in the context of MPN, display similar frequencies across disease stages, and can co-exist with JAK2V617F, KITD816V, MPLW515L and PML-RARA.

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MULTIPLE JAK2 V617F MUTATIONS ARE PRESENT IN MOST PATIENTS WITH ESSENTIAL THROMBOCYTHAEMIA: A NEW DISEASE PARADIGM

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Background. Essential thrombocythaemia (ET) is a myeloproliferative disorder characterised by chronic thrombocytosis in the absence of a recognisable cause and is usually an indolent disease with a low rate of transformation to acute leukaemia. The JAK2 V617F mutation is present in approximately 50% of patients with ET and, although its role in the pathogenesis of the disease remains controversial, detection of the mutation is often considered to indicate the presence of a monoclonal process. In most cases, however, the mutation is restricted to a subpopulation of neutrophils and platelets, and production of JAK2 wild-type (WT) platelets remains at normal levels. Non-mutated precursor cells may, therefore, be susceptible to the acquisition of further JAK2 mutations, resulting in multiple independent V617F-positive clones. *Aims*: To investigate the possibility that more than one JAK2 V617F-positive clone is present in patients with V617F-positive ET. *Design and Methods*: We used a common single nucleotide polymorphism (SNP) in exon 19 of the JAK2 coding sequence to genotype JAK2 V617F-positive alleles in 11 ET patients. For each patient, a PCR product spanning exons 14-20 of the JAK2 gene underwent restriction enzyme digestion (RED) to cut only JAK2 WT alleles. The V617F-positive products were then re-amplified and these second round PCR products were used to determine the exon 19 SNP allele (A or G) present. In 5 patients the exon 14-20 PCR products were cloned and V617F status and SNP genotype of individual clones examined. We also determined the inactivated X-allele in *in vitro* cultured JAK2 mutant-positive erythroid colonies from one patient using the human androgen receptor assay. *Results*. Both SNP alleles, in varying proportions, were detected in JAK2 mutant-positive alleles from neutrophils of 10 out of 11 ET patients studied using PCR and RED, indicating the occurrence of at least 2 separate JAK2 mutation events in the majority of ET patients investigated. These results were confirmed in cloned products from all 5 patients analysed. In 3 of these 5 patients, the majority of clones had allele A, in one the A clones and G clones were approximately equal, and in one the majority of clones had allele G. Neutrophil samples taken from one patient 23 months apart showed the ratio of A to G clones remained stable over this period, and in 2 patients the ratio of A to G clones was similar in platelet and neutrophil samples. In a further patient, mutant-positive erythroid colonies with either X-allele inactivated were detected (38% inactivated one allele, 62% inactivated the other), indicating that they could not have arisen from a common clonal precursor. *Conclusions*. These results indicate that the majority of JAK2 V617F-positive ET patients undergo at least two independent JAK2 mutation events and these can occur on a polyclonal background, suggesting that such individuals may have an increased susceptibility to acquiring this mutation. The identification of more than one V617F-positive clone in many mutant-positive ET patients implies an oligoclonal process (analogous to PNH), and the presence of the mutation should not, therefore, be equated with a malignant disease.

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TWO ROUTES TO LEUKAEMIC TRANSFORMATION FOLLOWING A JAK2 MUTATION-POSITIVE MYELOPROLIFERATIVE NEOPLASM

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Background. Transformation to acute myeloid leukaemia (AML) may follow a JAK2-mutant myeloproliferative neoplasm (MPN), although the mechanisms of disease evolution, which may involve loss of the JAK2 mutation, remain obscure. *Design and Methods*. Comprehensive clinical information and blood samples were obtained from 16 patients with AML and a preceding JAK2-mutant MPN (essential thrombocythaemia (ET), polycythaemia vera (PV), refractory anaemia with ringed sideroblasts and thrombocytosis (RARS-T) or primary myelofibrosis (PMF); 15 JAK2 V617F and 1 JAK2 exon 12; see table). Leukaemic blasts (and progenitor colonies where indicated) were assessed for JAK2 mutations, loss of heterozygosity by analysis of single nucleotide polymorphisms within or adjacent to the JAK2 locus, AML-associated mutations (N/KRAS, FLT3, EVI1, NPM, CEBPA, RUNX1 and TP53) and mutations in TET2.

Table 1. Features of 16 patients with post-MPN AML.

Patient	Age/sex	MPN	*JAK2* mutation	Therapy	At transformation to AML	
					JAK2 mutation	Other mutations
#1	65 M	PMF	*JAK2* V617F	HC, AN	heterozygous	NRAS G12S *RUNX1* D171N
#2	63 M	ET->PMF	*JAK2* V617F	HC	homozygous	*NRAS* G12D ↑*EVI1* expression
#3	57 F	PV->PMF	*JAK2* V617F	HC, IFN	homozygous	*RUNX1* N119K
#4	59 M	PMF	*JAK2* V617F	none	homozygous	*RUNX1* G138V
#5	70 M	PMF	*JAK2* V617F	none	homozygous	*RUNX1* D171N
#6	80 M	PMF	*JAK2* V617F	HC	homozygous	-
#7	73 M	ET->PMF	*JAK2* V617F	HC	homozygous	*TP53* R248Q
#8	69 M	PV	*JAK2* N542-E543del	P32, BU, HC	wild-type	*TET2* D1242V *RUNX1* H215X
#9	82 M	PMF	*JAK2* V617F	HC	wild-type	-
#10	87 F	PV	*JAK2* V617F	HC	wild-type	*FLT3*-ITD *RUNX1* Q127X
#11	78 F	PV	*JAK2* V617F	HC	wild-type	*TP53* S90X
#12	84 M	RARS-T	*JAK2* V617F	HC	wild-type	*TP53* V173M
#13	93 M	PV	*JAK2* V617F	P32, BU, HC	wild-type	*TP53* C238F
#14	75 M	PV	*JAK2* V617F	HC	wild-type	-
#15	61 F	PV	*JAK2* V617F	HC	wild-type	*TET2* P1549X
#16	86 F	ET	*JAK2* V617F	HC, BU	wild-type	-

M: male; F: female; PMF: primary myelofibrosis; ET: essential thrombocythaemia; PV: polycythaemia vera; RARS-T: refractory anaemia with ringed sideroblasts and thrombocytosis; HC: hydroxycarbamide; AN: anagrelide; IFN: interferon; BU: busulphan

Results. Analysis of highly purified leukaemic blasts demonstrated 6 V617F-homozygous, 1 V617F-heterozygous and 9 JAK2 wild-type leukaemias. Loss of the mutant JAK2 allele by mitotic recombination, gene conversion or gene deletion was excluded in all 9 JAK2 wild-type leukaemias. All 7 patients with JAK2-mutant AML were diagnosed with PMF or myelofibrotic transformation of ET/PV. In marked contrast, only 1 of 9 patients with JAK2 wild-type AML had evidence of preceding myelofibrosis, with the remaining 8 cases transforming directly from ET, PV or RARS-T (p=0.001). This difference in preceding MPN phenotype, coupled with a strong association with the JAK2 mutation status of the leukaemia, indicates the existence of two distinct pathways to leukaemia. No consistent differences were found, however, in the pattern of AML-associated mutations between the two groups: both JAK2-mutant and wild-type leukaemias harboured mutations in RUNX1 (4/7 and 2/9 respectively) or TP53 (1/7 and 3/9 respectively); EVI1 overexpression or oncogenic RAS mutations were detected in JAK2-mutant leukaemias (EVI1: 1/7, RAS: 2/7) and a single JAK2 wild-type leukaemia harboured a FLT3-ITD (see Table 1). Mutations in TET2 may precede acquisition of a JAK2 mutation and could potentially represent a shared clone of origin for JAK2-mutant MPN and JAK2 wild-type AML. Analysis of a patient with a biclonal MPN (JAK2 V617F and MPL W515L mutations in separate clones) identified the same TET2 mutation in JAK2-mutant, MPL-mutant and JAK2/MPL wild-type erythroid colonies, demonstrating that a TET2-mutant founder clone may give rise to phylogenetically related daughter clones bearing dissimilar patterns of genetic mutation. Although TET2 mutations were present in 2/9 JAK2 wild-type leukaemias, in both cases the TET2 mutation was absent from all JAK2-mutant erythroid colonies, demonstrating that in these two patients the TET2 and JAK2 mutations were present in separate clones. *Conclusions*. hese data demonstrate the existence of two routes to AML transformation that are strongly associated with the JAK2 status of leukaemic blasts. Our results are consistent with a model in which JAK2-mutant AML is preceded by accumulation of mutations that give rise to the phenotype of PMF or myelofibrotic transformation of ET/PV. By contrast, JAK2 wild-type AML usually follows directly from ET or PV, implying that different pathogenetic mechanisms underlie transformation to JAK2 wild-type versus JAK2-mutant leukaemia.

Chronic myeloid leukemia - Biology II

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GENOME-WIDE SCREENING OF CHRONIC MYELOID LEUKEMIA PATIENTS BY SNP ARRAYS: ALTERATIONS ASSOCIATED WITH DISEASE PROGRESSION

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BACKGROUND. The expression of BCR-ABL fusion gene in Chronic Myeloid Leukemia (CML) is necessary for malignant transformation but the biologic basis of the progression from Chronic Phase (CP) through accelerated phase to Blast Crisis (BC) is poorly understood. *AIM*. Identifying the genomic imbalances involved in the transition into BC, before clinical features, may provide diagnostic markers of progression; for this reason we planned to use SNP arrays to perform a high-resolution mapping of BC CML patients genomes. *Design and Methods*. We analyzed 11 patients affected by myeloid or lymphoid BC CML disease. Genomic DNA were extracted from bone marrow or peripheral blood mononuclear cells archived both at the time of diagnosis and progression. SNP array-based karyotyping was carried out using Affymetrix GeneChip Human Mapping 250K Nsp arrays. Copy number (CN) and loss of heterozygosity (LOH) analyses were performed using Hapmap normal individuals as reference set and two different softwares: Partek Genomics Suite 6.4 and Affymetrix Genotyping Console 3.0. Genes were recognized according to the March 2008 human reference sequence (NCBI Build 36.3). *Results*. After exclusion of genomic copy number variations (CNVs) by comparison to recorded CNVs in the Genome Variation Database (*http://projects.tcag.ca/variation/*), the following results were achieved: five patients showed huge amplifications and deletions, ranging from 30Mb to 160Mb, on chromosomes 9, 7, 3 and 6. We also found several heterozygous micro-deletions and micro-amplifications spreading all over the genome. This analysis has identified abnormalities in genes involved in apoptosis (e.g., GADD45A, FOXO3A, GAS6), DNA damage response (e.g., MYST as known as Hmof, XRCC2), tumor suppression (e.g., C/EBPdelta, LATS1), chromatin regulation (e.g., HDAC9), and genes belonged to ABC transporters (e.g., ABCB1), cytoskeletal and adhesion molecules (e.g., ITGAL as known as LAF1, SDK1), cytochromes (e.g., CYP3A5), tyrosine kinase (e.g., JAK2), ras family, transcriptional/ translational factors (e.g., ETV1), and zinc finger proteins. Moreover were found copy number changes (gain/loss) in genes associated with malignancy, in particular TNFRSF17, MET, IGFBPL1, EVI1, PTENP1. Other alterations affected key pathways including cell cycle regulation, WNT signaling, and proteasome. Interestingly, we also observed a high frequency of LOH on chromosomes 1, 4, 7,9,11,17 and 19 but in different patients; in addition, inside the altered regions of chromosomes 9, 7, 17, 19, the loss of 12 well-known miRNAs was highlighted. *Conclusions*. The use of the newly genomic tool Genome-Wide Human SNP array 250K allowed us to identify, at submicroscopic level, genetic lesions in patients affected by CML in BC. Our results will be further validated by real-time PCR for the altered genes involved in biological pathways, while sequencing and mutation analysis will be performed on the remaining allele of putative tumor suppressor genes to identify their residual activity. All these validations and the increased number of patients analyzed both at time of diagnosis and progression will provide new insights into the genetic profiling that lead disease progression from CP to BC and consequently new opportunities to develop specific target therapies.

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ICSBP DEFICIENCY IN CML CONFERS DIRECT BCR/ABL-INDEPENDENT IMATINIB RESISTANCE BUT RESTRICTS BCR/ABL KINASE POINT MUTATION DEVELOPMENT

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Background. Chronic myelogenous leukemia (CML) is a myeloproliferative disorder that is caused by the constitutively activated BCR-ABL tyrosine kinase. Interferon Consensus Sequence Binding Protein (ICSBP) is a transcription factor which is expressed in the bone marrow compartment and predominantly in the lymphatic system. Its expression is down regulated in the peripheral blood of CML patients. Current therapeutic strategies in CML include ABL-specific tyrosine kinase inhibitors (TKI) such as Imatinib (IM), Dasatinib (DA) and Nilotinib (NI). However, resistance and persistence of leukemic (stem) cells under TKI therapy still remains a significant clinical problem. Mutations in ABL kinase domain account for TKI-resistance in about 90% of BCR/ABL positive ALL, but only in ~50% of CML patients. Still little is known about mutation-independent mechanisms of TKI resistance and the nature of kinase mutation emergence. *Aims*. To elucidate mechanism of IM resistance development, we here analyzed the impact of ICSBP deficiency for BCR/ABL-dependent and/or -independent IM and TKI resistance. *Design and Methods*. 32D-BCR/ABL and 32D-BCR/ABL-ICSBP cell lines were generated as described previously. Lineage depleted primary bone marrow (BM) cells of ICSBP-wt and ICSBP-null (-/-) background were transduced with BCR-ABL to yield wt-BA and ICSBP-/- BA cells. Apoptosis was measured with propidium iodide (PI) staining. Viable colonies (CFU) were counted in methylcellulose after one time exposure to TKI. Phosphorylation was analyzed by intracellular staining and immunoblotting. BCR/ABL kinase mutagenesis was studied using the N-ethyl-N-nitrosourea (ENU) mutagenesis assay. *Results*. We demonstrate that wt-BA, when compared with ICSBP-/- BA, are significantly more sensitive to apoptosis induction by IM 4um (40% vs. 19%), DA 150nm (91% vs. 48%), and NI 4um (58% vs. 34%). Similarly, we observed less CFU in wt BA as compared to ICSBP-/- BA when treated with IM 4um (14±5 vs. 66±13), DA 150nm (10±6, 44±4) and NI 4um (8±8, 57±4). The mechanism of reduced sensitivity to TKI treatment in ICSBP-/- BA cells was clearly BCR/ABL-independent, because intracellular FACS analysis revealed no significant differences in the inhibition of phosphorylation (inactivation) of BCR/ABL and its downstream targets Stat5 and CrkL in an ICSBP-/- and ICSBP-wt context after treatment with IM, DA or NI. Likewise, significantly more apoptosis was observed in the myeloid 32D-BA cell system when ICSBP was coexpressed (~80% cell death after 48h) as compared to 32D-BA cells (~25% cell death after 48h). Increased apoptosis induction in 32D-BA-ICSBP cells was associated with a more rapid and more sustained phosphorylation of p38 MAPK - a proapoptotic gene, which has recently been implicated in the control of oncogene addiction. In order to address, whether an increased apoptosis response in the presence of ICSBP also translates into a lower frequency of kinase mutation evolution, we quantitated the rate of IM-induced BCR/ABL-kinase mutations in presence and absence of ICSBP using an established TKI mutagenesis screen. Unexpectedly, significantly more IM-resistant colonies emerged in 32D-BA-ICSBP cells as compared to 32D-BA cells, but only if cells were treated prior to IM-exposure with the randomly acting mutagen, ENU. This suggests that absence of ICSBP expression limits the development kinase point mutations and *vice versa*. *Conclusion*. Lack of ICSBP expression in CML may thus confer BCR/ABL-independent IM drug resistance, but also limit the frequency of point mutation development in a myeloid cell context. This would explain the lower rates of point mutations found in IM-resistant CML as opposed to BCR/ABL positive, IM-resistant ALL.

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OMACETAXINE CYTOTOXIC ACTIVITY IN CHRONIC MYELOID LEUKAEMIA STEM CELLS

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Chronic myeloid leukaemia (CML) is maintained by a rare population of malignant stem cells which are insensitive to tyrosine kinase inhibitors (TKI) including imatinib, nilotinib and dasatinib, the standard drugs of choice in this disease. We are therefore interested in investigating compounds that inhibit Bcr-Abl independent pathways important for CML stem cell survival. Our long term aim is to find a drug that can be combined with a TKI to improve overall disease response in chronic phase (CP) CML. Omacetaxine mepesuccinate (formerly homoharringtonine), a first in class cetaxine, has been evaluated by clinical trial in TKI insensitive / resistant CML. Omacetaxine is thought to inhibit synthesis of anti-apoptotic proteins of the Bcl-2 family including Mcl-1 and Bcl-XL, leading to cell death. We investigated the *in vitro* response of CP CML CD34+ versus normal stem cells to omacetaxine. Total CD34+ cells and more primitive CD34+38- subpopulations sorted by flow cytometry, were assessed for cell viability (trypan blue exclusion), apoptosis (Annexin-V / Viaprobe), protein expression (Western blotting), stem cell function (colony forming cell and long term culture initiating cell (LTCIC) assays), and cell division (CFSE tracking). For continuous drug exposures, concentration-effect curves established the IC50 for cell viability in total CML CD34+ cells (n=3) to be 62 and 2.8nM at 24 and 72h, respectively, which are clinically achievable concentrations. Following treatment with 10 or 100nM omacetaxine for 0.25, 2, 6, 24, 48 and 72h, the percentage of apoptotic cells (Annexin V+ / Viaprobe +) increased to a maximum of 90% at 72h. It was evident, however, that a minimum exposure time of 24h was sufficient to diminish cell counts at 72h to 30 and 4% of input with 10 and 100 nM omacetaxine, respectively. As we were interested in the mechanism of action as a secondary endpoint, and to ensure sufficient protein for analysis, we selected time points <24h for further investigation. Initial Western results show a reduction in Mcl-1 after 2h drug treatment of CML CD34+ cells and Bcl-2 protein levels also decreased progressively with increasing exposure to 10nM omacetaxine from 6 to 24h. Analysis of protein from the primitive CD34+38- sorted cell population also indicates reduced levels of Mcl-1 compared with no drug control. With respect to untreated CD34+ cells, omacetaxine inhibited colony formation at day 12 by approximately 50% in a dose dependent manner (10 to 100 nM). The effect of this drug on cell division was analysed using the fluorescent marker, CFSE. Omacetaxine exerted an anti-proliferative effect, holding the cells back in early divisions with respect to untreated control. However, when the drug was washed out, some stem cells appeared to recover their proliferative potential and could expand in culture on days 5 to 6. Although omacetaxine is not leukaemia stem cell specific as non-CML CD34+ control cells were found to be sensitive to the drug's activity, its apoptotic inducing functions on leukaemia stem cells differentiates it from TKIs and create the potential of a curative strategy. Further results for LTCIC and mechanism of action will be presented.

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BCR-ABL PEPTIDE VACCINATION IN HEALTHY SUBJECTS: IMMUNOLOGICAL RESPONSES ARE EQUIVALENT TO THOSE IN CHRONIC MYELOID LEUKAEMIA PATIENTS

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Chronic Myeloid Leukaemia (CML) is characterised by the BCR-ABL oncoprotein. We and others have previously reported that vaccination of CML patients with BCR-ABL junctional peptides can elicit anti-BCR-ABL T cell responses, and that these immune responses correlate with a decrease in BCR-ABL transcript levels. However, the anti-BCR-ABL immune responses we observed were only moderate and transient. To determine whether CML patients may be tolerised to BCR-ABL, here we vaccinated healthy subjects with the same protocol as we have previously reported for CML patients, and compared the intensity of the immune responses obtained in patients and healthy subjects. Vaccinations consisted of a cocktail of 3 BCR-ABL peptides: (1) a 9-mer spanning the e14a2 region, (2) this same 9-mer linked to a PADRE (a 15-mer non-natural peptide shown to activate $CD4^+$ T cells, to which all subjects are immunologically naive), and (3) a 13-mer consensus e14a2 junctional peptide linked to PADRE, administered intradermally at a dose of 600mg each with sargramostim (GM-CSF), on 6 occasions over 2 months. Immune responses were monitored by IFN-γ, Granzyme-B and IL-5 ELISPOT, and proliferation assays to the vaccination peptides by flow cytometry on peripheral mononuclear cells. No subjects showed detectable anti-BCR-ABL or anti-PADRE immune response at entry. All 5 subjects developed a proliferative response to the PADRE-containing peptides used in the vaccine. IFN-α and Granzyme-B production to PADRE was detected in all healthy subjects, demonstrating that the vaccine is capable of eliciting immune responses to novel antigens. Specific IFN-γ and Granzyme-B production to BCR-ABL was also detected in all healthy subjects post vaccination demonstrating that vaccination can elicit anti-BCR-ABL cells in healthy subjects. No IL-5 production was detected, indicating type 1 bias of the immune responses elicited by the vaccine. The specific IFN-γ production to PADRE and BCR-ABL peptides observed in all 5 healthy subjects were compared with those in all 19 CML patients in our previously reported EPIC study. For each timepoint the specific IFN-γ production was compared using a Mann-Whitney test between the healthy subject and patient group. No statistical differences in the intensity or duration of the immune response detectable post-vaccination were found. The present data indicate that CML patients are unlikely to be tolerised to BCR-ABL. The data also suggest that BCR-ABL may be a weak antigen, and this underlines the importance of measuring the immunogenicity of the antigen to be used in immunotherapy. If BCR-ABL has a role in immunotherapy, it may be preferable to use it as part of a multi-epitope vaccination strategy.

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BCR-ABL ENHANCES DIFFERENTIATION OF LONG-TERM REPOPULATING HEMATOPOIETIC STEM CELLS

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Background. Recently, we developed an inducible transgenic mouse model in which stem cell-targeted induction of BCR-ABL expression leads to chronic phase CML-like disease. *Aims*: To define the consequences of BCR-ABL expression in hematopoietic stem cells (HSC) *in vivo*. *Design and Methods*. Transplantation studies using inducible transgenic mice. *Results*. Here, we demonstrate that the disease is transplantable using BCR-ABL positive LSK cells (lin-Sca-1+c-kit+). Interestingly, the phenotype is enhanced when unfractionated bone marrow (BM) cells are transplanted. However, progenitor cells (lin-Sca-1-c-kit+) or cells displaying the immunphenotype of mature granulocytes (CD11b+Gr-1+) were not able to transmit the disease or alter the phenotype. The phenotype was largely independent of BCR ABL priming prior to transplant. However, BCR-ABL abrogated the potential of LSK cells to induce full blown disease in secondary recipients. Subsequently, we found that BCR-ABL increased the fraction of multipotent progenitor cells (MPP) at the expense of long term HSC (LT-HSC) in the BM of donor mice. Interestingly, while CML-like disease was reversible and re-inducible in transplanted mice, LSK cells transplanted from reverted BM were not able to re-induce disease in secondary recipients. *Conclusions*. Our results suggest that BCR-ABL induces differentiation of LT-HSC and decreases their self renewal capacities. In addition, they suggest that, although induction of disease is restricted to LSK cells, further cell populations contribute to the phenotype of CML.

Bleeding disorders, platelets and thrombocytopenia

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ESTABLISHMENT OF A EUROPEAN NETWORK OF RARE BLEEDING DISORDERS (EN-RBD DATABASE): PRELIMINARY RESULTS ON 190 PATIENTS

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In 2007, a European Network of Rare Bleedings Disorders (RBDs) was established and funded by the European Community. Ten Treatment Centers were enrolled to collect coordinated and consistent clinical, genetic and treatment data using a database with open-source engine and a web-based application. *Design and Methods*. A year after establishment of the project, complete data on 190 patients were analysed. Since the severity of RBDs is not always correlated with the level of coagulant activity, we arbitrary classified the patients as severe (<5%), moderate (6-10%) or mild (>10%). Chi-square or Fisher's tests were used to compare severe patients with moderate or mild ones in order to understand whether <5% of coagulant activity could prevent severe bleeding. A p value <.05 was considered to be statistically significant. *Results*. 43% of patients were severe, 11% moderate and 46% mild; 24% of patients were asymptomatic (10% of severe, 20% of moderate, 36% of mild). The frequency of mucocutaneous (cutaneous, epistaxis, menorrhagia, oral cavity) and GI bleedings were 45%±20 and 27%±8, respectively in all severe RBDs. Hematomas, haemarthrosis and CNS bleeding were more frequent in afibrinogenemia and severe FXIII deficiency. In mild/moderate RBDs, mucocutaneous bleedings were 33%±23, while more severe bleedings were significantly less frequent, but still present in few patients. A significant difference in the number of severe bleeds (haematoma, haemarthrosis, GI and CNS) was observed in severe patients compared to moderates (p<0.05). Comparing severes versus milds, mucocutaneous bleedings were also significantly different (p<0.007). Surprisingly menorrhagia and epistaxis were similar in the three groups. *Conclusions*. These results, coming from the analysis of a large amount of homogeneous data allow us to report that 5% of residual coagulant activity seems to be sufficient to prevent severe types of bleeds, while a higher amount of coagulation factor is required to prevent mucocutaneous bleeds.

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RETROSPECTIVE EVALUATION OF DYSFIBRINOGENEMIC PATIENTS AT A SINGLE CENTER: CLINICAL FEATURES AND LABORATORY FINDINGS

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Background. Dysfibrinogenemia is a very rare bleeding disorder in which the clinical phenotype is unpredictable. The literature consists predominantly of collections of case reports. A relatively recent compilation of over 250 patients, revealed that 53% were asymptomatic, 26% had hemorrhages and 21% had thromboses, some of whom also had hemorrhages. *Aims*. To retrospectively investigate the clinical features and laboratory findings of our dysfibrinogenemic patients and to compare them with the literature data. *Design and Methods*. Over the last 10 years, 27 dysfibrinogenemic patients were diagnosed at our center: 12 were males and 15 females, with a median age at diagnosis of 39.9 years (6.1-80.9); they were grouped in 15 families. The reasons for admission were: reduced fibrinogen activity in routine screening in 10 patients, pre-operative coagulation screening in 7, coagulation screening for bleeding in 1, ischemic symptom in 1, familial study in 8. The laboratory findings were as follows:

	Mean value	Median value	Range
Fibrinogen activity (n.v. 200-400 mg/dL)	37	38	0-112
Fibrinogen antigen (n.v. 200-400 mg/dL)	251	250	140-470
PT ratio (n.v. 0.90-1.14)	1.2	1.2	0.9-1.74
aPTT ratio (n.v. 0.92-1.16)	1.1	1.09	0.9-1.3

Results. "Full" mutational screening of the 3 fibrinogen genes (FGA, FGB, FGG) has, so far, led to a genetic diagnosis in 5 of the 14 studied patients: 1 was heterozygous for the novel FGG Asp330Val (g.7641A>T) mutation, whereas the other 4 were heterozygous for previously reported mutations; 3 carried the FGA Arg16His (g.1203G>A) mutation and 1 the FGG Arg275Cys (g.7475C>T). Sixteen of the 27 patients experienced hemorrhagic symptoms, mostly mild: traumatic cutaneous bleeding in 9; gastro-intestinal bleeding in 3; epistaxis in 7; gum bleeding in 2; heavy menses in 7. One patient experienced cerebral ischemia (concomitant disease: acleisto-cardia). Twenty one patients underwent surgery and 15 dental extractions. Prior to diagnosis carried out at our center, tranexamic acid and plasma infusions were used as prophylactic anti-hemorrhagic treatment before surgery in 2 and 1 cases, respectively. Only 1 patient bled after dental extraction. Eleven spontaneous deliveries and 8 cesarian sections were carried out without any prophylaxis treatment. No hemorrhagic or thrombotic complications were reported. No spontaneous abortions occurred. *Conclusions*. In our case series the prevalence of asymptomatic patients is inferior to literature data (37% vs 53%). Only 1 patient (4%) had a thrombotic event. Most hemorrhagic patients (59%) experienced only mild symptoms. None of them needed red blood cell transfusions. Anti-hemorrhagic prophylaxis of surgery with tranexamic acid and plasma was administered in other institutions, before diagnosis, on the basis of the low fibrinogen activity in pre-operative coagulation screening.

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THE CONFORMATIONAL CHANGES OF THE VON WILLEBRAND FACTOR (VWF) TESTED BY NANOBODY AU/VWF-A11 IN A COHORT OF 86 PATIENTS WITH DIFFERENT TYPES OF VON WILLEBRAND DISEASE: AN ADDITIONAL TOOL TO LOCALYZE MOLECULAR DEFECTS OF VWF

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Background. von Willebrand disease (VWD) is the most common inherited bleeding disorder that is caused by quantitative (VWD1 and VWD3) or qualitative (VWD2A. VWD2B, VWD2M, VWD2N) defects of von Willebrand factor (VWF). Several laboratory tests are usually required for a correct diagnosis of these VWD types. A llama-derived antibody fragment (nanobody) recognizing the glycoprotein Iba-binding conformation of VWF (VWF-GPIb:BC) has been developed (Blood 2005;106:3035) and values higher than normal have been found in patients with VWD2B. *Aims, Patients and Methods*. To further explore the usefulness of VWF-GPIb:BC in VWD diagnosis we have tested the nanobody in the plasma obtained from 86 patients with VWD1 (12),

VWD1/2MVIC (6); VWD1/2BNY (12), VWD2A (14); VWD2B (32), VWD2M (10), all characterized by mutations within or outside VWFA1 domain. All the 86 patients enrolled in the study were diagnosed by ristocetin induced platelet aggregation (RIPA) in the platelet rich plasma, ristocetin cofactor activity (VWF:RCo), VWF antigen (VWF:Ag), multimeric structure of VWF. Mutations within or outside VWFA1 domain were searched for and confirmed by sequencing. VWF-GPIb:BC was tested in 40 normal individuals (% of normal plasma = 0.70+0.13), in all VWD (86) at baseline and in 12 cases with VWD1, VWD2A and VWD2M before-1-2 hours after desmopressin (DDAVP). *Results*. VWF-GPIb:BC was high in all VWD2B but the 16 cases with P1266L/Q, R1308L mutations not associated with thrombocytopenia, with values up to 6 times higher than those of healthy controls. Interestingly, high VWF-GPIb:BC was also found in VWD2M(10) patients with mutations (D1277-78EdelInsL, R1315C/L, Y1321C, R1374H) within VWFA1 domain. Values lower than normal were measured in VWD1 and VWD2A, all characterized by mutations outside VWFA1. VWF-GPIb:BC was also measured before-1-2 hours after DDAVP in 12 patients [known mutations] and mean results were as follows: VWD1(6) [R202W, R275C, R1205H, R1583Q, C1927R]=0.5-1.7-1.5; VWD2A(3) [S1506L, V1607D, V1665E]=0.1-0.5-0.4;VWD2M(3) [D1277-78EdelInsL,R1315L,R1374H]=2.2-4.0-3.6. VWF-GPIb:BC levels increased after DDAVP because of the VWF released from endothelial cells, but remain lower than normal in VWD1 and VWD2A. *Conclusions*. Patients with VWD1 and VWD2 showed different results on VWF-GPIb:BC which might reflect the effect of mutations in adjacent domains (D3 and A2) on the GpIb-binding conformation of the A1 domain. Based on these results we conclude that VWF-GPIb:BC can be a useful tool for a rapid identification of different VWD types.

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ASSOCIATION OF HEALTH-RELATED QUALITY OF LIFE (HRQOL), BLEEDING, AND PLATELET LEVELS IN CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA (ITP): RESULTS FROM RAISE AND EXTEND

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Background. Chronic ITP is characterized by autoantibody-induced platelet destruction and reduced platelet production, leading to a low platelet count (<150 Gi/L). Bleeding and bruising signs and symptoms are largely dependent on the platelet count. Eltrombopag treatment is aimed at elevating and maintaining platelets above 50 Gi/L to minimize the risk of bleeding and to improve HRQoL and functioning. *Aim.* To evaluate the association among HRQoL outcomes, bleeding, bruising, and platelet counts in RAISE and EXTEND, long-term trials of eltrombopag. *Design and Methods*. The strength of the correlation among mean HRQoL outcomes, platelet levels, and frequency and severity of bleeding was estimated from linear models using generalized estimating equations methodology in previously reported RAISE and EXTEND studies (Cheng et al. Blood. 2008;112:400; Bussel et al. Blood. 2008;112:3432). Bleeding was evaluated using the WHO Bleeding Scale (Grade 0 = no bleeding through Grade 4 = debilitating blood loss). HRQoL was self-reported at baseline and various points during the study using: 1) the SF-36v2; 2) FACIT-Fatigue subscale; and 3) 6-item subset of the FACT-Thrombocytopenia (FACT-Th), which focuses on concern with bruising and bleeding, and limits to daily physical and social activities. *Results*. RAISE data from 197 evaluable patients suggest that as platelet counts rise and bleeding is reduced, HRQoL improvement is perceived by patients with chronic ITP (Table). All associations between HRQoL and bleeding, and between HRQoL and platelet counts, are statistically significant at $p<0.05$; coefficients are directionally correct. Similar results are observed in EXTEND with 144/207 patients evaluable (data not presented). The increases in platelet counts, decreases in bleeding, and increases in HRQoL were previously reported as independent outcomes in the long-term trials of eltrombopag; selected results are presented below as context. The statistically significant associations presented above suggest that these outcomes are linked. In RAISE, the median platelet count began to rise after 1 week in eltrombopag-treated patients and remained >50 Gi/L throughout the 6-month treatment period, whilst median platelet counts for the placebo group did not rise above 30 Gi/L. Fewer patients in the eltrombopag group had clinically significant bleeding compared with the placebo group. HRQoL improvements were observed in vitality, emotional role, and physical role domains during eltrombopag therapy, whereas no improvements were observed in the placebo group. In EXTEND, approximately 80% of evaluated patients achieved platelet counts >50 Gi/L during the study. In EXTEND, as in RAISE, elevations in platelet counts and reductions in bleeding were accompanied by improvements in HRQoL across multiple domains, measures of fatigue, and activities of daily living. *Conclusions*. HRQoL improvements in the RAISE (and EXTEND) studies are associated with elevation in platelets and reduction in bleeding and bruising symptoms, and improves one or more dimensions of HRQoL.

Table.

HRQoL Domain or Component Score	Covariate	Coefficient[a]	95% CI
SF-36v2			
Vitality	Platelet Count	1.90	0.36 , 3.45
	WHO Bleeding vs No Bleeding	-6.77	-10.28 , -3.26
Physical Function	Platelet Count	2.07	0.98 , 3.16
	WHO Bleeding vs No Bleeding	-6.79	-10.05 , -3.53
Bodily Pain	Platelet Count	1.71	0.16 , 3.25
	WHO Bleeding vs No Bleeding	-7.99	-11.94 , -4.04
General Health	Platelet Count	1.48	0.22 , 2.74
	WHO Bleeding vs No Bleeding	-5.30	-8.18 , -2.42
Social Function	Platelet Count	2.35	0.26 , 4.44
	WHO Bleeding vs No Bleeding	-7.20	-11.41 , -2.99
Emotional Role	Platelet Count	2.38	0.65 , 4.10
	WHO Bleeding vs No Bleeding	-8.75	-12.86 , -4.64
Mental Health	Platelet Count	1.47	0.19 , 2.76
	WHO Bleeding vs No Bleeding	-4.13	-7.23 , -1.04
Physical Role	Platelet Count	2.58	1.13 , 4.02
	WHO Bleeding vs No Bleeding	-8.81	-13.44 , -4.19
Mental Component Summary	Platelet Count	0.95	0.07 , 1.82
	WHO Bleeding vs No Bleeding	-2.89	-4.67 , -1.11
Physical Component Summary	Platelet Count	0.75	0.31 , 1.19
	WHO Bleeding vs No Bleeding	-2.77	-3.99 , -1.55
FACIT-Fatigue	Platelet Count	1.43	0.78 , 2.07
	WHO Bleeding vs No Bleeding	-3.70	-5.22 , -2.18
FACT-Th (6 items)	Platelet Count	0.99	0.62 , 1.37
	WHO Grade >0 vs 0	-2.68	-3.56 , -1.80

CI, confidence interval.
a. The coefficient is estimated from an unadjusted linear longitudinal model and describes the change in mean outcome associated with a one unit increase in the covariate, including only subjects completing baseline assessments. Platelet counts are expressed as log transformations.

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EFFICACY AND SAFETY OF ROMIPLOSTIM VERSUS MEDICAL STANDARD OF CARE AS CHRONIC THERAPY FOR NONSPLENECTOMIZED PATIENTS WITH IMMUNE THROMBOCYTOPENIA (ITP)

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Background. Chronic ITP is an autoimmune disease characterized by low platelet counts due to both increased platelet destruction and suboptimal platelet production. Conventional ITP therapies that target the immune system have variable response rates and issues regarding toxicity. Romiplostim is an Fc-peptide fusion protein (peptibody) that increases platelet counts by a mechanism similar to thrombopoietin, and has recently been approved in the EU, US, Canada, and Australia for the treatment of chronic ITP. *Aims*. To evaluate the ability of romiplostim versus medical standard of care (SOC) to reduce the incidence of splenectomy and treatment failures in adult nonsplenectomized ITP patients during a 52-week treatment period. *Design and Methods*. Patients provided informed consent and were randomized (2:1) to romiplostim or SOC and stratified by geographic region. Eligible patients either had a platelet count $<50\times10^9$/L or had their platelet count fall to $<50\times10^9$/L during or after a clinically-indicated taper or discontinuation of current ITP therapy. Once-weekly subcutaneous romiplostim was administered with dose adjustments based on platelet count. SOC treatments were prescribed by the investigator according to standard institutional practices or therapeutic guidelines; the only treatments not allowed were investigational agents (rituximab was allowed) or other thrombopoietic agents. The first primary endpoint was the incidence of splenectomy or study discontinuation. The second primary endpoint was the incidence of treatment failure (defined as: platelet count 20×10^9/L for 4 consecutive weeks at the highest recommended dose and schedule, or major bleeding event, or change in therapy due to intolerable side-effect or bleeding symptoms) or study discontinuation. *Results*. In total, 234 patients were randomized (SOC, 77; romiplostim, 157). In romiplostim-treated patients, there was a significantly lower incidence of splenectomy or study discontinuation (OR, 0.169; 95% CI: 0.081, 0.351; $p<0.0001$) and treatment failure or study discontinuation (OR, 0.374; 0.188, 0.744; $p=0.0039$) than in patients receiving SOC. A sensitivity analysis, applied to patients who did not discontinue the study, showed a consistent trend in the incidence of splenectomy and treatment failure with both of the

primary endpoint results. Safety analyses included all patients who received ≥1 dose of romiplostim or 1 type of SOC for ITP. Adverse events were experienced by 92% (69/75) of patients receiving SOC and by 95% (146/154) of patients receiving romiplostim. The most common adverse events in the SOC group were epistaxis (23%), nasopharyngitis (19%), and contusion (19%); and in the romiplostim group were headache (35%), fatigue (27%), and nasopharyngitis (23%). Treatment-related serious adverse events were reported by 8% (6/75) of SOC and 5% (7/154) of romiplostim patients. Three patients died during the 52-week treatment period: 2 SOC patients (cardio-respiratory arrest, hepatic failure), and 1 romiplostim patient (pneumonia). None of the deaths were deemed related to study treatment. Bleeding events with grade ≥3 severity were reported by 8% (6/75) of patients in the SOC group, compared with 3% (5/154) in the romiplostim group. *Conclusions*: Romiplostim significantly reduced incidences of splenectomy and treatment failure in nonsplenectomized ITP patients compared to standard of care. The safety profile was comparable between patients receiving romiplostim and those receiving SOC.

Table. Incidence of splenectomy and treatment failure.

	SOC N = 77	Romiplostim N = 157
Primary endpoints		
Splenectomy or study discontinuation[A]		
Overall	27/77 (35%)	13/157 (8%)
North America	12/31 (39%)	5/64 (8%)
European Union	11/37 (30%)	7/75 (9%)
Australia	4/9 (44%)	1/18 (6%)
Odds ratio, romiplostim/SOC (95% CI)	0.169 (0.081, 0.351)	
p-value	< 0.0001	
Treatment failure or study discontinuation[B]		
Overall	21/77 (27%)	19/157 (12%)
North America	11/31 (36%)	5/64 (8%)
European Union	8/37 (22%)	13/75 (17%)
Australia	2/9 (22%)	1/18 (6%)
Odds ratio, romiplostim/SOC (95% CI)	0.374 (0.188, 0.744)	
p-value	0.0039	
Sensitivity analysis[C]		
Splenectomy	15/77 (19%)	2/157 (1%)
Odds ratio, romiplostim/SOC (95% CI)	0.05 (0.01, 0.24)	
Treatment failure	9/77 (12%)	8/157 (5%)
Odds ratio, romiplostim/SOC (95% CI)	0.4 (0.14, 1.10)	

[A] Patients who received romiplostim or SOC and who discontinued study before reporting a splenectomy were scored as having had a splenectomy.
[B] Patients who received romiplostim or SOC and who discontinued study before reporting a treatment failure were scored as having had a treatment failure.
[C] Sensitivity analysis compared the incidence of patients who actually either reported a splenectomy procedure or met the treatment failure definition, excluding patients who discontinued the study.
Change in therapy to splenectomy, due to intolerable side-effects or bleeding symptoms, was counted as both treatment failure and splenectomy.

Acute lymphoblastic leukemia - Biology II

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PAX5 GENE IS FREQUENTLY REARRANGED IN A LARGE COHORT OF BCR-ABL1-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA: ON BEHALF OF GIMEMA AL & MDS WP

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Background. Recently, in genome-wide analyses of DNA copy number abnormalities using single nucleotide polymorphism (SNP) microarrays, a high frequency of genetic alterations of regulators of B-lymphoid development (Mullighan et al., Nature 2008) was described in B-progenitor ALL. Genetic alterations targeting the B lymphoid transcription factor PAX5 were identified in over 30% of cases. *Aim*. To characterize, by high resolution SNP arrays, the rearrangements on 9p involving the PAX5 locus. *Patients and Methods*. Affymetrix Human Mapping 250K NspI and Genome-Wide Human SNP 6.0 arrays and FISH assay using fosmid probes were used to profile the genomic of 98 adult BCR-ABL1-positive ALL patients. *Results*. The most frequent somatic copy number alterations affecting B-lymphoid development were deletions on 7p12 involving the IKZF1 gene (76/98, 78%), which encodes the transcription factor Ikaros, and mono-allelic copy number changes on 9p chromosome involving the PAX5 gene (31/98, 32%). Overall mono-allelic loss of PAX5 was identified in 28 patients (29%), whereas internal amplification in only 3 cases (3%). Four major PAX5 losses occurred: 1) focal deletions involving only the PAX5 gene in 3 cases (3%) with the minimal overlapping region of 101 kb from 37021876 to 36920536; 2) deletions involving only a portion of PAX5 and flanking genes in 8 (8%) with a median size of 364 kb (range: 154 kb-16395 kb) and ranging from 36603003 on 9p13.3 to 20553365 on 9p21.3; 3) broader deletions involving PAX5 and a variable number of flanking genes in 11 patients (11%) with a median size of 947 kb (range: 567 kb-18208 kb) and ranging from 36948016 on 9p13.3 to 20553365 on 9p21.3; 4) deletion of all chromosome 9 or 9p in 6 patients (6%). In 23 patients (23%) we identified the deletions of both IKZF1 and PAX5; in 51 patients (52%) only the deletion of IKZF1 was found, while the presence of deletion or rearrangements of only PAX5 gene was found in 5 patients (5%). In two cases we identified the loss of IKZF1 and the gain of an internal region of PAX5. According to the type of deletion we could have PAX5 haploinsufficiency or PAX5 mutants with impaired DNA-binding or transactivating activitiy. All these alterations are predicted to result in attenuation, but not complete abrogation of PAX5 activity, suggesting that PAX5 is a haploinsufficient tumor suppressor. FISH analysis with three overlapping BAC probes encompassing the whole PAX5 gene was performed confirming what obtained by SNP-array analysis. To investigate the consequences of genomic PAX5 alteration in BCR-ABL1-positive ALL patients, quantitative PCR (q-PCR) was used to assess the expression of PAX5 in cases with copy number changes on 9p13.2. Q-PCR confirmed the SNP results demonstrating that genomic alterations on 9p13.2 leads to a significant down-modulation at the transcript level of the Pax5. *Conclusions*. PAX5 rearrangements occurred at an incidence of about 30% in adult BCR-ABL1 ALL and its impairment may be associated with the development of this for this poor prognosis subtype of leukemia.

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MICRORNA SIGNATURES IN GENETIC SUBTYPES OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes that accounts for about 15 percent of ALL cases. Leukemic transformation of immature thymocytes is caused by a multistep pathogenesis involving numerous genetic abnormalities providing uncontrolled cell growth. Accumulating evidence suggests the presence of at least 5 different molecular-cytogenetic subgroups in T-ALL, ie. TAL/LMO, TLX1, TLX3, HOXA and MYB. Recently, non-coding microRNAs were discovered as important regulators of gene and/or protein expression and subsequently shown to be directly implicated in cancer. Nevertheless, it is currently unclear in which way deregulated miRNA expression may contribute to the pathogenesis of T-cell acute leukemia. In this study, we investigated whether different genetic subgroups in T-ALL are characterized by distinct miRNA expression patterns. Therefore, we profiled a total of 430 miRNAs using high-throughput quantitative stem-loop RT-PCR in a genetically well characterized T-ALL patient cohort (n=52), including 11 HOXA (3 MLL rearranged, 5 inv(7)(p15q35) and 3 CALM-AF10), 16 TAL/LMO (7 LMO2 rearranged, 8 SIL-TAL1 and 1 LMO2/TAL1 rearranged), 11 TLX3 and 5 TLX1 rearranged patient samples. Since T-ALL blasts originate from maturating T lymphocytes, we also profiled different subsets of sorted T-cell populations ($CD34^+$, $CD4^+/CD8^+/CD3^-$, $CD4^+/CD8^+/CD3^+$, $CD4^+$ single positive and $CD8^+$ single positive). These miRNA profiles of normal T-cells served as a negative control for the identification of deregulated miRNA expression that may be truly leukemia associated. SAM analysis (t-test and Wilcoxon, FDR=0) identified significant and differentially expressed miRNAs between the HOXA, TLX3 and TAL/LMO subgroups whereas no significant differentially expressed miRNAs were obtained for the TLX1 subgroup, probably due to the limited number of patient samples for this latter group. The HOXA subgroup showed specific up-regulation of miR-196a and miR-196b, which are encoded at the HOXB and HOXA cluster, respectively, but no significantly down-regulated miRNAs could be identified in this subgroup. The TLX3 subgroup was uniquely characterized by the up-regulation of miR-99a, miR-125b, let-7c, miR-508 and miR-509, and down-regulation of miR-127 and miR-182. Finally, specific up-regulation of miR-424, miR-148a, miR-422, miR-362, miR-148a, miR-502, miR-10a, miR-200c, miR-31, miR-660 and miR-15b, was identified in the TAL/LMO rearranged subgroup, which was also characterized by the specific down-regulation of miR-99b, miR-155, miR-125a, miR-153, miR-135a, miR-34a and miR-193b. Next, we evaluated the expression pattern of all significant and differentially expressed microRNAs in the different subsets of sorted normal T-cell populations. The expression patterns of these microRNAs could be classified into consistently active, completely absent or temporally regulated during T-cell development. For the microRNAs showing a temporal regulation during T-cell maturation, their differential expression in T-ALL subtypes may reflect differences in the maturation arrest of the T-cell of origin, rather than pointing to an oncogenic event. Nevertheless, their constitutive (in)activation in primary T-ALL patients could still be of oncogenic relevance, similar to transcription factors like TAL1 or LMO2 which also show a temporal regulation during T-cell maturation. In contrast, some other microRNAs showed no expression in any of the T-cell populations, providing strong evidence that their activation in specific T-ALL subtypes may contribute to T-ALL pathogenesis. In conclusion, this study shows that molecular-cytogenetic subgroups in T-ALL are characterized by a specific miRNA expression signature. In addition, correlation of our findings to the expression of these miRNAs in normal T-cell subsets may guide us to the miRNAs with true oncogenic potential. This report paves the way for further investigation directed at the role of these miRNAs in the pathogenesis of T-ALL, which may provide us with further insight in the oncogenic pathways that are (in)activated in different T-ALL subgroups. Ultimately, these deregulated miRNAs may offer new targets for therapeutic intervention.

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WILMS' TUMOR 1 GENE MUTATIONS IN ACUTE T-LYMPHOBLASTIC LEUKEMIA

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The wilms tumor 1 gene (WT1) encodes a transcriptional regulator involved in normal hematopoietic development and is aberrantly expressed in a subset of acute myeloid leukaemia (AML). When it is used for MRD monitoring, failure to normalize WT1 transcript levels after treatment predicted a significantly increased relapse risk. In addition, mutations of WT1 have been found in about 10% of AML patients (pts) and have recently been shown to predict poor outcome. Although Heesch et al. (ASH 2008) reported WT1 mutations in 8% of 239 adult T acute lymphoblastic leukemias (T-ALL), little data is available regarding the frequency, characteristics and clinical impact of WT1 mutations in this disorder. We searched for WT1 mutations in 204 T-ALL pts (128 adults and 76 children). 111 out of 128 adult T-ALLs were enrolled in the French GRAALL or LALA protocols. Diagnostic bone marrow specimens were studied for WT1 mutations by DNA sequencing. All pts were screened for exon 7 and 9 mutations and 46 pts were screened for all WT1 exons (1-10). Mutation status was compared with immunophenotype, T-Cell receptor (TCR) status, WT1, HOX11/TLX1, HOX11L2/TLX3, SIL-TAL, CALM-AF10, NUP-ABL transcript levels by real-time RT-PCR and NOTCH1 and FBXW7 mutational status. Sixteen out of 204 pts (8%) were found to have a WT1 mutation; 13 in exon 7 and 3 in exon 9. WT1 exon 7 mutations were frameshift mutations predicted to result in a truncated WT1 protein whereas exon 9 mutations were missense mutations. All identified mutations were heterozygous. No mutations were detected outside exon 7 and 9 in the 46 pts tested. Frequency of mutation did not differ significantly between adult and pediatric pts. WT1mut and WT1 wildtype (WTwt) pts did not significantly differ with respect to clinical parameters at diagnosis. WT1 was over-expressed in 75% of T-ALL, particularly those of the TCRαβ lineage (p=0.001) and all WT1mut pts demonstrated high WT1 expression levels [WT1mut median: 88.6% (range 17.2%%-557%) vs. WT1wt median: 7.8% (range: 0.001%-774%); p=0.001]. WT1mut were significantly (p=0.02) associated with deregulation of HOXA or TLX1/3 genes (4 TLX3 , 2 TLX1 , 2 CALM-AF10 , 1 MLL-AF6) and 3/9 WT1mut were NUP214-ABL positive vs 7/117 WT1wt (p=0.02). 10/12 WT1mut were NOTCH1 mutated (p=0.05). WT1mut cases were not characterized by particular immunophenotypic features in the EGIL classification , but were preferentially associated with expression of CD79a and with the TCRγδ lineage (5/51 IMδ/IMγ/TCRγδ vs. 2/92 IMb/pre ab/TCR ab cases, p=0.04). No WT1mut were seen in the 8 immature IM0 T-ALLs. Amongst adult T-ALLs, no significant differences were observed in the complete remission rate or overall survival., although 5/7 WT1mut relapsed vs 47/99 WT1wt pt and the DFS tended to be shorter when WT1 is mutated (median 10.8 months) vs. WT1wt (28.5 months). In conclusion, WT1 mutations occur in 8% of adult and pediatric T-ALL and are located in the same region as in AML. WT1mut T-ALLs express relatively high level WT1 transcripts and are found preferentially in T-ALLs of the TCRγδ lineage and/or in those which express HOXA or TLX1/3. Their prognostic impact needs to be tested in a prospective fashion.

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ANALYSIS OF GENOMIC BREAKPOINTS INDICATES THAT BCR-ABL IN ACUTE LYMPHOBLASTIC LEUKAEMIA MAY ARISE IN A COMMITTED LYMPHOID PROGENITOR CELL IN A SUBSET OF CASES

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Chromosomal translocations in haematological malignancies are believed to result from non-homologous end joining (NHEJ) following two double stranded DNA breaks. In lymphoid disorders, DNA breaks may be a consequence of normal or aberrant RAG and/or AID activity, whereas in myeloid disorders the reasons for breakage are unknown. The paradigm for fusions genes in leukaemia is BCR-ABL, produced as a consequence of the t(9;22). In chronic myeloid leukaemia (CML), breaks within BCR are located in the 5.8 kb major breakpoint cluster region and a region of at least 200 kb in ABL, resulting in a p210 BCR-ABL protein. In Philadelphia chromosome positive acute lymphoblastic leukaemia (Ph+ ALL) the breaks within BCR are frequently located further upstream in the 40 kb minor breakpoint cluster region, resulting in a smaller p190 protein. To determine if the mechanism giving rise to the Ph chromosome is different in CML vs ALL, or p210 vs p190, we developed a FISH and long range PCR strategy to amplify and sequence t(9;22) genomic breakpoints. Forward breakpoints were characterized from 25 cases with p190 ALL, 25 with p210 ALL and 32 with p210 CML and reciprocal breakpoints were identified in a subset of these cases. Statistical analysis of the forward BCR breakpoints revealed 2 distinct clusters in p210 ALL but no significant clusters in CML or p190 ALL. Analysis of the forward ABL breakpoints showed evidence for 2 clusters in p210 ALL and one cluster in p190 ALL, but no clustering in CML. When the forward breakpoints were compared to the proximity of sequence motifs, the only significant association was a deficit of breakpoints falling in repeat regions for p210 BCR breakpoints. No significant differences were found in the distribution of forward breakpoints in ABL between the different subtypes of leukaemia. However, comparison of the patterns at reciprocal breakpoints showed differences between p190 and p210, suggesting that they may be formed by distinct mechanisms. To explore this in more detail, we performed extra-chromosomal recombination assays to test the possibility of RAG involvement in selected cases in which breakpoints were found to be close to cryptic recombination signal sequence (RSS) sites. Of 5 p190 cases tested, one had a 'specific' RSS 4 bp from the forward breakpoint but at the exact location of the reciprocal breakpoint. Involvement of RAG was confirmed by the formation of both coding joints and signal joints, which is the defining feature of V(D)J recombination. In addition 1 of 3 p210 ALL cases tested had a 'specific' RSS at the forward breakpoint, however only a hybrid joint and signal joints were formed therefore the significance of this is uncertain. Neither of the p210 CML cases tested gave 'specific' RSSs. Our data therefore shows that aberrant RAG mediated recombination is involved in a subset of BCR-ABL translocations in ALL, supporting the hypothesis that the fusion may arise in a committed lymphoid progenitor cell, at least in some cases.

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IKZF1 (IKAROS) DELETIONS IN BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA ARE ASSOCIATED WITH AN IMPAIRED B-CELL DIFFERENTIATION AND POOR OUTCOME

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Introduction. The BCR-ABL1 fusion gene defines the subgroup of acute lymphoblastic leukaemia (ALL) with the worst clinical prognosis. *Aim and Methods*. To identify oncogenic lesions that combine with BCR-ABL1 to cause ALL, we used Affymetrix Genome-Wide Human SNP arrays (250K NspI and SNP 6.0) and genomic PCR to study 106 cases of adult BCR-ABL1-positive ALL. *Results*. The most frequent somatic copy number alteration was a deletion on 7p12 of IKZF1, which encodes the transcription factor Ikaros and was identified in 80 of 106 patients (75%). Two major deletions occurred: the first one (Δ4-7) was characterised by a loss of exons 4 through 7 (44/106, 42%), and the second one (Δ2-7) that we cloned was characterised by removal of exons 2 through 7 (20/106, 19%). A variable number of nucleotides (patient-specific) were inserted at the conjunction and maintained with fidelity at the time of relapse. The extent of the Δ4-7 deletion correlated with the expression of a dominant-negative isoform with cytoplasmic localisation and oncogenic activity, while the Δ2-7 deletion resulted in a transcript lacking the translation start site. The IKZF1 deletion was also identified in the progression of chronic myeloid leukaemia (CML) to lymphoid blast crisis (66%), but never in myeloid blast crisis or chronic phase CML, or in acute myeloid leukaemia patients. Known DNA sequence and structural features were mapped along the breakpoint cluster regions including heptamer recombination signal sequences (RSS) recognised by RAG enzymes during V(D)J recombination, suggesting that IKZF1 deletions could arise from aberrant RAG-mediated recombination. Using gene-set enrichment analysis to compare the gene-expression signatures of patients with IKZF1 deletion versus not-deleted patients, we identified a unique signatures characterized by down-regulation of genes involved in pre-B-cell differentiation (e.g. VPREB1, VPREB3, IGLL3, BLK) and up-regulation of genes involved in cell-cycle progression (STK17B, SERPINB9, CDKN1A). These findings suggest that genetic alterations of IKZF1 influence the transcriptome and contribute to an impaired B-cell differentiation. We next investigated whether the IKZF1 deletions associated with a poor outcome. Univariate analysis showed that the IKZF1 deletion is a negative prognostic marker influencing the cumulative incidence of relapse (10.1 months for pts with deletion vs 56.1 months for wild-type pts, p=0.0103) and disease-free survival (DFS) (10.1 months vs 32.1 months, respectively, p=0.0229). The negative prognostic impact of the IKZF1 deletion on DFS was also confirmed by multivariate analysis (p=0.0445). *Conclusions*. Deletion of IKZF1 is an important event in the development of BCR-ABL1 B-progenitor ALL which significantly influences clinical outcome. It is likely that Ikaros loss combines with BCR-ABL1 to induce lymphoblastic leukaemia, arresting B-lymphoid maturation.

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Acute myeloid leukemia - Clinical

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A MULTICENTER PHASE I-II STUDY OF TOSEDOSTAT IN THE TREATMENT OF PATIENTS WITH RELAPSED OR REFRACTORY ACUTE MYELOID LEUKAEMIA

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The aminopeptidase inhibitor, tosedostat (TSD, CHR-2797,) selectively depletes amino acid pools in malignant cells, causing anti-proliferative, pro-apoptotic and anti-angiogenic effects. In this open label, single-arm, phase I-II study of TSD elderly/previously treated patients with AML/high risk MDS were treated with escalating doses (60-180 mg) of once daily TSD in phase I, and with the recommended dose (130 mg once daily) for 84 days or longer in phase II. Clinical responses were assessed by monthly bone marrow aspirates and weekly haematological assessments. The primary efficacy endpoint was bone marrow complete response (CR). In total, 35 patients with relapsed/refractory AML and 1 patient with relapsed 'high risk' MDS were enrolled in this trial, 10 in phase I, and 26 in phase II. Twenty five patients were male and 11 were female, with a median age of 68 years (range 34-78). The median performance status (ECOG) at baseline was 1 (range 0-2). Of the AML patients, 10 (29.4%) had either secondary leukaemia or adverse cytogenetics or both. For 10 patients (28.6%), treatment with TSD was a second or later salvage attempt. Half of the patients completed the formal 84-day study period, and 17 (16 AML, 1 MDS) continued treatment with TSD after 84 days. MTD (phase I) was 130 mg with 2 out of 4 patients on 180 mg developing DLT (>75% drop in platelet count). Eleven (31.4%) relapsed/refractory AML patients achieved a bone marrow response: 6 CR (<5% blasts in bone marrow), and 5 partial responses (PR, 5-15% blasts); four patients achieved a complete haematological recovery (in remission for up to 12 months), and another patient reached a cytogenetic response. The single patient with MDS RAEB-1 achieved a morphological bone marrow response, and remained stable for more than 6 months. All responders were >60 years at the time of the first dose. Median overall survival was 131.5 days (range 33 - 623) in all AML patients, and 253 days (range 130-470) in AML patients with CR. The most frequently reported adverse events were: thrombocytopenia (61.1%), fatigue (47.2%), diarrhoea (41.7%) and anaemia (38.9%). Three patients (8%) withdrew due to a drug related toxicity. In summary: this study in patients with relapsed and refractory AML/MDS has shown anti-leukaemic activity of TSD in a substantial number of AML patients with very poor prognosis. TSD at 130 mg once daily is also very well tolerated over a long period of exposure (6-12 months). TSD will be tested in a pivotal study in relapsed AML.

Disclosures. Two co-authors are employed by Chroma Therapeutics Ltd; co-author employees may carry stock options

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5' AZACITIDINE IN COMBINATION WITH VALPROIC ACID INDUCES COMPLETE REMISSIONS IN PATIENTS WITH HIGH RISK ACUTE MYELOID LEUKAEMIA BUT DOES NOT ERADICATE CLONAL LEUKAEMIC STEM/PROGENITOR CELLS

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Background. Epigenetic therapies in the form of demethylating agents or histone deacetylase inhibitors demonstrate significant clinical activity in patients with acute myeloid leukaemia (AML) and myelodysplasia (MDS) and represent an important new therapeutic modality in myeloid malignancies. Responses however are rarely durable and the great majority of patients will relapse. There is increasing interest in the contribution of leukaemic stem/progenitor cells to disease relapse in AML but the impact of epigenetic therapies on this cellular population has not been examined. *Aims*. We wished to correlate clinical response to combined treatment with a demethylating agent and a histone deacetylase inhibitor with changes in committed and multi-potent leukemic stem/progenitor cell populations in patients with high risk AML and MDS. *Design and Methods*. 45 patients with high risk AML/MDS (30 relapsed/refractory AML, 6 *de novo* AML, 9 high risk MDS) were treated with azacitidine (75 mg/m^2 5 days every 28 days), sodium valproate, all-trans retinoic acid and theophylline. The median age of the patient cohort was 66 years (range 32-85 years). 33 patients had good/intermediate risk cytogenetics and 12 adverse risk cytogenetics. Detailed immunophenotypic analysis, clonal assays (methyl cellulose and cobblestone-area forming colony assays, CAFC) and FISH analysis were performed on stem cell enriched (CD34+CD38-), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP) and megakaryocyte erythroid progenitor (MEP) compartments at diagnosis and during therapy. *Results*. Major clinical responses were observed in 15 patients; 7 achieved a complete remission (CR) or complete remission with incomplete blood count recovery(CRi) and 8 a partial response (PR). The median time to maximal clinical response was 2 cycles (range 1-6). 40% (6/15) of patients achieving CR, CRi or PR had adverse risk cytogenetics.In univariate and multivariate analysis blast percentage at presentation was the only significant predictor of response rate and overall survival. In all diagnostic samples abnormal immunophenotypic CD34$^+$CD38$^-$, CMP, GMP and MEP myeloid progenitor compartments were present. There was an anticipated failure of normal colony and CAFC growth from sorted CD34$^+$CD38$^-$ and myeloid progenitor populations. In responding, but not non-responding, patients there was partial or complete restoration of normal immunophenotypic populations accompanied by normal haemopoietic colony output. Restoration of normal immunophenotypic populations and colony output occurred in advance of clinical responses. In one patient who achieved a CR we were able to demonstrate persistence, in sorted stem/progenitor populations, of clonally abnormal cells. Morphologic relapse was preceded by an increase in clonally abnormal cells in the GMP compartment by 7 months. *Conclusions*. This is the first study to correlate clinical and biological response to epigenetic therapies at a stem/progenitor level and has implications for the use of these agents in the treatment of AML and MDS. These data provide original insights into the cellular kinetics of response and relapse in patients with AML treated with epigenetic therapies and identify potential biomarkers of response. The experimental approach outlined has more general implications for understanding the biological basis of disease relapse in patients with AML and MDS treated with both conventional chemotherapy and novel agents.

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NOVEL PROGNOSTICS SUBGROUPS IN CHILDHOOD 11Q23/MLL-REARRANGED ACUTE MYELOID LEUKEMIA AS DEFINED BY TRANSLOCATION PARTNERS: A RETROSPECTIVE INTERNATIONAL STUDY

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Background. In pediatric acute myeloid leukemia (AML), one important subgroup is characterized by translocations of chromosome 11q23, which accounts for 15 to 20% of all cases with an evaluable chromosome analysis. In most cases, 11q23 abnormalities involve the rearrangement of the mixed lineage leukemia (MLL) gene. More than 50 different fusion translocation partners of the MLL gene have been identified. In 11q23/MLL-rearranged childhood AML, the most common translocations are t(9;11)(p22;q23), t(11;19)(q23;p13.1), t(11;19)(q23;p13.3), t(6;11)(q27;q23), and t(10;11)(p12;q23). The 11q23/MLL rearrangements are considered to be associated with poor outcome. However, conflicting data concerning specific 11q23/MLL rearrangements have been reported; for instance some collaborative groups reported better clinical outcome for patients with AML and t(9;11)(p22;q23). Aim. Our aim was to identify differences in outcome between the different translocation partners in 11q23/MLL-rearranged AML in a large cohort of patients collected retrospectively from various collaborative groups. This could lead to a better risk group stratification of these patients in current and future pediatric AML treatment protocols. *Design and Methods*. A total of 756 11q23/MLL-rearranged AML cases were collected from 11 international study groups, representing nearly all recent pediatric AML trials (1993-2005). Survival analysis of the 11q23/MLL subgroups was performed, after central review of karyotypes and/or FISH and PCR results. Patients were assigned to subgroups based on their translocation partner. At least 10 patients had to be included per subgroup; otherwise, they were assigned to the "11q23/MLL-other" group. *Results*. Between the subgroups, age at diagnosis, white blood cell count (WBC), and morphology differed significantly ($p<0.001$). The 5y-pEFS, pOS, and cumulative incidence of relapse (CIR) of the total group with 11q23/MLL-rearranged pediatric AML were 44%, 56%, and 35%, respectively. However, the outcome between the different subgroups varied greatly. Patients with a t(1;11)(q21;q23) had a favorable 5y-pEFS of 92%, whereas those with a t(6;11)(q27;q23) had the worst outcome (5y-pEFS, 11%). Also the subgroups t(10;11)(p12;q23), t(10;11)(p11.2;q23), and t(4;11)(q21;q23) showed a worse 5y-pEFS of 31%, 17%, and 29% respectively (Table 1). Multivariate Cox-regression analysis identified the following independent poor-prognosis indicators for pEFS: t(6;11)(q27;q23) (HR 2.2, $p<0.001$), t(10;11)(p12;q23) (HR 1.5, $p=0.005$), and t(10;11)(p11.2;q23) (HR 2.5, $p=0.005$). The t(1;11)(q21;q23) (HR 0.1, $p=0.004$) was identified as a favorable prognostic indicator. Patients with t(9;11)(p22;q23) represented a heterogeneous group, but the subset with FAB-M5 (HR 0.4, $p<0.001$) was an independent favourable prognostic indicator. *Conclusions*. This retrospective study identified novel independent prognostic subgroups within 11q23/MLL-rearranged pediatric AML It is currently unknown whether other (as yet unidentified) secondary genetic abnormalities play a role in the differential treatment outcome of 11q23/MLL-rearranged AML, or that this is the mere result of the variation in translocation partners. This requires further studies to elucidate the underlying biology. We conclude that identifying the translocation partners in pediatric 11q23/MLL-rearranged AML at diagnosis is essential for effective risk group stratification, especially including t(1;11)(q21;q23), t(10;11)(p12;q23), a t(10;11)(p11.2,q23) and t(6;11)(q27;q23). The patients with a t(9;11)(p22;q23) represent a heterogeneous subgroup, and inclusion of FAB-type in diagnostics is strongly recommended for risk group stratification of patients with a t(9;11)(p22;q23).

Table 1. Survival analysis of subgroups of 11q23/MLL-rearranged AML and prognostic factors for those patients.

	5y-pEFS (%, SE)	*P-value (Logrank)	5y-pCIR (%, SE)	*P-value (Gray)	5y-pOS (%, SE)	*P-value (Logrank)
MLL- rearrangement						
All	44 (2)		35 (2)		56 (2)	
t(1;11)(q21;q23)	92 (5)	*<0.001*	4 (4)	*<0.001*	100 (0)	*<0.001*
t(9;11)(p22;q23)	50 (3)		29 (3)		63 (3)	
t(11;19)(q23;p13)	49 (9)		34 (8)		49 (10)	
t(11;19)(q23;p13.1)	46 (9)		42 (9)		61 (9)	
t(11;19)(q23;p13.3)	46 (10)		21 (9)		47 (11)	
t(11;17)(q23;q21)	42 (14)		41 (15)		67 (14)	
t(10;11)(p12;q23)	31 (5)		52 (5)		45 (5)	
t(4;11)(q21;q23)	29 (13)		56 (16)		27 (13)	
t(10;11)(p11.2;q23)	17 (11)		50 (16)		27 (13)	
t(6;11)(q27;q23)	11 (5)		54 (9)		22 (7)	
Other	39 (4)		40 (5)		54 (5)	
Additional cytogenetic aberrations						
No	48 (3)	*0.01*	31 (3)	0.05	61 (3)	*0.002*
Yes	39 (3)		39 (3)		48 (3)	
White blood cell count						
<20 x 10^9/L	48 (3)	*0.004*	45 (3)	0.60	59 (3)	*0.004*
20-<100 x 10^9/L	43 (4)		42 (4)		57 (4)	
≥100 x 10^9/L	36 (4)		46 (4)		47 (4)	
Age						
<10 y	46 (2)	*0.006*	43 (3)	0.048	60 (2)	*0.001*
≥10 y	34 (4)		51 (4)		42 (4)	

*P-values indicate if the differences are significant between the subgroups.

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AZACITIDINE (AZA) IMPROVES OVERALL SURVIVAL (OS) IN WHO ACUTE MYELOID LEUKEMIA (AML) IN ELDERLY PATIENTS WITH LOW BONE MARROW BLAST COUNTS

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Background. A subanalysis of the phase-III CALGB 9221 study compared AZA with best supportive care (BSC) in patients with WHO AML, and showed a trend for improved OS: 19.3 vs 12.9 months (Silverman JCO 2006;24:3895). Statistical significance was not achieved (hazard ratio [HR] = 0.70 [95%CI: 0.40-1.23], $p=0.2$), possibly due to small patient numbers and crossover design, patients with progression could crossover from BSC to AZA. A more recent phase-III study, AZA-001, showed AZA significantly prolongs OS in patients with higher-risk MDS vs conventional care regimens (CCR) (Fenaux, Lancet Oncol, 2009). AZA-001 employed FAB, but most RAEB-t patients met WHO criteria for AML. This subanalysis of the AZA-001 study sought to confirm and further elucidate the positive OS trend with AZA in WHO AML patients observed in the CALGB 9221 trial. *Design and Methods*. Patients with higher-risk MDS (FAB: RAEB, RAEB-t, CMML and IPSS: Int-2 or High) were enrolled. Before randomization, investigators preselected (based on clinical status and regional prescribing patterns) 1 of 3 CCR: BSC; low-dose ara-C (LDaraC), or intensive chemotherapy (IC). Subsequently, patients were randomized to AZA (75 mg/m^2/d SC x 7d q 28d) or CCR. If randomized to CCR, patients received their investigator preselected regimen. Karyotypes were reclassified using AML criteria into unfavorable (-7/del7q, 3q abnormality, complex); favorable (t(8;21) and inv16) (none in this analysis); and intermediate (others). OS was analyzed with Cox regression models and Kaplan-Meier (KM) methods. All patients were followed until death or study completion. *Results*. 113/358 (32%) patients met WHO AML criteria (median 23% marrow blasts); 55 were randomized to AZA and 58 to CCR. 53 CCR patients (5 withdrew before treatment) were treated as follows: BSC 47% (25/53), LDaraC 34% (18/53), and IC 19% (10/53). AZA and CCR groups had comparable baseline characteristics. Median age (both arms) was 70 years, with 24% and 72% having an unfavorable or intermediate karyotype (including 46% normal), respectively. Median (range) numbers of treatment cycles were 8 (1-39) for AZA; 2.5 (1-3) for IC; 5.5 (1-14) for LDaraC; and 6 months (2-19) for BSC. Median follow-up for OS was 20.1 months. KM median OS was 24.5 vs 16.0 months in the AZA and CCR groups, respectively, HR=0.47 [95%CI: 0.28-0.79], $p=0.004$ (Figure).

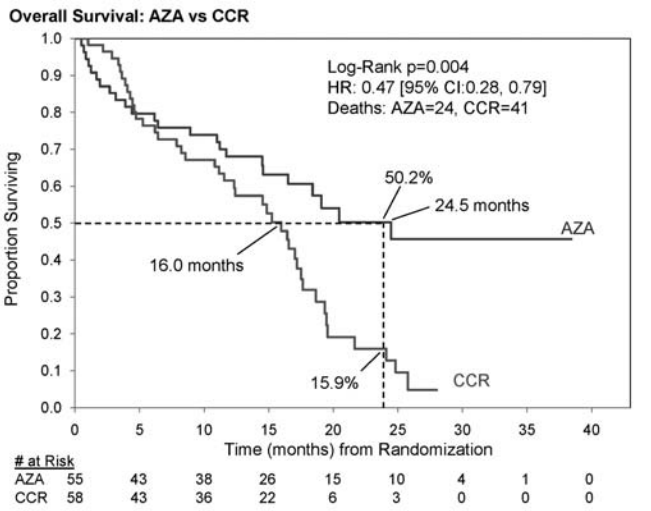

Figure.

At 2 yrs, 50% and 16% of the AZA and CCR groups, respectively, were alive, p=0.0007. OS results in cytogenetic intermediate patients showed a significant HR favoring the AZA group (N=38) over CCR (N=43), HR=0.47 [95%CI: 0.24-0.91], p=0.024. The HR was nonsignificant in patients with unfavorable cytogenetics: AZA (N=14) vs CCR (N=13), median 12.3 months vs 5.3 months, respectively, HR=0.66 [95%CI: 0.26-1.68], p=0.38. *Conclusions*. In the AZA-001 study, outcomes in WHO AML patients are consistent with and support the trend for improved OS observed in CALGB 9221. AZA demonstrates significant activity in elderly patients with WHO AML with low marrow blasts and prolongs OS vs CCR. AZA is currently being more widely evaluated in AML.

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PROGNOSTIC SIGNIFICANCE OF CEBPA MUTATIONS IN A LARGE COHORT OF YOUNGER ADULT PATIENTS WITH AML: IMPACT OF DOUBLE CEBPA MUTATIONS AND THE INTERACTION WITH FLT3 AND NPM1 MUTATIONS

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Prognostic markers identified in AML are becoming increasingly important as a means of risk-stratification for therapy. Mutations in the transcription factor CCAAT/enhancer binding protein α (CEBPA) are associated with a favourable prognosis, but a recent study has suggested this may be limited to patients with biallelic mutations. To investigate this we used dHPLC to screen the CEBPA gene in samples from 1049 young adult AML patients, excluding APL, who were entered into the UK MRC AML10 and 12 trials. Three PCR products were analysed, Fragment 1 covered the N-terminus (both start codons and transactivation domain [TAD] 1), Fragment 2 included TAD2 and Fragment 3 covered the C-terminus (DNA-binding and basic leucine zipper domains). Two common polymorphisms (synonymous G/T SNP rs34529039 and p.P194_H195 dup) were clearly identified by their chromatogram and were scored as Wild-type (WT), other samples with abnormal chromatograms were sequenced. Overall, 92 patients (9%) had one/more mutations (MUT); 43 (47%) had a single mutation and 49 (53%) had two mutations, including three that were homozygous. Mutant level ranged between 21% and 59%, median 45% (n=57). The full-length gene was cloned in samples from 11 biallelic MUT patients. In each case the majority of clones (86% overall) had only one mutation, indicating that the two mutations were on different alleles, but a small number (4%) were homozygous MUT. Presence of CEBPA mutations did not correlate with age, white blood cell count or type of leukaemia (de novo or secondary). Cytogenetics was available in 864 patients and the presence of mutations was strongly associated with the intermediate risk group (67 of 73 MUT cases, p=0.003), in particular a normal karyotype (50 of 73 MUT cases, p=0.01). The distribution of monoallelic and biallelic mutations did not differ in the normal versus other intermediate karyotypes (p=0.2). There was some evidence for a lower frequency of FLT3/ITDs in CEBPA MUT patients (29% were CEBPA-/ITD+, 22% CEBPA+/ITD+, p=0.05), which was more apparent in patients with biallelic mutations (32% vs. 13% for monoallelic vs. biallelic). There was a much lower frequency of NPM1 mutations in biallelic CEBPA MUT patients (44% vs. 39% vs. 4% NPM1+ in 0 vs. 1 vs. 2 MUT patients, p<0.0001). In the total cohort, a favourable outcome was restricted to biallelic MUT patients, 5 year overall survival (OS) 37% vs. 41% vs. 61% for 0 vs. 1 vs. 2 mutants (p=0.009) and relapse-free survival (RFS) 36% vs. 41% vs. 55% (p=0.02). This difference was less obvious if only patients in the intermediate risk group were analysed: OS 39% vs. 48% vs. 55% (p=0.05) and RFS 36% vs. 48% vs. 48% (p=0.06) respectively. There was some evidence that the favourable effect of CEBPA MUT was only present in FLT3/ITD- cases (p=0.08 for interaction), OS 41% vs. 46% vs. 68% in FLT3/ITD-, 29% vs. 23% vs. 17% in FLT3/ITD+ patients. These results indicate that the outcome in CEBPA mutated patients is dependent not only on the number of CEBPA mutations, but also on karyotype and other co-operating mutations.

Novel therapeutics and drug resistance

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PX-171-006: PHASE IB MULTICENTER DOSE ESCALATION STUDY OF CARFILZOMIB (CFZ) PLUS LENALIDOMIDE (LEN) AND LOW DOSE DEXAMETHASONE (LODEX) IN RELAPSED AND REFRACTORY MULTIPLE MYELOMA (MM) – PRELIMINARY RESULTS

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Background. CFZ a highly specific proteasome inhibitor that demonstrates promising single-agent activity in relapsed/refractory MM (ASH 2008). The purpose of this study is to evaluate the safety and activity of CFZ in combination with LEN and loDex. *Design and Methods*. This phase Ib trial evaluates 4 dose levels (≥3 subjects each) to define the maximum tolerated dose (MTD) of CFZ/LEN/loDex in relapsed/refractory MM patients who failed 1-3 prior therapies, including prior LEN or bortezomib (BTZ). CFZ is administered intravenously (IV) 15-27 mg/m2 (d1, 2, 8, 9, 15, 16), LEN 10-25 mg po (d1-21) and loDex 40 mg po (d1, 8, 15, 22) in 28-day cycles (C). An additional 10-15 subjects will be evaluated at the highest dose level reached. Overall response (complete response [CR] /stringent complete response [sCR], very good partial response [VGPR] / partial response [PR]) was assessed by the International Working Group Criteria, with secondary assessment by modified European Bone Marrow Transplant Criteria which includes minimal response (MR). *Results*. As of 17 February 2009, 19 subjects have been enrolled in the first 3 cohorts: 8 of the 19 patients were evaluable for response and toxicity. Prior therapies (median = 2) in the evaluable subjects included dexamethasone (8/8), bortezomib (6/8), lenalidomide (7/8), alkylators (6/8), anthracyclines (5/8), stem cell transplant (5/8), and thalidomide (1/8); 6/8 subjects had received both LEN and BTZ. MTD has not yet been reached after the first 3 dose cohorts. No drug-related serious adverse events have been reported. Responses were rapid and occurred within the first 28-day cycle. Dosing regimens and responses to date with a median of 2 cycles (range 1-4) are as follows: i. In cohort 1 (CFZ 15 mg/m2 / LEN 10 mg) all 6 patients were evaluable: 3 PR, 1 stable disease (SD) and 2 progressive disease (PD). ii. In cohort 2 (CFZ 15 mg/m2 / LEN 15 mg) 2 out of 6 subjects were evaluable: 1 PR and 1 SD. iii. Cohort 3 (CFZ 15 mg/m2 / LEN 20 mg) is in dose escalation and enrollment is ongoing. *Conclusions*. CFZ, LEN, and loDex in combination is well tolerated in the first 2 cohorts. There have been no DLTs. The combination has achieved early encouraging responses in subjects who had failed both LEN and BTZ at doses well below the single-agent MTDs of either LEN or CFZ. The overall results from this study support the dosing regimen selected for the Phase 3 study.

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POST TRANSPLANT CONSOLIDATION WITH BORTEZOMIB, THALIDOMIDE AND DEXAMETHASONE INDUCES A CLINICALLY SIGNIFICANT SHRINKAGE OF RESIDUAL TUMOR BURDEN MEASURED BY REAL TIME PCR

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Background. In multiple myeloma (MM) molecular remission (MR) is not achieved with autologous transplantation (auto-SCT) as opposed to allogeneic transplantation where it associates to an improved outcome. *Aims*. To assess, by nested and real time-quantitative PCR (RQ-PCR), the impact of a consolidation treatment, including Bortezomib/Thalidomide/Dexamethasone (VTD) on residual MM cells after a good response

to auto-SCT. *Design and Methods*. Inclusion criteria were: complete or very good partial response (CR/VGPR) following first-line auto-SCT; no previous treatment with Bortezomib/Thalidomide; presence of a molecular marker based on the immunoglobulin heavy chain rearrangement (IgH-R). The VTD consolidation regimen has been described (*Palumbo et al., EHA2008*). Minimal residual disease was assessed on bone marrow samples at diagnosis, study entry, after two VTD courses, at the end of treatment and then at six months intervals. Nested and RQ-PCR analysis were performed using IgH-R-derived patient-specific primers as described (*Voena et al,Leukemia1997; Ladetto et al,Biol.BoneMarrow-Transpl.2000*). Results. Thirty-nine patients were enrolled. Twenty percent of patients did not receive the whole planned treatment. Fifty-four percent of patients experienced at least one grade 3-4 toxic event. At a median follow-up (MFU) of 42 months (range 27-75), five patients have died (13%) and 11 have relapsed (28%). Nested-PCR has been performed on the whole population. Eighteen percent of patients converted to stable MR and none of them experienced clinical or molecular relapse at MFU (opposed to 11 clinical relapses among those remaining PCR-positive). RQ-PCR has been performed on the whole population. Following auto-SCT a median tumour burden reduction of 5.51 ln was observed. However a further major decrease of 3.85 ln was recorded following VTD, resulting in an overall median tumour shrinkage of 9.36 ln. Both the tumour reduction attributable to auto-SCT and to VTD consolidation proved statistical significant ($p<0.001$). Considering separately patients in clinical remission from those who relapsed, the level of tumour reduction after VTD was remarkably different: the decrease of marginal medians of ln-PCR was 5.15 ln in the former group but just 0.14 ln in the second one. Moreover the level of response measured by RQ-PCR resulted of clinical relevance as patients showing a tumor burden above the median of ln-PCR values after two VTD courses, at the end of consolidation and after six months were clearly associated to a worse PFS (at MFU 65% vs 94%, $p=0.02$, 57% vs 100%, $p<0.01$, and 77% vs 100%, $p=0.02$, respectively). *Conclusions*. We here document for the first time that persistent MR can be obtained in a proportion of non-allotransplanted MM. We observed a major tumour load reduction by RQ-PCR after VTD consolidation and we proved that the best performances in terms of molecular kinetics are associated to an improved outcome. These findings indicate that impressive levels of tumour cell reduction can be achieved in MM thanks to the introduction of new non-chemotherapeutic drugs.

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MECHANISMS OF MSC (MESENCHYMAL STEM/STROMAL CELL) ACCUMULATION AT THE SITES OF TUMORS

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Background. Mesenchymal stem/stromal cells (MSCs) have the ability to accumulate at the sites of tumors when injected into tumor-bearing mice, and therefore MSCs are considered to be an appropriate cellular vehicle for cancer-targeted gene therapy. Intravenous injection of engineered MSCs expressing interferon-β was reported to inhibit the growth of pulmonary metastasis of melanoma and breast cancer in mice, and it also prolonged the survival of mice with glioma xenografts. It is considered that efficient delivery of engineered MSCs to tumor sites improves therapeutic efficacy through reinforced production of antitumor cytokines (transgene products) *in situ*. *Aims*. Although various growth factors and chemokines such as PDGF, HGF, and SDF-1α may be involved, the detailed molecular mechanisms of MSC accumulation at tumors are poorly understood. In this study, we focused on MSC-endothelial cell (EC) adhesion following TNF-α stimulation as a clue to elucidate the mechanisms. *Design and Methods*. To assess tumor tropism of MSCs, GFP-expressing MSCs or fibroblasts transduced with fiber-modified adenovirus vectors were injected into the left ventricular cavity in tumor-bearing nude mice. In addition, luciferase-expressing MSCs or fibroblasts were also injected to the mice, and optical bioluminescence imaging was performed to periodically trace the cells using an *in vivo* imaging system (IVIS). Migration capacity of MSCs and fibroblasts toward growth factors and chemokines was examined by transwell migration assay. The MSC adhesion to ECs was tested with or without TNF-α stimulation *in vitro*. Luciferase-expressing MSCs were injected to the mice treated with parthenolide for reducing TNF-α production, and IVIS imaging was performed to periodically trace the cells. *Results*. Injected GFP-labeled MSCs were detected in tumor tissues, but labeled fibroblasts were not. *in vivo* imaging also revealed that the administration of luciferase-expressing MSCs caused marked accumulation of the bioluminescence signal at the sites of tumors, whereas no accumulation was observed in mice injected with fibroblasts. On the other hand, the factors that induced MSC migration *in vitro* enhanced fibroblast migration as well. It is worthy of note that the stimulation of MSCs with TNF-α enhanced MSC adhesion to ECs *in vitro*. This adhesion was partially inhibited by blocking antibodies against VCAM-1 and VLA-4. The similar phenomenon was not observed for fibroblasts. Furthermore, in the mice treated with parthenolide, the TNF-α level in tumor tissues was reduced, and MSC accumulation at the tumors was significantly inhibited. *Conclusions*. Our data suggest that the MSC-EC adhesion following activation by TNF-α may play a crucial role in MSC accumulation at tumor sites. The present findings would be important to develop efficient MSC-based cancer gene therapy.

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FOXO REACTIVATION AS A CONSEQUENCE OF IMATINIB TREATMENT IN CML INDUCES QUIESCENCE IN PH+ PROGENITOR CELLS

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Background. The FoxO family of transcription factors is regulated by PI3K/Akt induced phosphorylation resulting in nuclear exclusion and degradation. Nuclear FoxO transcribes proapoptotic molecules and cell cycle inhibitors. In CML cells the TK activity of Bcr-Abl leads to the abnormal activation of downstream effectors including PI3K/Akt. The aim of this study was to investigate the role of FoxO3 in Bcr-Abl induced apoptotic arrest and cell growth and the effect of imatinib (IM) induced re-activation of FoxO3 activity in CML progenitor cells. *Design and Methods*: BM cells were collected from 52 CML patients and 20 healthy donors. The expression level of FoxO3 was tested by RQ-PCR. The protein amount and localization was analyzed by Western blot and immunofluorescence, DNA binding activity was measured by EMSA. In addition, FoxO3 was analyzed in CML primary cells and $CD34^+$ cells after IM incubation. Cell cycle and the expression levels of CD47, which has been demonstrated to increased during progression through the cell cycle and stem cell mobilization, was measured by FACS in $CD34^+$ cell population. In addition K562 cells was transfected with pECE-FoxO3 to clarify FoxO3 effects on cell growth and apoptosis. Finally we used our already set up model of Drosophila melanogaster (Dm) transgenic for human Bcr-Abl to study the pathway leading to FoxO3 inactivation. Results: We found that, despite either FoxO3 mRNA levels or protein amount are similar in CML cells compared to controls, FoxO3 protein is equally distributed in the nucleus and cytoplasm in controls but it is completely cytoplasmatic in CML cells and it enters the nucleus during *in vivo* IM treatment or *in vitro* IM incubation. Additionally, FoxO3 DNA binding activity in CML patients is completely absent at diagnosis and reappears after IM treatment. Moreover FoxO3 overexpression in transfected cells results into a 49±9% reduction of proliferation which was further reduced of 75±5% after IM incubation. Furthermore, we demonstrated that IM incubation results into the reactivation of FoxO3 in Ph+ $CD34^+$ cells inducing quiescence into this population as demonstrated by the comparison of cell cycle kinetics and by a decreased expression of CD47. Finally, the progeny obtained from the crossbreeding of Bcr-Abl flies and flies transgenic for FoxO showed a rescue of FoxO phenotype demonstrating that FoxO inactivation is Bcr-Abl mediated. *Conclusions*. Overall, these *in vitro* and *in vivo* experiments suggest that FoxO3 is inactivated in CML cells and its delocalization is mainly dependant from Bcr-Abl activity. The antiproliferative activity of IM may be mediated by FoxO3 re-localization. On the other side, FoxO3 re-activation induced by IM results into a quiescence of Bcr-Abl $CD34^+$ progenitor cells, which raises a hypothesis that FoxO3 could play a role in IM resistance.

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PGP - DEPENDENT RESISTANCE TO IMATINIB AT ADVANCED CML IS EXCLUSIVELY DEVELOPED IN AGGRESSIVE MINOR BLAST SUBSET AND CAN BE REVERSED BY PGP MODULATORS

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Background . Although very effective in chronic phase CML, imatinib (IM) and second generation Bcr-Abl tyrosine kinase inhibitors are usually less effective in advanced CML (accelerated and blast crisis phases) since drug-resistant clones inevitably shortly emerge. In a recent study (Simanovsky M et al, Differentiation. 76: 908-922, 2008), we have found that at blast crisis CML (CML-BC), 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. Briefly, we found that these minor subsets (MS) of blasts (both from patients and human CML-BC cell lines) have highly repopulating ability, increased clonogenicity, and commonly over express BCR-ABL and few other cancer-related genes. *Aims*. To evaluate whether the MS blasts also exhibit differential drug resistance mechanisms toward IM, we also compared the two blast subsets for the level of resistance to IM in relation to expression of a functional Pgp, an ABC transporter that is the product of the ABCB1 (MDR1) gene. *Design and Methods*. RNA expression levels of MDR1 were measured by quantitative RT-PCR. Protein and drug-efflux activity levels of the ABCB1 (Pgp) multidrug transporter were evaluated by Western blotting and by flow cytometry measurements. 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). *Results*. The MDR1 gene is significantly (5-7 fold) upregulated in the MS blasts, relatively to the major population. Moreover, while Pgp could not be detected on the cell surface of the major blast subsets, Pgp is exclusively highly expressed in the MS blasts. Moreover, functional Pgp assays in the MS blasts (efflux, dose-dependent competitions, and UIC2 Pgp-specific shift assays) indicated unequivocally that IM is a substrate for Pgp. While IM efficiently inhibited the proliferation of the major blasts in dose-dependent manner, the proliferation rate of the MS blasts was essentially not affected. Furthermore, the anti-proliferative effect of IM on the MS blasts could be restored by addition of the Pgp inhibitor, R-verapamil. While relatively long, gradual selection in culture of the major CML-BC subsets resulted in some Pgp-independent IM-resistant clones, Pgp activity levels were shortly further elevated (by 1-order magnitude) in the MS blasts. Interestingly, FACS analyses, using different monoclonal antibodies that bind specifically to different known extra cellular epitopes of Pgp, indicated differential antibodies-epitopes binding ratios after IM selection. These stoichiometric changes suggest a topological folding shift of Pgp between its moderate to high activity (proposed model, Figure 1).

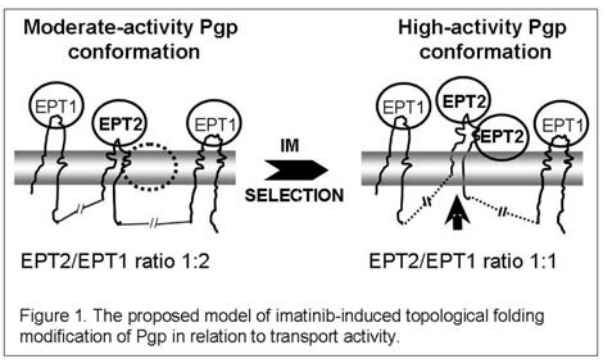

Figure 1. The proposed model of imatinib-induced topological folding modification of Pgp in relation to transport activity.

Figure 1.

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 IM. Moreover, as both IM, dasatinib and nilotinib are transported by Pgp, this study suggests that their combination therapy with Pgp-modulators might also be clinically effective in targeting the MS aggressive blast population.

Red blood cells, iron and granulocytes

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NRAMP1 PROMOTES EFFICIENT IRON RECYCLING FOLLOWING ERYTHROPHAGOCYTOSIS IN VIVO

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Background. Natural resistance-associated macrophage protein 1 (Nramp1) is a divalent metal transporter expressed exclusively in phagocytic cells such as macrophages and neutrophils. *Aims*. Based on our earlier *in vitro* study (*Soe-Lin et al. Exp Hematol. 2008;36:929-937*), we intended to determine whether Nramp1 participated in the recycling of iron acquired through the phagocytosis of senescent red blood cells by macrophages *in vivo*. *Design and Methods*. We employed Nramp1+/+ and Nramp1-/- mice and created conditions of accelerated erythrophagocytosis either by the induction of hemolytic anemia, or by hypertransfusion of ospsonized erythrocytes. *Results*. In preliminary characterization experiments of the iron parameters of wild-type (Nramp1+/+) and Nramp1 knockout mice (Nramp1-/-), we found that untreated knockout mice exhibited greater serum transferrin saturation and splenic iron content, with higher duodenal ferroportin (Fpn) and divalent metal transporter 1 (DMT1) expression. Furthermore, hepatocyte iron content and hepcidin mRNA levels were dramatically lower in knockout mice, indicating that hepcidin levels can be regulated by low hepatocyte iron stores despite increased transferrin saturation. In addition, we observed significant iron loading of the reticuloendothelial organs of knockout mice that increased with age. After injection of 59Fe-labeled heat damaged reticulocytes, knockout animals accumulated erythrophagocytosed 59Fe within their liver and spleen, whereas wild-type animals were able to efficiently recycle the phagocytosed 59Fe to the marrow for incorporation into newly formed erythrocytes. In order to further examine the effect of Nramp1 on iron recycling *in vivo*, accelerated erythrophagocytosis was induced in wild-type and knockout mice by administration of the hemolytic agent phenylhydrazine. Following acute phenylhydrazine treatment, Nramp1-/- mice experienced a significant decrease in serum iron levels and hematocrit, while their Nramp1+/+ counterparts were relatively unaffected. Following a month-long phenylhydrazine regimen, Nramp1-/- mice retained markedly increased quantities of iron within the liver and spleen, and exhibited greater splenomegaly and reticulocytosis than wild-type mice. *Conclusions*. The data presented in this report suggest that in the absence of Nramp1, iron accumulates to a greater degree within iron-recycling macrophages in the liver and spleen following erythrophagocytosis. We hypothesize that hepatocytes iron stores are released to compensate for the lack of iron release from macrophages, resulting in a lower production of hepcidin mRNA. Our observation of increased DMT1 and ferroportin within the duodenums of the Nramp1-/- animals imply that the increase in transferrin saturation despite the impaired iron release from erythrophagocytosing macrophages occurs due to a compensatory increase in iron absorption from the diet. These findings are consistent with our hypothesis that Nramp1 promotes the efficient recycling of iron in erythrophagocytosing macrophages.

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PREVENTION OF CARDIAC IRON ACCUMULATION WITH ONCE DAILY ORAL DEFERASIROX THERAPY IN REGULARLY TRANSFUSED PATIENTS WITH B THALASSAEMIA MAJOR

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Background. Myocardial siderosis as a result of regular blood transfusion therapy is a key cause of cardiac morbidity. Iron chelation therapy with the once-daily oral iron chelator, deferasirox, reduces cardiac iron

levels in patients with cardiac siderosis. Subgroup analysis from the EPIC trial has also allowed assessment of the efficacy of deferasirox in preventing cardiac iron accumulation. Aim. To assess the longitudinal effect of deferasirox over 1 year in preventing myocardial siderosis in non-cardiac iron-overloaded patients with β-thalassaemia major and normal cardiac function. *Design and Methods*. Patients aged ≥10 years with normal myocardial iron content (cardiovascular magnetic resonance [CMR] imaging of myocardial T2* ≥20 ms), serum ferritin (SF) >2500 ng/mL; MR (R2) liver iron concentration (LIC) >10 mg Fe/g dry weight (dw); left ventricular ejection fraction (LVEF) ≥56%; and a lifetime minimum of 50 previous packed red blood cell transfusions were included. Deferasirox was initiated at 20 or 30 mg/kg/day (subsequent dose adjustments of 5-10 mg/kg/day were based on changes in SF, month-6 myocardial T2*, and safety parameters). The primary endpoint was change from baseline in myocardial iron at 1 year. *Results*. 78 patients were enrolled (35 male:43 female; mean age 20.2±7.5 years). Mean baseline (±SD) LVEF was 67.7±4.7%, LIC 28.8±10.2 mg Fe/g dw, median SF 4367 ng/mL, and geometric mean cardiac T2* 32.0 ms ± 25.6%. Transfusion requirements were 133.7 mL/kg in the year prior to enrolment. 76 patients (97%) had received prior chelation therapy (deferoxamine 69.2%; combination deferoxamine/deferiprone 28.2%). Mean deferasirox dose over 1 year was 27.6±6.0 mg/kg/day. After 12 months of therapy, geometric mean myocardial T2* levels remained unchanged (32.5 ms ± 25.1%, +2.0%, p=0.57) and T2* did not fall below 20 ms in any patients. LVEF increased to 69.6% (p<0.0001). Median SF was significantly reduced from baseline at 12 months by 1048 ng/mL (p<0.0001; 20% relative reduction). LIC was also significantly reduced from baseline by 7.2 mg Fe/g dw (p<0.0001; 30% reduction). Change from baseline in T2* was inversely correlated with change in SF (r=-0.2, p=0.05). 75 patients (96.2%) completed the 12-month study; three patients (3.8%) discontinued because of adverse events (AEs) [n=2] or because they no longer required study drug (n=1). Investigator-assessed drug-related AEs were generally mild (78%) and were reported in 31 patients (39.7%), including diarrhoea (n=8, 10%), and rash (n=7, 9%). One patient (1.3%) had a non-progressive increase in serum creatinine >33% above baseline and the upper limit of normal (ULN) on two consecutive visits. No patients had an increase in alanine aminotransferase >10xULN on two consecutive visits. No patients died and no drug-related serious AEs were reported. *Conclusions*. This is the first study to show that iron chelation therapy with deferasirox in patients with β-thalassaemia major and normal baseline cardiac iron levels can prevent accumulation of cardiac iron, whilst significantly reducing body iron burden and improving cardiac function (LVEF). These results demonstrate that prevention of myocardial iron accumulation, in patients who are currently free from cardiac iron loading, is achievable with deferasirox.

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RITUXIMAB IN AUTO-IMMUNE HAEMOLYTIC ANAEMIA AND IMMUNE THROMBOCYTOPENIC PURPURA: A BELGIAN RETROSPECTIVE MULTICENTRIC STUDY

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Background. Auto-immune haemolytic anemia (AIHA) and immune thrombocytopenic purpura (ITP) may respond to the chimeric anti-CD20 monoclonal antibody rituximab, even when refractory to conventional therapy. *Aims*. To better characterize the effect of anti-CD20 therapy in AIHA and ITP. *Design and Methods*. We retrospectively analysed the use of rituximab in Belgian patients experiencing AIHA and ITP. Belgian hematology centers were invited to fill a questionnaire specifying the major characteristics and quality of response of ITP and AIHA patients given rituximab. For AIHA complete response (CR) was defined by achievement of a normal haemoglobin concentration. Partial response (PR) was defined by transfusion independency in a previously transfused patient or by a 2 gr increase of hemoglobin concentration in patients not given blood transfusion. Other situations were reported as failure (F). In patients with ITP a CR was defined by achievement of a platelet count superior to 100x10^9/L or by maintaining a platelet count >100x10^9/L without immunosuppressive drugs and a PR if the platelet count was 50-100x10^9/L. A F was reported for all other situations. *Results*. 17 centers elected to join the study. 68 courses of rituximab in 53 patients with AIHA and 43 courses in 40 patients with ITP were analyzed. All patients were given rituximab after failing at least one previous line of treatment, including splenectomy in 19% and 72.5% of AIHA-patients and ITP-patients respectively. Overall response rates were 79.2% in AIHA and 70% in ITP, with a median follow up since first rituximab administration of 15 months (range 0.5-62) in AIHA and 11 months (range 0-74) in ITP. As shown in Figure 1 Progression Free Survival (PFS) at one and two years were 72% and 56% in AIHA and 70% and 44% in ITP. In this retrospective analysis we were not able to identify pre-treatment characteristics predictive for response to rituximab. Nine patients with AIHA and three patients with ITP were given one or more additional courses of rituximab. Most of these patients, who had responded to a previous course, experienced a new response comparable to the previous one, both in terms of quality and of duration of response. Finally, the outcome of patients who failed to respond to rituximab therapy was poor both in terms of response to subsequent therapy and in terms of survival. *Conclusions*. This study confirms that rituximab induces responses in a majority of previously treated patients with AIHA and ITP. Response duration generally exceeds one year. Retreatment with rituximab in responding patients is most often successful. The outcome of patients who fail on rituximab is poor. We were not able to identify pre-treatment patient characteristics predicting for response.

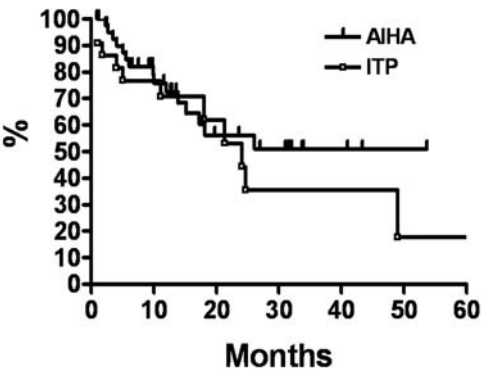

Figure 1. Progression free survival in AIHA and ITP.

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INVOLVEMENT OF MATRIX METALLOPROTEINASES AND CXC CHEMOKINES IN LPS-INDUCED AIRWAY INFLAMMATION IN SICKLE MICE

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Transgenic mice expressing exclusively human sickle hemoglobin (SS) are well-established models for the study of vascular inflammation. Previous studies have shown that systemic lipopolysaccharide (LPS) challenge causes exaggerated inflammation, including increased serum and broncoalveolar lavage (BALF) TNF-α and IL-1 cytokines and sVCAM-1 in sickle mice. The goal of this study was to evaluate the contribution of cytokines, CXC chemokines and matrix metalloproteinases (MMPs) in the acute airway inflammation induced by LPS in SS mice. Acute lung inflammation was induced by intranasal administration of LPS (50 mL of 250 mg/mL) in C57BL/6 (control) and Berkeley (SS) mice. BALF was performed 4h (early) and 24h (late) after LPS challenge. qRT-PCR analysis was used to examine gene expression and ELISA protein production. Intranasal instillation of LPS in control and SS mice induced acute airway inflammation after 4 and 24h, characterized by increases in total leukocytes numbers (WBC) and was almost exclusively accounted for by neutrophils (NS); however this influx was significantly greater in SS

mice, when compared with control mice (4h: 1.4±0.06 vs 0.66±0.12 WBC; 1.06±0.1 vs. 0.40±0.12 NS, 24h: 2.9±1.3 vs 1.4±0.2 WBC; 2.6±0.5 vs 1.2±0.2 NS, respectively). Baseline levels, KC and MIP-2 are significantly higher in BAL fluid of SS mice compared to control mice (105.2±14.1 vs 14.1±5.8; 41.2±7.9 vs 11.4±7.3, respectively). After 4h of LPS challenge KC, MIP-2, TNF-α and MMP-9 were significantly higher in SS BALF compared to control mice (2491±454 vs 798.1±98.2; 1726±307 vs 887.3±149.5; 4250±636 vs 1585±263; 187.5±16.7 vs 103.9±16.9, respectively). After 24h of LPS inhalation, BALF levels of these chemokines/cytokine and MMP-9 were significantly decreased in SS mice (217.4±32.5; 125.5±55.6; 30.6±12.1; 29.2±12.8, respectively) and controls (105.1±18.5, 133.5±25.95, 121.7±45.05, 84.07±27.8, respectively) when compared to 4h after LPS inhalation. mRNA levels of KC, TNF-a, IL-1-β, MMP-8, MMP-9 and TIMP-1 in the lungs of control and SS mice in early (4h) LPS-induced lung inflammation were significantly higher when compared to animals that received PBS instead of LPS, whereas LPS-induced KC, MMP-8 and MMP-9 expression was significantly higher in SS mice lung (0.42±0.1; 0.42±0.06; 0.49±0.11, respectively) compared with the control group (0.19±0.047; 0.2±0.06 vs 0.016±0.004; 0.22±0.03, respectively). MMP-2, MMP-12 and TIMP-2 gene expressions were not significantly different between SS and control mice. Increased KC, TNF-α, IL-1-β, MMP-8, MMP-9 and TIMP-1 mRNA levels were detected in the lungs from both 24h mice groups after LPS challenge. Lung MMP-12 expression was significantly increased in SS mice compared to control mice (0.69±0.12 vs. 0.12±0.03). In contrast, TIMP-1 mRNA levels were significantly lower in lung from SS mice compared with control mice (0.08±0.03 vs. 0.19±0.017). Our results provide evidence that local inflammatory mediators and neutrophil recruitment in response to LPS were significantly increased in SS mice and demonstrate that the development of acute LPS-induced airway inflammation in SS mice is associated with enhanced expression of chemokines and MMPs expression in lungs. The findings from this study indicate that these mediators could participate in the lung vaso-oclusive complications in SCD and give further support to the claim that a proinflammatory state is present in SCD.

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PROLONGED ADMINISTRATION OF HYDROXYUREA REDUCES MORBIDITY AND MORTALITY IN ADULT PATIENTS WITH SICKLE-CELL SYNDROMES: FINAL ANALYSIS OF A 17-YEAR, SINGLE CENTER, TRIAL

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Background. Hydroxycarbamide (hydroxyurea; HU) is now considered as the main pharmacological agent used for the management of patients with sickle-cell disease (SCD). Whether HU can prevent the severe chronic complications or modify the mortality of these patients remains still an interesting unresolved question. *Aim*. The present prospective study aims to evaluate the effects of HU in a large number of SCD patients who received HU over long period of time and were followed in a single Center. *Design and Methods*. Since 1991, 330 patients were evaluated: 34 with sickle cell anemia (HbS/HbS), 131 with HbS/β0-thal and 165 with HbS/β+-thal (107 with HbS/IVSI-110 and 58 with HbS/IVSI-6). According to study inclusion criteria 131 patients received HU, while 199 patients were conventionally treated. The usual dosage of HU was 20 mg/kg/day, except for some patients, where toxicity or lack of effectiveness imposed modifications of the dosage in the range of 15- 35 mg/kg/day. The median follow-up period was 8 years for HU patients and 5 years for non-HU patients. *Results*. All HU patients responded to therapy with a median 5-fold increase of HbF at 6-month. Patients who received HU showed a dramatic reduction of: 1) the frequency of severe painful crises (mean±SD: from 7.34±6.5 episodes/year pre-HU to 0.25±0.44 episodes/year post-HU; $p<0.001$); 2) transfusion requirements (from 1.5±5.9/year pre-HU to almost zero during HU treatment; $p<0.001$); 3) hospital admissions (from 2.11±2.96/year pre-HU to 0.07±0.18/year post-HU; $p<0.001$); and 4) the frequency of chest syndrome (from 6.1% pre-HU to 0.8% during HU; $p=0.016$). HU administration produced a significant increase of total Hb and HbF at 6, and 12 months post-HU initiation and at last follow-up along with a significant reduction of leukocyte, platelet and reticulocyte counts, serum bilirubin and LDH levels. The death rate of HU patients was significantly lower than that observed among non-HU patients (13 *vs.* 49 deaths; 9.9% vs. 24.6%; $p=0.001$). The probability of 10-year survival was 86% and 65% for HU and non-HU patients, respectively ($p=0.001$; Figure). The 10-year probability of survival for HbS/HbS, HbS/β0-thal, and HbS/IVSI-110 patients was 100%, 87% and 82%, respectively for HU patients and 10%, 54% and 66%, for non-HU patients. The multivariate analysis showed that HbF values at baseline and percentage change of LDH between baseline and 6-month were independently predicted for survival in the HU group, while in non-HU patients increased levels of Hb and low levels of bilirubin at baseline were independent predictive factors for superior survival. Complications of HU were predictable and easily manageable. Fertility data will be reported in the congress. *Conclusions*. Our study suggests that adult SCD patients who received HU for a long period of time reduce the incidence of acute and chronic complications of SCD, while they have a survival advantage. This seems to be more prevalent in patients with HbS/HbS or HbS/β0-thal, but patients with HbS/β+-thal may be also benefited from HU administration. These results highlight the beneficial effect of HU which seems to modify the natural history of SCD and raise the issue of expanding its use in all SCD patients.

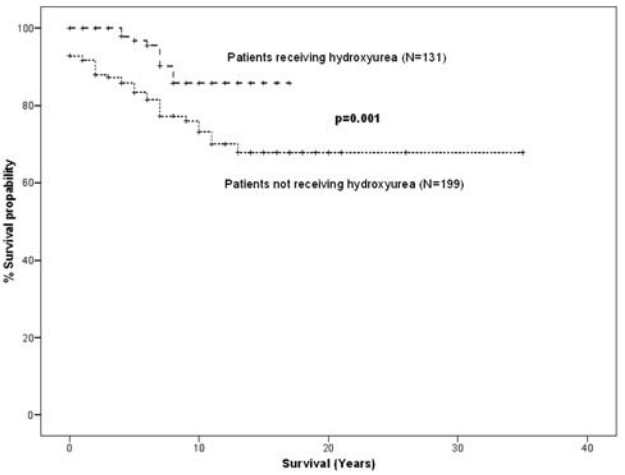

Figure.

SIMULTANEOUS SESSION IV

Aggressive non-Hodgkin lymphoma - Clinical

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SEX-SPECIFIC EFFECTS OF RITUXIMAB ON TREATMENT OUTCOME OF ELDERLY PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA: A RETROSPECTIVE ANALYSIS OF THE RICOVER-60 TRIAL OF THE GERMAN HIGH-GRADE NON-HODGKIN LYMPHOMA STUDY GROUP (DSHNHL)

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Background. In the RICOVER-60 trial, where 1222 elderly (61-80 year-old) patients with untreated CD20-positive aggressive NHL were randomized to receive 6 or 8 cycles of CHOP-14 with or without 8 applications of rituximab, best results were obtained with 6xR-CHOP-14 in all subgroups including patients who did or did not receive additional radiotherapy to bulky disease. *Aims*. To study the impact of sex on treatment outcome, patient characteristics and results were analyzed according to the patients´gender. *Design and Methods*. A retrospective analysis has been performed to compare the outcome from female and male patients treated in the RICOVER-60 trial. *Results*. Female patients within the rituximab arms presented a significantly higher LDH and lower performance status compared to the male counterparts (Elevated LDH: 57.2% vs 43.1%, p=0.001; ECOG >1: 18.6% vs. 11.1%, p=0.009). This significance was transferred into the whole population even though the two patient groups did not differ within the non rituximab arms. Female patients experienced more grade 3&4 leukocytopenias than men (64.5% of cycles vs. 45.9%, p<0.001) and the time with median of leukocytes < 2000/mm3 was longer (2 vs. 0 days). This was associated with more grade 3&4 infections (7.4% of cycles vs. 6.0%, p=0.020), but there was no difference with respect to therapy-associated deaths rates (7.2% vs 7.8%, p=0.654). Female patients had a higher 3-year PFS (67.5% vs. 61.0%; p=0.062) and OS (74.2% vs. 68.4% p=0.086). These differences were largely due to a greater improvement by the addition of rituximab on the outcome of females: while the difference in 3-year PFS between female and male patients was 5.2% (p=0.448) in patients receiving CHOP-14 only, this increased to 7.6%(p=0.053) when rituximab was added. Although a less favourable outcome would have been expected for female patients according to the described differences in LDH and performance status. In a multivariate analysis adjusting for the IPI-relevant risk factors LDH, ECOG performance status, advanced stage and >1 extranodal involvement, the relative risk for progression in male compared to female patients was not significantly elevated after CHOP-14 only (1.127; p=0.348), but was significantly higher when rituximab was added (1.592: p=0.004). *Conclusions*. Rituximab improves outcome both in elderly female and male patients treated with CHOP-14; however, the positive effect of rituximab is more pronounced in female patients and renders male sex a significant risk factor. This observation together with the low toxicity of rituximab justifies a trial with higher doses of rituximab for male patients.

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A PHASE II TRIAL OF THE ORAL MTOR INHIBITOR EVEROLIMUS IN RELAPSED NON-HODGKIN LYMPHOMA (NHL) AND HODGKIN DISEASE (HD)

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Background. The phosphatidylinositol 3-kinase/mammalian target of rapamycin (mTOR) signal transduction pathway integrates signals from multiple receptor tyrosine kinases to control cell proliferation and survival. Everolimus (RAD001, Novartis Pharmaceuticals) is an oral investigational antineoplastic agent that targets mTOR. *Aims*. To learn the anti-tumor activity and toxicity of single-agent RAD001 in pts with relapsed/refractory aggressive or indolent NHL and HD. *Design and Methods*. Patients were eligible if they had measurable disease, a platelet count >75,000, an absolute neutrophil count >1,000, and a creatinine and bilirubin <2x laboratory upper limit of normal. Patients received everolimus 10 mg PO daily and were evaluated monthly. Dose reductions to 5 mg daily and 5 mg every other day were allowed. Response was assessed after 2 cycles and periodically thereafter. Patients could remain on drug until progression or toxicity. *Results*. 145 pts were treated - 77 with aggressive NHL (47 DLBCL; 19 MCL, 8 follicular grade 3, 1 transformed, and 2 high grade); 41 indolent (16 follicular; 22 SLL and 3 marginal zone); 8 T-cell NHL (4 CTCL; 3 PTCL and 1 anaplastic) and 17 HD. The median age was 67 years (range, 27-92). Patients had received a median of 4 prior therapies (range, 1-15). The overall response rate (ORR) was 33% (48/145; 95% CI: 26-41%) with 5 complete responses and 43 partial responses. ORR by disease type is summarized in the Table below. Patients have received a median of 3 cycles (range, 1-29) of therapy. The median time to progression for all 145 patients is 4.3 months (95% CI; 3.6-5.9 months). The median duration of response for the 48 responders is 6.8 months (95% CI; 5.4-11.0 months) and 19 responders remained progression free at 6 months. 18 patients are currently maintaining a response with a median time of 5.5 months (range, 0-32.1 months). Everolimus was well tolerated. The incidence of grade 3/4 anemia, neutropenia, and thrombocytopenia in this heavily pretreated pt population was 16%, 17%, and 35%. Grade 2 hypercholesterolemia occurred in 8% and grade 3 in 1%; grade 2 hyperglycemia in 8%, grade 3 in 4% and grade 4 in 1%; 1 pt had grade 4 hypertriglyceridemia. *Summary*. Everolimus has single-agent activity in relapsed NHL and HD and provides proof-of-concept that targeting the mTOR pathway in NHL is clinically relevant.

Table 1. Overall response rate by disease type.

Disease	n	ORR (95% CI)	CR	PR
DLBCL	47	30% (17-45%)	2%	28%
MCL	19	32% (13-57%)	11%	21%
FL	16	50% (25-75%)	6%	44%
SLL	22	18% (5-40%)	0%	18%
T-cell NHL	8	63% (24-91%)	0%	63%
HD	17	53% (28-77%)	6%	47%

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DOSE-DENSE INDUCTION FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANT (ASCT) LEADS TO SUSTAINED REMISSIONS IN A LARGE FRACTION OF PATIENTS WITH PREVIOUSLY UNTREATED PERIPHERAL T-CELL LYMPHOMAS (PTCLS) - OVERALL AND SUBTYPE-SPECIFIC RESULTS OF A PHASE II STUDY FROM THE NORDIC LYMPHOMA GROUP

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Systemic PTCLs are uncommon malignancies and carry, with the exception of ALK-positive anaplastic large cell lymphoma (ALCL), a poor prognosis when treated with conventional chemotherapy. The role of intensified treatment schedules consolidated by upfront ASCT as 1st line therapy in PTCLs is debated. Therefore, the NLG designed a prospective multicenter phase II study to evaluate the impact of a dose-intensified induction schedule (6 courses of two-weekly CHOEP) consolidated in 1st PR/CR with high-dose therapy (BEAM) followed by ASCT in previously untreated systemic PTCL. With a total of 166 patients enrolled between Oct 2001 and Oct 2007, this is, by far, the largest prospective PTCL clinical trial completed to date. Newly diagnosed systemic PTCL cases aged 18-67 yrs were included. Cases of ALK-positive ALCL, primary cutaneous, primary leukemic and lymphoblastic subtypes were excluded. Of the 166 PTCL cases, 160 were histologically confirmed at referral center level: PTCL unspecified (n=62; 39%), ALK-neg ALCL (n=31; 19%), angioimmunoblastic type (AIL) (n=30; 19%), enteropathy-type (n=21; 13%), panniculitis-like (n=6; 4%), T/NK nasal-type (n=5; 3%), hepatosplenic (n=5; 3%). The M/F ratio was 2.0 and the median age 57 yrs (range 22-67 yrs). The majority of the cases presented with advanced-stage disease (81%), B-symptoms (59%) and/or elevated s-LDH (62%). Nevertheless, 71% of all patients had a good performance score (WHO 0-1) at inclusion. Of the 155 patients with available response assessment after completed induction schedule, 132 (85%) were either in CR/CRu (n=81) or in PR (n=51). Disease refractory to induction treatment was observed in 23 patients. From the original cohort of 160 patients, a total of 112 (70%) had undergone ASCT at the time of the present analysis. Of these, 88 (78%) were in CR/CRu at first assessment 3 months after ASCT, 11 (10%) were in PR and 13 (12%) experienced early post-transplantation disease progression. The reasons for not undergoing ASCT in 20 of the 132 responders were: (i) disease progression between last course of induction and ASCT, (ii) toxicity, (iii) mobilization failures, and (iv) other causes. Treatment-related toxicity following induction and high-dose treatment was predictable and manageable. No subtype-specific toxicity patterns were observed. Of all 160 patients, 87 were alive at last available follow-up. The median follow-up for survival in this cohort is 3 years and 9 months (range 10-81 months). The median follow-up for the deceased patients (n=73) is 8.4 months. Causes of death were: lymphoma n=55 (75%), toxicity n=7 (10%), second malignancy n=2 (3%), other causes n=6 (8%), unknown n=3 (4%). Three- and five year overall survival (OS) for the entire patient cohort was 57% (CI 49%-65%) and 50% (CI 41%-58%), respectively. The corresponding PFS figures were 48% (CI 40%-56%) and 43% (CI 34%-52%). Subtype-specific analysis revealed particularly favorable OS and PFS values for ALCL cases (3yrs: OS 77% and PFS 64%; 5yrs: OS 73% and PFS 64%). AIL and PTCLu had an OS at 3 yrs of 57% and 51%, respectively. The corresponding PFS values were 54% and 42%. As a unique feature, this study included a large number of enteropathy-type PTCL cases (n=21). Their 3-yr OS and PFS were 52% and 47%, respectively. In conclusion, the present data shows that a time- and dose-intensified schedule consolidated by autologous hematopoietic stem cell transplant is effective in previously untreated systemic PTCL. Early treatment failures remain an unsolved problem and novel induction strategies are needed. However, long-term remissions are achieved in a substantial fraction of patients with best PFS values obtained for the ALK-negative ALCL subtype.

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8X BEACOPPESC COMPARED TO 4X BEACOPPESC FOLLOWED BY 4X BEACOPPBASE WITH OUR WITHOUT RADIOTHERAPY FOR ADVANCED STAGE HODGKIN LYMPHOMA PATIENTS: FINAL ANALYSIS OF THE HD12 TRIAL OF THE GERMAN HODGKIN STUDY GROUP

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Background. The German Hodgkin Study Group (GHSG) trial HD9 had established a new standard of care for advanced-stage Hodgkin Lymphoma (HL) patients by showing significant superiority of BEACOPP escalated (BE) in terms of failure-free survival (FFTF) and overall survival (OS) over COPP/ABVD and BEACOPP baseline (BB) (each 8 cycles) at the cost of increased toxicity. *Aims*. HD12 aimed to reduce toxicity by deescalating chemotherapy comparing 8 cycles BE with 4 cycles BE followed by 4 cycles BB, and to assess additional radiation (RT) to initial bulk and residual disease. *Design and Methods*. Patients with HL in stage IIB with large mediastinal mass and/or E-lesions or stage III-IV were randomised according to a 2x2-factorial design between: 8BE + RT, 8BE no RT, 4BE+4BB + RT, 4BE+4BB no RT. Reviewing CT-images before and after chemotherapy, fields for RT were centrally planned by a multidisciplinary diagnostic panel blinded for the randomisation arm. Primary endpoint of the trial was FFTF. *Results*. Between 9/1999 and 1/2003, a total of 1.670 patients aged 16-65 were randomized. For this final analysis, 99 patients were excluded (42 HL not confirmed, 20 revision of stage, 20 no study treatment or documentation, 17 other) resulting in 1.571 eligible patients equally distributed between the 4 study arms. Patient characteristics in the 4 groups were comparable with 49% of patients in stage III, 35% in stage IV, 68% reporting B-symptoms and 28% having a large mediastinal tumor. As expected, chemotoxicity was high with 97% of patients receiving at least one WHO grade III or IV toxicity. Most prominent differences between pooled chemotherapy arms were anemia (65% 8BE vs 51% 4BE+4BB) and thrombopenia (65% vs 51%). Death due to acute toxicity was 3% (sepsis, cardiac, pulmonary, infection), with 20 deaths in the 8BE arms and 27 in the 4BE+4BB arms. Treatment outcome was complete remission for 92.4% of patients with another 2.2% experiencing early progression. Total progression/relapse rate was 7.8% (n=52 vs 71) with a median follow up of 78 months. A total of 156 deaths (72 vs 84, 9.9%) have been observed (22 vs 32 due to acute or salvage treatment toxicity; 15 vs 24 HL, 22 vs 13 secondary neoplasia). Secondary neoplasias were observed in 77 patients (4.9%): 12 vs 11 AML/MDS, 11 vs 5 NHL and 20 vs 18 solid tumors/others. OS after 5 years was 91%, 5-year FFTF was 85.5% and 5-year progression free survival (PFS) was 86.2% (Kaplan-Meier estimates). Estimates for the difference at 5 years are -1.8% [-4.7%, 1.1%] for OS, -2.3% (95% CI [-5.9%, 1.3%]) for FFTF and -2.7% [-6.2%, 0.8%] for PFS. The confidence intervals show that while 4BE+4BB is clearly not significantly different from 8BE in all 3 long-term outcome parameters ($p>0.19$, log rank test), a difference of up to -5.9% in FFTF cannot be ruled out statistically. *Conclusion*. BEACOPPescalated remains standard of care for patients with advanced stage HL in our group.

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A PROSPECTIVELY RANDOMIZED PLACEBO-CONTROLLED TRIAL OF EPOETIN-A IN PATIENTS WITH ADVANCED-STAGE HODGKIN LYMPHOMA: FINAL ANALYSIS OF THE GHSG HD15-EPO TRIAL

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Background and aims. Anemia is frequent among cancer patients and impacts organ function and quality of life of those affected. Anemia has been shown to be a negative prognostic factor in several malignancies including Hodgkin Lymphoma (HL). In addition, the advent of more aggressive treatment regimen such as BEACOPP escalated has resulted in more HL patients needing red-blood-cell transfusion. *Design and Methods*. Thus, apart from the chemotherapy-related question of the GHSG HD15 trial, ie 8 cycles of BEACOPP escalated or 6 cycles of BEACOPP escalated or 8 cycles of BEACOPP14, patients in HD15 were randomized between Epoetin-α and placebo. Study drug was given at 40.000 I.E. weekly with a hemoglobin target level of 12-13g/dL. Primary endpoint was fatigue after the end of chemotherapy (CT) and 6 months later measured with the EORTC QLQ-C30 questionnaire (0-100 scale). With respect to the more recent safety discussion related to erythropoetins (EPOs) in general, we also analysed efficacy and safety endpoints although the trial was not sufficiently powered for time-to-event analyses. *Results*. Between1/2003 and 12/2006 a total of 1.379 patients were randomized for the EPO question of whom 1.329 were eligible. Of these 1.286 (96.8%) are fully evaluable. Patient characteristics were balanced between the placebo and the EPO arm. In those 688 patients who were randomized before 7/2005 and eligible for analysis, the CR/CRu rate was 90,6% with a median follow-up of 28 months, a total of 58 progressions/relapses (8.4%) and 33 deaths (4.8%) without differences between study arms. There was also no difference in terms of freedom from treatment failure (hazard ratio (HR) 0.9, 95%-confidence interval (CI) 0.6-1.4) and overall survival (HR 1.1, 95% CI 0.6-2.3). 51 serious adverse (7.4%) and 35 thrombovascular events (5.1%) were observed with no significant difference between arms. The median number of red blood cell units given significantly favored patients receiving Epoetin-α (2 vs 4; test for trend $p<0.001$). The interim analysis for fatigue gave an average value of 61±25.8 (mean±SD) at the end of treatment and a reduced fatigue score of 33±25.3 at six months after CT. *Conclusions*. This is the largest prospectively randomized, placebo-controlled trial with EPO in cancer patients. So far we found no difference in terms of fatigue, response, relapse or side effects between those advanced-stage HL patients receiving Epoetin-a or placebo. The number of RBC units given was significantly reduced in the EPO group.

Myeloproliferative disorders - Clinical

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RISK FOR AML/MDS TRANSFORMATION IN PHILADELPHIA NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS - A POPULATION-BASED NESTED CASE-CONTROL STUDY IN SWEDEN

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Background. The clinical course of Philadelphia negative chronic myeloproliferative neoplasms (MPN)s, including polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF), is characterized by a variable risk (1-20% after 10 years of disease) for disease transformation into acute myeloid leukemia/myelodysplastic syndromes (AML/MDS). The risk is higher in IMF, intermediate in PV and low in ET. There is no apt way to predict which patient is likely to develop this ominous complication. The use of alkylators and P32 carries a well established increased risk for leukemic transformation. Hydroxyurea (HU) is known to be less leukomogenic. However, its potential long-term effect on AML/MDS development remains controversial. This is due to the relative rarity of MPNs, late appearing transformation events in a long-term disease course, and lack of randomized trials. *Aims*. To define risk factors (treatment, disease and host related factors) for AML/MDS development in a large nested case-control study with a special focus on the leukemogenic potential of HU. *Design and Methods.* 11,039 patients with MPN were identified from the Swedish Cancer Registry and major hematology/oncology units. Through record-linkage with the Cancer Registry patients who developed AML (n=271) and MDS (N=21) were identified. For each patient with a subsequent AML/MDS diagnosis (cases) two control patients (without AML/MDS) matched for MPN disease, age, gender, and calendar period were defined (controls). Information was collected regarding treatment (agent, cumulative dose, duration), disease related factors (laboratory variables) and host-related factors (demographics, co-morbidity). Cases and control patients with prior chemotherapy/radiotherapy for non-MPN, misdiagnosis, no proper match and lack of relevant medical records were excluded from the analysis. Data was analysed by logistic regression conditioned on follow-up time, MPN diagnosis and age. Analyses focusing on treatment effects were mutually adjusted for each other. Results are presented as odds ratios (OR) with 95% confidence interval (CI). *Results*. The final study population consisted of 206 MPN cases (193 AML, 13 MDS; 43% females; PV n=138; ET n=32; MF n=21; MPD NOS n=15) and 225 matched control patients (42% females; PV n=166; ET n=36; MF n=19; MPD NOS n=4). Median time from MPN diagnosis to AML/MDS was 7 years, range 0.5-35 years. Compared to no HU exposure the ORs for 1-499g, 500-999 g, >1000 g of HU were 1.07 (0.42-2.70), 0.90 (0.28-2.89), and 1.01 (0.28-3.60), respectively for AML/MDS development (not significant). In contrast, MPN patients who received P32 >1000 MBq and alkylating agents >1 g had a 3.39-fold (1.28-8.99; $p=0.01$) and 4.46-fold (1.22-16.31; $p=0.03$) increased risk of developing AML/MDS, respectively. Lower exposures to P32 and alkylators were not associated with a significantly increased risk of AML/MDS. *Conclusions*. In this large population-based nested case-control study, the risk of AML/MDS was strongly associated with high exposures of P32 and alkylator treatment. In contrast, HU treatment did not significantly increase the risk for transformation to AML/MDS.

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TREATMENT OF PROGRESSION OF MYELOPROLIFERATIVE NEOPLASM TO MDS/AML BY AZACYTIDINE : A REPORT ON 44 PATIENTS

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Background. MDS and AML occurring during the course of myeloproliferative neoplasms (MPN) carry a very poor prognosis and current treatments have very limited efficacy. Azacytidine (AZA) significantly

improves survival in higher risk MDS (Lancet Oncol, 2009,online) and in some AML, but its use has not been extensively reported so far in MDS and AML post MPN. *Patients*. The French health agency (AFSSAPS) designed a still ongoing pt named program (ATU) of AZA (75 mg/m²/d x7d every 4w) in higher risk MDS and poor risk AML. 44 pts with MDS or AML post MPN, included in this program in 16 centers, before October 08 and having completed at least 1 cycle of AZA are analysed here. Diagnostic of MPN was based on WHO 2008 criteria, and response to AZA on IWG 2006 criteria for MDS and IWG-AML 2003 criteria for AML. *Results*. Median age was 69.5y (range 37-89), M/F: 27/17. The initial MPN was PV in 17 pts, ET in 16 pts, primary myelofibrosis (MF) in 5 pts, MPN unclassifiable (MPN,U) in 6 pts. 19/44 (43%) pts had JAK2-V617F mutation. Median interval from diagnosis to progression was 6.1 years (3 months-34 years). At inclusion, diagnosis was AML in 21 pts, MDS in 22 pts (RAEB-2 in 16 pts, RAEB-1 in 6 pts) and MDS/MPN U in 1 pt (WHO 2008 classification). Karyotype at transformation was normal in 7 pts, failure in 4 pts, 9 pts had isolated chromosome 7 abn, 5 had +8, 5 had del 20q, and 16 had complex karyotype. Among the 19 pt with JAK2-V6127F mutation at the onset of MPN, 12 were still positive at the time of transformation. The median number of cycles of AZA administered was 6 (range 1-20). Early AZA discontinuation (< 4 cycles) was due to death in 9 pts, restoration of features of MPN in 4 pts and patient decision in 1. Overall response rate (ORR, including CR, PR, CRi, marrow CR, HI for MDS) was 17 (39%). Among the 35 pts who received at least 4 cycles of AZA, 22 (63%) were MDS and 13 (37%) were AML. In MDS pts, 8 (36%) achieved CR (including 2 cytogenetic CR), 2 (10%) achieved PR, and 2 (10%) HI-E leading to an ORR of 54%. In AML pts, 1 achieved CR and 3 achieved CRi (ORR 31%). Median follow up from inclusion was 6 months (2-31). 6 of the 15 responders relapsed after a median time of 9.5 months (5-13) and 9 maintained their responses after a median time of 8 months (4+-26+). 26 pts were still alive after 3 to 26 months (median 7.5). Median survival was 13 months. OS was better in MDS pts than in AML pts (73% vs 15% at 18 months, p=0.0072). Interestingly, in 8 pts with a long history of MPN (median 14 years) response to AZA was associated to recurrence of initial features of MPN requiring restart of cytoreductive therapy in 4 of them. *Conclusions*. 5 AZA gives encouraging results in MDS or AML occurring in the course of MPN

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PHASE II STUDY OF CEP-701, AN ORALLY AVAILABLE JAK2 INHIBITOR, IN PATIENTS WITH PRIMARY MYELOFIBROSIS AND POST POLYCYTHEMIA VERA/ESSENTIAL THROMBOCYTHEMIA MYELOFIBROSIS

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Background. Myelofibrosis (MF; primary or post-essential thrombocythemia/post-polycythemia vera) is one of the Philadelphia chromosome-negative myeloproliferative disorders. There are few treatment options available for patients with MF. Activating mutations of the JAK2 tyrosine kinase (JAK2V617F) are found in 50% of patients with MF, leading to a constitutive autophosphorylated JAK2 protein. CEP-701 is an orally available tyrosine kinase inhibitor that has activity as a JAK2 inhibitor both *in vitro* and *in vivo*. *Aims.* This is a single-center phase II trial evaluating therapy with CEP-701 in patients with JAK2V617F-positive MF. *Design and Methods*. Patients were eligible for this trial if they were at least 18-years of age and had a diagnosis of JAK2V617F-positive MF requiring therapy, including previously treated patients that were relapsed, intolerant, or refractory to therapy or if newly diagnosed then with intermediate or high risk according to Lille scoring system, or with a symptomatic splenomegaly ≥10 cm below costal margin. Patients were treated with CEP-701 at an initial dose of 80 mg twice daily. Responses were evaluated using the criteria developed by the International Working Group on Myelofibrosis Research and Treatment (IWG-MRT). *Results*. A total of 22 patients were treated. Their median age was 61 years (range 38-83 years). Median time from diagnosis to therapy was 28 months (range 0-184 months). Most patients (90%) were previously treated, and the median number of previous therapies was 3 (range 0-6). Splenomegaly was present in 90% of patients, and the median size from left costal margin was 19 cm (range 0-30 cm). Median JAK2V617F / total JAK2 ratio (allele burden) was 53.5% (range 13.5-96.6%). Fourteen out of 21 evaluable patients presented with abnormal cytogenetics. Eight patients (36%) were transfusion dependent. Median time on study was 4 months (range 1-19 months). Responses were seen in 6 patients (27%). All responses were defined as Clinical Improvement by IWG-MRT criteria and consisted of reduction in spleen size alone in 3 patients, transfusion independency in two patients, and reduction in spleen size together with improvement in neutrophils and platelets in one patient. Median time to response was 3 months (range 1-9 months). JAK2 allele burden was unchanged in responders. No patient had improvement in bone marrow fibrosis. No patient had a cytogenetic response. The median duration of response was 14 months (range 3-17 months). Three patients are still on study and have maintained their response for 17, 16 and 6 months, respectively. Main toxicities were anemia (grades 3-4: 18%), thrombocytopenia (grades 3-4: 18%) and diarrhea (all grades: 68%; grades 3-4: 9%). Six patients (27%) required CEP-701 dose reductions because of toxicity. Median time to dose reduction was 3 months (range 1-6 months). Eight patients (36%) had their dose of CEP-701 escalated to 100 mg twice daily. Only one achieved a response after increasing the dose. *Conclusions*. Therapy with CEP-701 was relatively well tolerated and had modest efficacy in patients with MF. Most responses consisted of the reduction in spleen size. There was no improvement in the JAK2 allele burden or bone marrow fibrosis.

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TG101348, A JAK2-SELECTIVE INHIBITOR, IS WELL TOLERATED IN PATIENTS WITH MYELOFIBROSIS AND SHOWS SUBSTANTIAL THERAPEUTIC ACTIVITY ACCOMPANIED BY A REDUCTION IN JAK2V617F ALLELE BURDEN

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Background. The discovery of JAK2V617F (VF) has spurred development of JAK2-selective small molecule inhibitors for the treatment of myeloproliferative neoplasms. TG101348 is a potent and selective orally bioavailable JAK2 inhibitor. *Aims*. To assess the safety, tolerability, and pharmacokinetic behavior of TG101348 in a Phase I dose-escalation study. The secondary objectives were evaluation of treatment responses, pharmacodynamic activity of TG101348, and drug effect on VF allele burden. TG101348 was administered orally once daily in 28-day cycles. For patients achieving less than a complete remission after 3 cycles of treatment, escalation was permitted to the highest tolerated dose in the absence of disease progression or unacceptable toxicity. *Results*. Twenty eight patients (median age=66 years) were treated in the dose escalation phase, at 8 dose levels from 30mg to 800mg daily. Nineteen patients had PMF, 7 post-PV MF, and 2 post-ET MF; 89% were VF-positive. Median palpable spleen size was 17cm and 10 patients were transfusion-requiring at the time of study enrollment. The median period of drug exposure to date is 21 weeks (range <1 to 55 weeks). Dose-linear plasma exposures were observed, with mean elimination T1/2 at steady state ranging from 23 to 52 hours across doses. Toxicity. At the highest dose level (800mg), 2 of 6 patients experienced dose-limiting toxicity (asymptomatic grade 3 or 4 amylasemia with grade 4 lipasemia in 1 patient) that was reversible upon holding drug (both patients currently being treated at a lower dose); consequently, the maximum tolerated dose (MTD) was declared at 680mg. The most frequent non-hematological toxicities were grade1/2 nausea/vomiting (64%) and diarrhea (50%), well controlled with anti-emetics and anti-diarrheals or resolving spontaneously; no neurologic toxicities were observed. Grade 3/4 thrombocytopenia was seen in 8 patients (29%; n=6 grade 3, n=2 grade 4); grade 3/4 neutropenia was seen in 3 patients (11%; n=2 grade 3, n=1 grade 4). Efficacy. Six (21%) of the 28 study patients have so far discontinued treatment due to competing comorbidities (n=1), investigator discretion/non-compliance (n=4), or toxicity (n=1; grade 4 neutropenia). The remaining 22 patients are currently at the following dose levels: 680mg (n=17), 520 mg (n=1), 360mg (n=3), and 240 mg (n=1). A reduction in spleen size was seen in all 22 patients (100%) including 5 (23%) whose spleen became non-palpable from a pre-treatment spleen size of 4 to 34cm. A total of 14 patients (64%) have experienced a greater than 50% decrease in spleen size. All 14 patients with leukocytosis at baseline (WBC range 20 to 103x10⁹/L) have experienced a marked reduction in their WBC count (range 6.6 to 28.3). Of the 25 VF-positive patients, 8 (32%) have experienced a greater than 50% reduction in granulocyte mutant allele burden during 2 consecutive readings. *Conclusions*. TG101348 is well tolerated in patients with myelofibrosis and the MTD

is now established at 680 mg/day. Already during the dose-escalation phase of the study, the drug has shown substantial activity in reducing spleen size, leukocyte count, and VF allele burden. The expansion phase of the study at the MTD is scheduled to open in March 2009.

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ANAHYDRET: A EUROPEAN MULTICENTER PROSPECTIVE PHASE 3-STUDY: NON-INFERIORITY OF ANAGRELIDE COMPARED TO HYDROXYUREA IN NEWLY WHO-DIAGNOSED ET PATIENTS

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Background. Based on the superiority of hydroxyurea & aspirin over anagrelide & aspirin in ET patients in the PT1-trial, guidelines recommend hydroxyurea as first line therapy for ET. However, the diagnosis of ET in the PT1-trial was based on the PVSG classification. Therefore, it remains questionable whether this recommendation is also true for ET patients diagnosed according to the WHO classification. *Aims*. In this European phase 3 study efficacy and tolerability of ANAgrelide and HYDRoxyurea were compared in high risk ET patients diagnosed according to the WHO classification. The study was designed as a non-inferiority trial because of the limited number of treatment naïve ET patients available and the expected low number of ET related events following treatment with a cytoreductive therapy. *Design and Methods*. 258 treatment naïve high risk patients with ET were randomized to anagrelide (n=122) or hydroxyurea (136). The patients characteristics were equally distributed with a median age of 58,1 years (range 19-90 years; 46 male, 76 female) in the anagrelide arm vs. a median age of 56,4 years (range 22-83 years, 47 male, 89 female) in the hydroxyurea arm. *Results*. A central blinded pathology review of 236 bone marrow biopsies revealed a high reproducibility of the ET diagnosis by applying the WHO classification: 194 (82.2%) of patients were classified as having ET, 16 patients (6,8%) were reclassified as PMF-0 and 16 patients (6,8%) were considered having early PVs with an ET-like clinical phenotype. Confirmatory proof of non-inferiority was achieved after a mean observation time of 2,1 years (comprising 539 patient years) based on predefined equivalence criteria for platelet counts, course of hemoglobin levels and white cell count during therapy as well as for the rate of ET related events. In the anagrelide arm 75,4% of the patients developed a complete response of platelet counts (<450.000/µL) compared to 81,7% in the hydroxyurea arm. Neutrophil counts remained unchanged in the anagrelide arm but were significantly reduced by hydroxyurea. No significant differences were observed for the rates of major and minor clinical ET releated events in the anagrelide group (4,29 %, and 16,8 %, resp.) compared to hydroxyurea (4,25 %, and 12,8 %, resp.). During the entire study period 11 major ET related complications occurred in the anagrelide group (5 arterial events, 2 venous thrombotic complications and 4 bleedings) and 12 major events in the hydroxyurea arm (5 arterial events, 5 venous thrombotic events and 2 bleedings). 43 minor ET related events were observed in the anagrelide arm as compared to 36 such events in the hydroxyurea arm. Adverse drug reactions or poor response were reasons for discontinuation of the study drug in 19 patients treated with anagrelide and in 10 patients treated with hydroxyurea. Transformations to myelofibrosis were not reported during the whole study period. The JAK2 mutation status was evaluated in 189 patients with 101 JAK2V617F positive (53,4%) and 88 (46,6%) JAK2V617F negative patients. *Conclusions*. Final analysis of the study proofs non-inferiority of anagrelide compared to hydroxyurea in the treatment of ET diagnosed according to the WHO classification.

Chronic myeloid leukemia - Clinical II

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NILOTINIB 800 MG DAILY IN EARLY CHRONIC PHASE CHRONIC MYELOID LEUKEMIA: 12-MONTHS RESULTS OF A PHASE 2 TRIAL OF THE GIMEMA CML WORKING PARTY

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Background. Imatinib (IM) 400 mg daily is the standard treatment for CML in early chronic phase (ECP). The cumulative rate of complete cytogenetic response (CCgR) in the IRIS trial for the IM 400 mg arm was 51%, 69% and 87% at 6, 12 and 60 months, respectively. One-fourth of all the IM treated pts fail to obtain a stable CCgR due to upfront resistance, refractoriness or intolerance. Nilotinib has a higher binding affinity and selectivity for Abl with respect to IM and is highly effective in IM resistant patients, across every disease phase. *Aims*. To investigate the safety and the efficacy of nilotinib 400 mg BID in ECP, Ph-pos CML patients, the GIMEMA CML Working Party is conducting an open-label, single-stage, multicentric, phase II trial (*ClinicalTrials.gov.NCT00481052*). *Design and Methods.* The primary endpoint is the CCgR rate at 1 year; the kinetic of MR is studied by Q-PCR baseline and after 1, 2, 3, 6, 9 and 12 month. *Results*. 73 patients have been enrolled from 20 Centres between June, 2007 and February, 2008. The median age is 51 years (range 18-83), 45% low, 41% intermediate and 14% high Sokal risk. Median follow-up is currently 410 days (range 185-548).The minimum follow-up is 6 months for all the pts and 12 months for 52 (71%) (complete 12-months data will be presented on site). The cumulative CCgR rate within 12 months is 100%. At 3, 6 and 12 months (ITT), the CCgR rate is 78%, 96% and 90%, respectively (at 12 months, 21 pts with pending results). The achievement of the first MMR, defined as a BCR-ABL:ABL ratio ≤0.1% according to the International Scale, after 1, 2, 3, 6, 9 and 12 months is 3%, 22%, 59%, 66%, 75% and 77%, respectively (15 and 21 pts at 9 and 12 months pending). One patient only progressed at 6 months to ABP with T315I mutation. The dose intensity was high: during the first and second trimester, 58% and 60% of the 73 pts received the full, 800 mg daily dose while 25% and 16% of the pts received a mean daily dose ranging between 600 and 799 mg daily, respectively. Adverse events (AEs) were mostly grade 1 and 2 and manageable with appropriate dose adaptations: the most frequent biochemical AEs were bilirubin increase (53% all grades, 16% grade 3), s-GPT increase (42% all grades, 8% grade 3) and lipase and amylase increase (29% and 18%, all grades, 8% and 4% grade 3+4, respectively); 2 pts went off treatment after 6 and 13 months due to recurrent episodes of amylase and/or lipase increase (no pancreatitis). Hematologic toxicity was recorded in 4 pts (5% - only 1 event grade IV neutropenia); *Conclusions*. The results that have been achieved in these unselected patients and within a multicentric trial, strongly support the hypothesis that in ECP, Ph-pos CML patients the response to nilotinib is faster than the response to IM standard and high dose.

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DASATINIB 100 MG ONCE DAILY FOR CHRONIC PHASE CHRONIC MYELOID LEUKEMIA (CML-CP) FOLLOWING IMATINIB FAILURE: LONG-TERM FOLLOW-UP FROM STUDY CA180-034

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Background. Dasatinib 100 mg once daily is the recommended and approved dose for patients with CML-CP following resistance, suboptimal response, or intolerance to prior imatinib, based on previous results from CA180-034 - a phase III dose-optimization study demonstrating similar efficacy and significantly minimized toxicity compared with other dose schedules. In a pharmacokinetic analysis, the dasatinib steady-state trough concentration (Cmin) was lower in the 100 mg once-daily arm than in other arms, and lower a Cmin was associated with less frequent key side effects (pleural effusion, neutropenia, and thrombocytopenia), dose reductions, and dose interruptions. *Aims*. To investigate the long-term efficacy and safety of dasatinib 100 mg once-daily treatment and to determine the association of early cytogenetic response with long-term endpoints. *Design and Methods*. Patients were randomized after informed consent using a 2x2 factorial design to one of four treatment arms: 100 mg QD (n=167), 70 mg BID (n=168), 140 mg QD (n=167), or 50 mg BID (n=168). Details of study design and endpoints have been described previously. *Results*. After minimum follow-up of 2 years, the 2-year rate of progression-free survival (PFS) with 100 mg once daily was 80% (vs 75-76% in other arms) and the overall survival rate was 91% (vs 88%-94%). Among responding patients in the 100 mg once-daily arm, excluding Ph-negative BCR-ABL-positive patients, median time to major cytogenetic response (MCyR) was 2.9 months (intolerant: 2.8 months; resistant/suboptimal: 2.9 months) and to complete cytogenetic response was 3.0 months (intolerant: 2.9 months; resistant/suboptimal: 3.1 months), with similar times to response observed in other arms. Among patients treated with dasatinib 100 mg once daily (vs other arms) who had any baseline BCR-ABL mutation following resistance or suboptimal response to imatinib, the 2-year MCyR rate was 57% (vs 47-55%), the CCyR rate was 43% (vs 32-45%), and PFS at 2 years was 75% (vs 58-65%). Dasatinib 100 mg once daily was well tolerated and rates of key side effects showed only a minimal increment in the second year. Across the four treatment arms, significant differences were observed in rates of drug-related pleural effusion (all grades: p=0.049) and cytopenia (p=0.003 for grade 3/4 thrombocytopenia), with lowest rates observed in the 100 mg once-daily arm. Compared with other arms, dasatinib 100 mg once daily resulted in the lowest rates of treatment interruption (62% vs 72-79%), reduction (39% vs 46-62%), and discontinuation (41% vs 47-50%). In addition to 3-year data for PFS, overall survival, and safety, the likelihood of achieving long-term endpoints based on landmark analysis of cytogenetic status at 6, 12, and/or 18 months will be presented. *Conclusions*. This is the first clinical study showing an advantage for intermittent over continuous tyrosine kinase inhibition in terms of maintaining efficacy and improving safety. Extended follow-up from the CA180-034 study confirm that dasatinib 100 mg once daily is the optimal dosing schedule for patients with CML-CP following resistance, suboptimal response, or intolerance to imatinib.

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IMATINIB AND DASATINIB TREATMENT RESULTS IN SELECTION OF A POPULATION OF QUIESCENT LEUKEMIC STEM CELLS SHOWING CROSS-RESISTANCE TO CELLULAR IMMUNOTHERAPEUTIC INTERVENTIONS

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Background. Tyrosine kinase inhibitors 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 contrast, allogeneic stem cell transplantation (allo-SCT) and application of donor T cells may be a curative treatment. *Aims*. The aim of this study was to investigate whether the leukemic stem cells residing during imatinib treatment are susceptible targets for immunological interventions, and whether allo-SCT performed for persistent CML should be combined with continuous treatment with tyrosine kinase inhibitors. *Design and Methods*. $CD34^+$ positive CML cells were isolated from bone marrow and labeled with the fluorescent dyes CFSE or PKH to allow monitoring of single cell proliferation upon addition of cytokines. CML cells were exposed to imatinib (200-10 μM) or dasatinib (200-10 nM) or to $CD8^+$ minor histocompatibility antigen (mHag) or allo-HLA antigen specific cytotoxic T lymphocyte (CTL) clones. The number, phenotype, and proliferative status of the CML cells residing after single and combined interventions were measured by quantitative flowcytometric analysis. *Results*. In the absence of therapeutic interventions the majority of $CD34^+$ CML cells entered proliferation as determined by dilution of CFSE or PKH. 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. Addition of imatinib or dasatinib 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 appeared to increase compared to the non-treated controls, indicating additional growth arrest of proliferating CML cells by exposure to tyrosine kinase inhibitors. We next 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 $CD34^+$ 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 the adhesion molecules CD49d and CD58 on these quiescent cells, probably resulting in the inability of these target cells to form a high avidity interaction with the T cells. Moreover, the tyrosine kinase inhibitors suppressed T cell proliferation. Removal of the tyrosine kinase inhibitors from the cultures resulted in conversion of part of the quiescent $CD34^+$ CML cells to susceptibility to CTL attack. *Summary and Conclusions*. Treatment with tyrosine kinase inhibitors results in selection of a population of quiescent leukemic stem cells showing cross-resistance to CTL-induced cell death, most likely due to their inability to form a high avidity interaction, indicating that treatment with tyrosine kinase inhibitors may not act synergistically with immunological interventions. The anti-proliferative effect of tyrosine kinase inhibitors on both the leukemic cells and the T cells may potentially even hamper the potentially curative immune response after allogeneic SCT.

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MOLECULAR RESPONSES OF THE SPIRIT PHASE III TRIAL OF THE FRENCH CML GROUP COMPARING IMATINIB 400 MG TO HIGHER DOSE IMATINIB OR COMBINATION WITH INTERFERON OR CYTARABINE FOR THE TREATMENT OF NEWLY DIAGNOSED CHRONIC PHASE (CP) CHRONIC MYELOID LEUKAEMIA (CML) PATIENTS

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Background. Imatinib (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-IFN2α (90 μg/wk) (n=159). *Design and Methods*. Pts were allocated at a 1.1.1.1 ratio, stratified by Sokal risk groups. Molecular assessments were centralized, blinded and calculated according to the international standardized scale (IS). *Results*. This 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 been planned in order to select the best experimental arm for further comparison to IM-400. Pts were recruited between 9/2003 and 10/2007, median age 51 yrs (18-78), 62% males; Sokal score was low 33%, intermediate 41% and high risk 27%. Median follow-up was 36 Mo. (range 12-62). Overall, at 3 Mo, 88% of pts achieved complete haematological

response. Complete cytogenetic response (CCyR), MMR and OMR rates are described in Table 1 MMR rates at 6 and 12 Mo were higher for IM-PegIFN as compared to IM-400 (p<10-3). At 18 MO the cumulative OMR rates were 22% (IM-400), 28% (IM-600), 25% IM-1ra-c), 43% (IM-PegIFN). Grade 3/4 neutropenia and/or thrombocytopenia occurred during the first year in 8% IM-400, in 14% IM-600, in 41% IM-Ara-C and in 40% IM-PegIFN arms respectively. No significant infection rates were observed between the 4 arms. Grade 3/4 non-haematological toxicities occurred in 19% IM-400 (oedemas, muscle cramps), in 30% IM-600, in 27% IM-Ara-C (diarrhoea) and in 31% IM-PegIFN pts (skin rashes, asthenia). Within the first 12 Mo, discontinuation of experimental treatment occurred in 8% IM-600, 39% IM-Ara-C and in 45% IM+PegIFN pts. *Conclusions*. Although a significant number of pts reduced or stopped PegIFN within the first year, highly significant improvements in the MMR rates were observed in the IM-Peg IFN arm and may translate into survival benefit.

Table 1.

% of 636 pts *(ITT analysis)*	IM-400	IM-600	IM-400 + Ara-C	IM-400 + Peg-IFN2a	P value *(overall)*
At 6 Mo.					
CCyR	49	68	56	56	0.0177
MMR	***20***	***31***	***23***	***39***	**0.0024**
At 12 Mo.					
CCyR	55	62	63	65	-
MMR	***38***	***49***	***46***	***57***	**0.0085**
OMR	***15***	***18***	***15***	***30***	**0.0019**

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EARLY MOLECULAR RESPONSE TO FIRST LINE IMATINIB THERAPY IS PREDICTIVE FOR LONG TERM PFS AND EFS IN CP-CML - AN INTERIM ANALYSIS OF THE RANDOMIZED GERMAN CML STUDY IV

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Background. The introduction of imatinib has significantly changed prognosis of CML patients. Despite favourable hematologic and cytogenetic response (CyR) data, patients (pts) on first line imatinib therapy may relapse. Thus, studies have been conducted to improve initial therapy by dose escalation or combination with other drugs. CML Study IV was designed to compare imatinib in standard dose (400 mg/d) vs high dose (800 mg/d) vs combinations with low dose cytarabine or interferon α. *Aims*. We sought to evaluate the predictive impact of early molecular response for long term progression free (PFS) and event free survival (EFS). *Design and Methods*. 531 pts (60% m, median age 54 years, range 16-84) randomized to imatinib based therapies by December 2005 were investigated, the median follow up was 42 mo (range, 0-74). At baseline, multiplex PCR was applied to determine the dominating BCR-ABL transcript: b2a2 (n=201), b3a2 (n=244), b2a2 and b3a2 (n=78), e1a2 (n=2), e19a2 (n=4), b3a3 (n=1) and e8a2 (n=1). Quantitative PCR from 5,260 peripheral blood samples was performed using the LightCycler technology in two central labs. PCR data were aligned to the international scale (IS) by introduction of conversion factors (Hughes et al., BLOOD 2006). For analysis of prognostic impact, events were defined as (i) loss of complete hematologic response, (ii) loss of major CyR following loss of complete CyR, (iii) accelerated phase, (iv) blast crisis, and (v) death for any reason. Criteria (iii), (iv) and (v) were further regarded as progression. Pts were censored at the time of allogeneic stem cell transplantation or switch to 2nd generation tyrosine kinase inhibitors because of imatinib intolerance or resistance. *Results*. Cumulative molecular response of 531 pts at 3-36 mo after randomization is summarized in Table 1.

Table 1. Cumulative achievement of molecular response levels.

Month	3	6	12	18	24	36
BCR-ABL^{IS}	achieved by % of pts					
≤10%	41	66	79	84	86	89
≤1%	15	40	64	74	77	80
≤0.1% (MMR)	2	16	36	50	59	69
≤0.01%	0	2	9	19	26	39

The molecular response cutoffs with best predictive impact were BCR-ABLIS of 10% after 3 mo (PFS, p=0.053), 10% after 6 mo (PFS, p=0.0095; EFS, p=0.012), 1% after 12 mo (PFS, p=0.011, EFS, p<0.0001), and 1% after 18 mo (PFS, p=0.0045; EFS, p=0.0002) of imatinib based therapies. The achievement of 0.1% BCR-ABLIS (MMR, major molecular remission) was not predictive after 18 mo. In order to investigate the reasons for unsatisfying responses BCR-ABL kinase domain mutations were assessed in 175 pts. 30 pts (17%) harbored 35 mutations affecting 18 different amino acids. *Summary*. Prospective molecular surveillance of CML shows early response predicting stable remissions on first line imatinib therapy. After 3 mo of treatment, residual BCR-ABL transcript levels start to be predictive for PFS. In pts with unsatisfactory response or molecular, cytogenetic and hematologic relapse, BCR-ABL mutations have been detected in 17% of pts. Calculation of molecular response rates dependent on the various imatinib based therapies will be performed after stop of randomization which is expected by the end of 2009.

Infectious diseases, supportive care

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RECOMBINANT ACTIVATED FACTOR VII FOR BLEEDING IN THE SETTING OF MALIGNANCY AND HEMATOPOIETIC STEM CELL TRANSPLANTATION.

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Background. Haemorrhage commonly complicates the course of patients with malignancies, resulting from either the disease process or its therapy. Recombinant activated Factor VII (rFVIIa) may have a role in augmenting conventional therapies in this setting. However its safety and impact on transfusion requirements and clinical outcomes has not been explored. *Aims*. To explore the safety and efficacy of rFVIIa in the treatment of haemorrhage associated with malignancy or hematopoietic stem cell transplantation (SCT). *Design and Methods*. Clinical registry data can provide important information, particularly where clinical trials are impracticable. The Australia and New Zealand Haemostasis Registry documents 'off-label' usage of rFVIIa in patients who do not have congenital haemophilia. More than 90 institutions currently participate, capturing an estimated 85% of 'off-label' doses in the region. We analysed episodes where the cause of bleeding was malignancy or SCT. *Results*. 240 eligible episodes were identified in 223 patients (10.7% all registry episodes). Median age was 57 (IQR 43-68); 143 (64.1%) were male. Primary diagnosis was hematological in 119 episodes (49.5%); non-hematological malignancy in 121 (50.4%). 207 (86.2%) were receiving disease-directed therapy, 11 (4.6%) supportive care, unreported in 22 (9.2%). Intent of care was 130 (62.2%) curative, 79 (37.8%) palliative, 31 (12.9%) unreported. 21 (8.8%) were undergoing allogeneic SCT and 4 (1.6%) autologous SCT. Primary sites of bleeding were gastrointestinal 124 episodes (51.6%), pulmonary 28 (11.7%), hepatic 25 (10.4%), genitourinary 23 (9.6%), intracranial 15 (6.2%), others 25 (10.4%). Causes of bleeding were 76 (31.6%) surgical (including resection of tumour or metastases), disease-induced coagulopathy 40 (16.7%), disease invasion 38 (15.8%), mucositis (including graft-versus host disease) 32 (13.3%), others 51 (21.2%); 3 (1.2%) received rFVIIa prophylactically prior to procedures. In 167 episodes (69.6%) a single dose was administered. Median dose was 90 µg/kg (IQR 75-99). Prior to administration median (IQR) haemoglobin was 82 g/L (69-93); platelet count 76 x 109/L (36-132), fibrinogen 2.1 g/L (1.4-3.1), INR 1.4 (1.2-1.9), pH 7.3 (7.2-7.4), temperature 36.8oC (36.0-37.2), and patients had received 5 (2-12) red cells, 2 (1-4) platelets, 4 (0-8) plasma, and 0 (0-8) cryoprecipitate. Bleeding stopped or decreased in 116 (60.7%) episodes where response was reported. Response varied with cause of bleeding, pH and temperature. Transfusion requirements fell to 2 (0-4) red cells, 0 (0-2) platelets, 0 (0-3) plasma, and 0 (0-1) cryoprecipitate following the first dose of rFVIIa. 18 thromboembolic adverse events were reported within 28 days of administration. 132 patients (59.2%) survived to 28 days. *Conclusions*. This is the first comprehensive exploration of rFVIIa in malignancy. LeveIs of 'off-label' use in this setting were higher than anticipated. Reported efficacy and safety were promising in this heterogenous group with difficult bleeding. Response rates were high, with an associated reduction in transfusion requirements following administration. Use of rFVIIa may be an effective adjunct to conventional therapies in uncontrolled malignancy-associated haemorrhage.

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INCIDENCE OF FEBRILE EPISODES DURING STEM CELLS MOBILIZATION AFTER HIGH DOSE CYCLOPHOSPHAMIDE CHEMOTHERAPY AND G-CSF (FILGRASTIM OR LENOGRASTIM) ADMINISTRATION IN MULTIPLE MYELOMA PATIENTS: STUDY UPDATE

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Introduction. The G-CSF, primary regulator of granulopoiesis, has shown its efficacy in reducing duration of neutropenia after chemotherapy or myelosuppressive therapy. In these situations G-CSF, accelerating the granulocytous reconstitution, may enable a significant reduction of the incidence, duration and severity of infection. Commercially formulations of rHu-G-CSF include lenograstim, a glycosylated form, and filgrastim, a non-glycosylated form. Glycosylation of the molecule contribute to pharmacokynetis advantages and to higher affinity to specific receptor. Additionally, lenograstim exposed neutrophils unchanged all their functions *in vitro*, while filgrastim exposed neutrophils present functional defects due to higher adhesivity, cytoscheletric alterations and a more immature phenotype. *Aim*. On these bases, we hypothesized that lenograstim may prevent febrile episodes (FE) and reduce their lasting in patients with chemotherapy derived neutropenia more efficiently than filgrastim. Primary endpoint is the incidence of FE. *Patients and Design and Methods.* Starting from April 2005, 132 multiple myeloma patients, achieving high dose cyclophosphamide for stem cells mobilization, were enrolled in 11 Italian centers. Treatment plan consisted in: high dose cyclophosphamide (3 or 4 g/sqm) on day 1, G-CSF (random 1:1 on the base of a generated random list: filgrastim or lenograstim) 30 MU/day from day +4 to +9, 60 MU/day from day +10 to the achievement of an optimal CD34+ cell count for staminoapheresis. FE, significant if equal or higher than 38 oC for at least 2 different determinations, were recorded till day +30. *Results*. All 132 patients underwent post-chemo grade 4 neutropenia and G-CSF was administered starting from day +4. FE were recorded in 23 pts, 14 in the filgrastim arm (66 total patients) and 9 in the lenograstim arm (66 total patients). The global fever incidence was 17.4%, 21.2% with filgrastim and 13.6% with lenograstim. However, to demonstrate functional block of filgrastim exposed neutrophils, FE have been related to neutrophil absolute count. Related to the neutropenia grade, 8 FE are recorded with filgrastim (12.1%) and 1 FE with lenograstim (1.5%) with absolute neutrophil count >500/µL (grade 3) (p=0.0328); this difference is still evident when neutrophils are >1000/µL (grade 2), with 7 episodes with filgrastim (10.6%) versus 1 (1.5%) with lenograstim. *Conclusions*. Lenograstim is associated with a reduced global incidence of FE in multiple myeloma patients undergoing to high dose cyclophosphamide and stem cells mobilization when compared to filgrastim. Additionally, excluding the time frame when neutrophils are not yet recovered (neutrophils <500/µL; grade 4 neutropenia) and G-CSF effects may not be demonstrated, filgrastim treated patients present an higher FE incidence at neutrophil absolute count recovery (12.1% and 10.6%, with neutrophils respectively > 500/µL and 1000/µL), confirming the functional block of filgrastim exposed neutrophils described *in vitro*. On the contrary, lenograstim allows to recovery normally functional neutrophils as demonstrated by the very low incidence of FE (1.5% with neutrophils >500/µL). On these evidences, patients' enrollment will continue to 180 to validate these results.

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ENZYME LINKED IMMUNOSPOT (ELISPOT) ASSAY FOR THE DIAGNOSIS OF INVASIVE ASPERGILLOSIS IN HIGH RISK PATIENTS

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Background. The high mortality rate of Invasive Aspergillosis (IA) in hematologic and transplant patients is due in part to the limitations of the current diagnostic tools, which do not permit a prompt and undoubted diagnosis. Recent studies in mice and humans have suggested that Aspergillus-specific interferon-γ producing T-cells (IFN-γ-TH1) are protective, while Aspergillus-specific inteleukin-10 producing T-cells (IL-10-TH2) are permissive versus IA. *Aims*. We have evaluated whether the enumeration of Aspergillus-specific IFN-γTH1 and/or IL-10-TH2 through an *ex vivo* ELISPOT assay may be effective in the diagnosis of IA in high risk patients. *Design and Methods*. ELISPOT has been performed, as described [*Potenza et al. Leukemia 2007; 21: 578-81*] on 70 consecutive patients (91% with hematologic malignancies, 9% undergoing solid organ transplantation), classified according to the EORTC/MSG criteria as: 14 proven, 16 probable, 10 possible IA. The remaining 30 patients were considered controls because they were affected with infectious diseases of etiology other than IA, proven by histological or cultural methods. *Results*. At some time during the disease, ELISPOT resulted positive in the 14 proven IA cases. In particular, 13 out of 14 were positive for Aspergillus-specific IL-10-TH2; in 5 out of 14, also for *Aspergillus*-specific IFN-γ-TH1, and in 1 out of 14 only for Aspergillus-specific IFN-γ-TH1. The sensitivity of the test was 100% in proven IA, 50% in probable IA, 20% in possible IA. In the twelve patients in whom ELISPOT was performed at the time of radiological diagnosis, the assay resulted positive in 10 out of 12 (83%). Two patients demonstrated non-reactive in the first collected sample, because their T cells could not be stimulated by phytohemagglutinin. In the 30 control patients, ELISPOT resulted negative in 29 out of 30, with only one patient resulting non-reactive. The overall comparison (considering also nonreactive samples) of proven IA with control patients showed 85.6% sensitivity and 96.6 % specificity of the assay, while the positive predictive value (PV) and the negative PV resulted 100%. Furthermore in 8 proven IA patients with two or more samples collected during the course of the infection, ELISPOT seems to provide the kinetics of the Aspergillus-specific human response in course of IA, *in vivo*, by showing: high number of IL-10-TH2 at the onset, and its persistence/increase in case of progression or death; growing number of IFN-γ-TH1 during stabilization/regression; sustained number of IL-10-TH2 during the growing of IFN-γ-TH1 number to avoid an excessive inflammatory response; persistence of a protective IFN-γ-TH1 response after the resolution of the lesions. *Conclusions*. Our findings demonstrate the potential of ELISPOT in the diagnosis of IA. The ELISPOT assay may complement the other diagnostic tools [e.g. galactomannan antigenemia], enabling a more consistent diagnosis of IA. A larger number of proven and probable IA is necessary to address definitely the diagnostic accuracy of the ELISPOT. The assay may also provide new insights about the dynamic skewing between the Aspergillus-specific IFN-γ-TH1/IL-10-TH2, *in vivo*, during the course of IA, with possible consequences in designing drug and/or cellular therapeutic strategies.

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MYELOABLATIVE CONDITIONING PREDISPOSES FOR TOXOPLASMA GONDII REACTIVATION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Toxoplasmosis in recipients of allogeneic stem cell transplants (HSCT) is a severe infectious complication and occurs mainly through reactivation of latent Toxoplasma gondii cysts. Monitoring of T.gondii DNA in peripheral blood (PB) by polymerase chain reaction (PCR) has been shown to be an appropriate tool to detect reactivation. *Aim*. To monitor recipients of allogeneic HSCT for T.gondii reactivation by PCR and to identify risk factors for T. gondii reactivation. We also designed this study to evaluate the effectiveness of preemptive treatment before the onset of organ involvement. *Design and Methods*. From November 2001 until December 2008, we prospectively monitored all patients undergoing allogeneic HSCT in our institution that were seropositive (IgG) for T.gondii or from which the donor was seropositive. Patients did not receive trimethoprim-sulfamethoxazole prophylaxis but were monitored with qualitative PCR for T.gondii DNA in PB, weekly until D+100, thereafter two-weekly until 1 year post-transplant. Toxoplasmosis infection (TI) was defined as two positive PCRs in PB without clinical, radiologic or microbiologic evidence of invasive infection. Patients with TI were preemptively treated with pyrimethamine and clindamycine until PCR results were negative in two consecutive samples. *Results*. Two-hundred and eight patients were prospectively monitored. Eighteen patients had toxoplasmosis reactivation (8.7%) and the median time after transplantation was 62 days (range 25-166). One third (6/18) had evidence of invasive disease at first diagnosis (5 central nervous system, 1 cardiac involvement). Three of these patients with invasive toxoplasmosis died, but in only one patient toxoplasmosis was the direct cause of death (two also had concomitant severe invasive pulmonary aspergillosis). Twelve patients (67%) had TI at first diagnosis. Only one of these 12 patients who were preemptively treated, subsequently developed invasive infection but this was only 11 months later (chorioretinitis). Overall, 5 patients had a second reactivation of toxoplasmosis with a median time of 33 days (range 10-318) between termination of treatment and relapse. All but one reactivation occurred in seropositive recipients. The incidence of toxoplasmosis infection/disease was significantly higher in patients conditioned with a myeloablative regimen including total body irradiation ($p<0.01$). All but one patient (94%) had a CD4 T cell count below 200/μL. In our series, the presence of graft-versus-host disease, conditioning with anti-thymocyte globulin or the presence of CMV reactivation did not influence the risk of T.gondii reactivation. Our series contained only 1 patient transplanted with a cord blood graft, but this patient developed fatal cardiac toxoplasmosis at day 64 post-transplant. Finally, HSCT recipients with grafts from seropositive donors had a significantly lower incidence of toxoplasmosis reactivation ($p<0.05$). *Conclusions*. This study represents the largest prospective study on the incidence of toxoplasmosis after allogeneic HSCT. Toxoplasmosis is common with an incidence of 8.7%. All reactivations occurred within the first 6 months after transplantation, except in patients with previous toxoplasmosis reactivation. The majority of reactivations occurred before reconstitution of CD4 T lymphocytes. We demonstrate that myeloablative regimens containing TBI predispose to toxoplasmosis reactivation. Seropositivity of the donor coincided with a lower incidence of toxoplasmosis reactivation in the recipient.

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PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY FOLLOWING RITUXIMAB THERAPY IN HIV NEGATIVE PATIENTS: A REPORT OF 56 CASES FROM THE RESEARCH ON ADVERSE DRUG EVENT AND REPORTS (RADAR) PROJECT

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Rituximab improves outcomes for persons with lymphoproliferative disorders and is increasingly used to treat immune-mediated illnesses. Recent reports describe two patients with systemic lupus erythematosus and one with rheumatoid arthritis, who developed progressive multifocal leukoencephalopathy (PML) following rituximab treatment. We reviewed PML case descriptions among patients treated with rituximab from the FDA, the manufacturer, physicians, and a literature review from 1997 to 2008. Overall, 52 patients with lymphoproliferative disorders, one patients with systemic lupus erythematosus, one patient with rheumatoid arthritis, one patient with an idiopathic autoimmune pancytopenia, and one patient with immune thrombocytopenia developed PML following treatment with rituximab and other agents. Other treatments included hematopoietic stem cell transplantation (7 patients), purine analogues (26 patients), or alkylating agents (39 patients). One patient with an autoimmune hemolytic anemia developed PML following treatment with corticosteroids and rituximab and one patient with an autoimmune pancytopenia developed PML following treatment with corticosteroids, azathioprine, and rituximab. Median time from last rituximab dose to PML diagnosis was 5.0 months. Median time to death after PML diagnosis was 2.0 months. The case-fatality rate was 89%. Awareness is needed of the potential for PML among rituximab-treated individuals.

Acute lymphoblastic leukemia - Clinical II

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PEG-ASPARAGINASE IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): EFFICACY AND FEASIBILITY ANALYSIS WITH FOCUS ON LIVER TOXICITY IN THE GERMAN MULTICENTER STUDY GROUP FOR ADULT ALL (GMALL) STUDY 07/2003

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Randomised pediatric trials have demonstrated that intensification of Asparaginase (ASP) treatment in ALL can contribute to improvement of outcome. In adult ALL the optimal preparation, schedule and intensity which are effective and feasible have to be defined. The optimisation of ASP treatment is therefore an essential aim of the GMALL. GMALL induction consists of dexamethasone, vincristine, daunorubicine, PEG-ASP (phase I), mercaptopurine, cyclophosphamide and cytarabine (phase II). In the pilot trial 06/99 7x5000U/m^2 *E.coli* ASP given over 14 days were replaced by 1x1000U/m^2 PEG-ASP (500U/m^2 >55yrs). A dose dependent duration of PEG-ASP activity was demonstrated. Based on this correlation in study 07/2003 (Amend 12/06) the dose for PEG-ASP was increased to 2000U/m^2 in induction and consolidation (1000U/m^2 >55 yrs) in order to improve outcome. 1066 pts with a median age of 36 (15-65) yrs were evaluable (8%>55yrs). 799 pts were treated with 1000U/m^2 (cohort 1) and 185 pts with 2000U/m^2 (cohort 2) (reductions >55yrs). 82 pts did not receive ASP in induction due to various reasons. In cohort 1 and 2 91%/92% achieved CR after induction (91%/93% resp. <55 yrs). Data on molecular response (MRD below 10-4) after induction are available in a subset. There is a trend towards earlier and higher molecular CR rate in cohort 2 vs cohort 1 (81% vs 70% after induction). Survival after 1 yr is similar in cohort 1 (79%) vs cohort 2 (81%). Probability of CCR after 1 year shows a trend to improvement with 95% in cohort 2 vs 78% in cohort 1. Toxicity reported here is focused on potentially ASP related oIII-IV (WHO scale) events (734/147 pts in cohort 1/cohort 2). Incidences are as follows: GOT or GPT (29%/29%), bilirubine (10%/14%), thrombosis (4%/3%), bleeding (3%/2%) and hypersensitivity (1%/1%). Details on AEs of all degrees are available in a subset of pts showing an increase of any WHO grade in for bilirubine (81%), GPT (80%), amylase (52%), lipase (29%) and glucose (51%). Bilirubine °III/IV occurred after median 16d, was correlated with age and infections, associated with treatment delays and poorer outcome. PEG-ASP was also feasible in Ph$^+$ ALL combined with imatinib with a trend towards a higher incidence of liver toxicity in cohort 2 and pts with imatinib starting from diagnosis. *Conclusions*. This is the largest cohort of adult ALL treated with PEG-ASP. Overall intensified PEG-ASP was feasible within intensive multidrug induction. Coagulation disturbances occurred frequently but bleeding or thrombosis were rare events. The rate of severe hepatotoxicity was stable after dose escalation however lead to significant treatment delays in individual pts which were prognostically relevant. Lab value changes occurred frequently; it remains open to what extent they are clinically relevant and require interruption of further chemotherapy. It would be an important goal to identify parameters to predict severe ASP related toxicities e.g. by pharmacogenomics. The molecular CR rates after dose escalation and the remission duration are promising and will hopefully turn out into an improved overall survival.

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MONITORING OF MINIMAL RESIDUAL DISEASE IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS WITHOUT KNOWN GENETIC ABNORMALITIES: IMPACT ON PROGNOSIS IN THE GIMEMA LAL-0904 PROTOCOL

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Background. The evaluation of minimal residual disease (MRD) is becoming always more important in the management of different hematologic malignancies. In childhood acute lymphoblastic leukemia (ALL), monitoring of MRD has a significant prognostic role and influences stratification of patients and treatment. Although MRD is currently incorporated in several clinical protocols for adult ALL patients, data confirming its prognostic significance is scarce and there is no consensus on the time-points for MRD evaluation. *Aims*. The LAL-0904 GIMEMA protocol was designed to evaluate the impact of MRD, at pre-defined times, on achievement of complete remission (CR) and disease-free survival (DFS). The protocol was opened in July 2004 and is still ongoing. We report the results on 123 patients negative for the most important genetic abnormalities (BCR-ABL, ALL1-AF4, E2A-PBX1), with a median follow-up of 22.4 months. *Design and Methods*. All patients were homogeneously diagnosed through a central handling of the samples at presentation and were investigated for morphology, immunophenotype, cytogenetics, molecular biology, and IgH and TCR gene rearrangements. MRD was assessed by both immunophenotype and RQ-PCR analysis of IgH and TCR gene rearrangements during induction at days +35 and +50, and at the end of the consolidation. *Results*. Of the 123 patients, 39 were females and 84 males, with a median age of 31.4 years (range: 15.8 - 59.5), a median white blood cell (WBC) count of $14.8x10^9$/L (range: 0.5 - 300.4); 82 patients had B-lineage ALL and 41 T-ALL. The immunologic subtypes defined according to the EGIL system were as follow: 7% pro-B, 38% common-B and 19% per-B; 3% pro-T, 11% pre-T, 19% cortical-T and 3% mature-T. At the end of the induction, 91.1% of patients were in CR. We failed to observe any significant effect of MRD on the achievement of CR. In univariate analysis, MRD, evaluated by immunophenotype (cut-off at 10-3) and by IgH and TCR gene rearrangement (cut-off at 10-4) at days +35 and +50, showed an impact on DFS: by immunophenotype p=0.067 and p=0.038, respectively, and by IgH and TCR gene rearrangement, p=0.0035 and p=0.0007, respectively. In multivariate analysis, only the results of MRD detected by IgH and TCR gene rearrangement at day +50 had a highly significant predictive impact on DFS (p=0.0013). The evaluation of MRD after consolidation does not seem to add additional information to that obtained after induction therapy. Patients who showed a reduction of at least 10-3 (by immunophenotype) and 10-4 (by IgH and TCR gene rearrangement) had a better outcome. *Conclusions*. In our adult ALL series, the reduction of MRD at the end of induction represents an important prognostic factor both by immunophenotype and by IgH and TCR gene rearrangements. The results of our LAL-0904 protocol clearly indicate that the MRD status during induction should be taken into account when planning the overall therapeutic strategy of adult ALL patients.

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PROGNOSTIC IMPLICATIONS OF THE WILMS TUMOR 1 (WT1) GENE MRNA EXPRESSION IN ADULT ACUTE T-LYMPHOBLASTIC LEUKEMIA

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The Wilms tumor 1 gene, WT1, encodes a transcription factor involved in normal and malignant hematopoiesis. The role of WT1 in acute leukemias has been underscored by mutations in WT1 found in 10-15% of acute myeloid leukemia (AML) identifying patients with inferior survival. In addition, aberrant expression of WT1 in AML has been associated with an increased relapse risk. Thus far, larger studies have not determined the prognostic impact of WT1 alterations in acute T-lymphoblastic leukemia (T-ALL). Therefore, we have analyzed WT1 mutations and WT1 expression levels in a large cohort of T-ALL that included 239 newly diagnosed adult patients treated on GMALL protocols 06/99 and 07/03. Diagnostic bone marrow specimens were studied for WT1 mutations by DNA direct sequencing and WT1 mRNA expression was determined by real-time RT-PCR. Twenty (8%) of the 239 analyzed T-ALL patients exhibited WT1 mutations (WT1mut) in exon 7. The presence of co-operating gene mutations in WT1mut patients was further investigated. WT1mut patients showed a higher frequency of FLT3 mutations (4/20; 20% of cases) compared to WT1wt T-ALL patients (3/108; 3% of cases). Significant differences were not observed in the complete remission (CR) rate as well as overall survival and relapse free-survival (RFS) between WT1mut and WT1wt patients. Expression levels of WT1 were analyzed in 223 of the 239 T-ALL patients. Three expression groups including patients with WT1 negative (n=97), WT1 intermediate (n=81), and WT1 high (n=45) expression levels were defined. Patients with high WT1 expression were characterized by immature features such as an early immunophenotype, high BAALC expression levels, and aberrant expression of CD13 and HOX11L2. WT1 negative and WT1 intermediate cases had predominantly a thymic phenotype. A strong correlation was observed between the WT1 expression and mutation status: WT1 mutations were predominantly found in the WT1 high expression group (13/41; 29% were WT1mut) compared to only 1/94 (1%) of WT1 negative and 4/74 (5%) of WT1 intermediate cases harboring WT1 mutations (p<0.001). T-ALL patients with aberrant (negative or high) WT1 expression levels showed a higher relapse rate and an inferior outcome as compared to patients with intermediate WT1 expression (4-year disease-free survival: 21.7% for WT1 high, 53.9% for WT1 negative, 75.5% for WT1 intermediate expressers; p<0.001). Interestingly, patients of the high risk groups (negative or high expression) showed an improved survival when consolidated with stem cell transplantation (SCT; 4-year survival: 62%) as compared to chemotherapy only (4-year survival: 36%; p=0.04). In multivariate analysis, WT1 expression was of independent prognostic significance (hazard ratio: 6.8; p=0.003). An interaction was observed demonstrating a favourable impact of SCT for WT1 high risk patients (negative/high expression). Taken together, WT1 gene mutations were identified in a similar frequency and at the same hotspot regions as previously identified in AML but of less prognostic significance in this cohort of T-ALL. Moreover, aberrant WT1 expression was of independent prognostic significance associated with inferior outcome, thus suggesting a role of WT1 in T-ALL. Furthermore, expression analysis could be applicable for a more defined molecularly-based treatment stratification including stem cell transplantation.

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GOOD GENERAL MEDICAL CONDITION IN LONG TERM SURVIVORS OF ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) BUT MORE HEALTH IMPAIRMENT IN OLDER PATIENTS AND PATIENTS AFTER STEM CELL TRANSPLANTATION (SCT): RESULTS OF A SYSTEMATIC ANALYSIS OF GMALL STUDIES

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Since 1981 the majority of adult pts with ALL in Germany was treated in 7 consecutive trials of the German Multicenter Study Group for Adult ALL (GMALL). Survival was step-wise improved to 50% in the most recent trial with an increasing number of long-term survivors. However so far there is no published evidence about health status and/or late effects in survivors of adult ALL. Therefore the GMALL initiated a retrospective analysis of pts from studies 2/84 - 6/99 alive more than 5 yrs after diagnosis. The questionnaire covered 8 organ systems and 1 category of "specific syndromes", known as potential late-effects of chemotherapy and was directed to treating physicians. It was intended to assess all diseases, appearing after end of therapy. 402 questionnaires were evaluable. Median age at diagnosis was 29 (15-64) years; median observation time was 10 yrs. Pts were included in GMALL-study 2/84 (10%), 3/87 (4%), 4/89 (15%), 5/93 (46%) and 6/99 (25%). 24% had received SCT (4% auto, 20% allo). In 94% of the pts the ECOG status was 0/1 (72/22%); 6% had ECOG 2-4. In 33% of the pts no disease was reported (Table 1). 67% presented a disease in ≥ one organ system; most frequently endocrinium (e.g. infertility, diabetes, osteoporosis), neurologic system (e.g. mood alteration, polyneuropathy, concentration) and skin (e.g.alopecia, erythema); cardiovascular diseases consisted mainly

of hypertension; only 2 pts had heart failure. The most frequently documented category was "specific syndromes" in 39%. Infections, fatigue and osteonecrosis were the most frequent ones. GVHD/sicca syndrome was observed in 10% of all pts and in 44% of SCT pts. Favourable ECOG (0-1) was significantly more often observed in chemo vs SCT pts (97% vs 86%;p=.0003). More pts with chemo had no disease compared to SCT pts (40% vs 9%; p<0.0001). All organ systems were more often involved in SCT pts. They also had more fatigue (p=0.01), and infections (p<.0001). Age was associated with poorer ECOG too. 97% of the pts with diagnosis in adolescent age (15-25 yrs) had ECOG 0-1 compared to 81% in pts > 55 yrs. Fertility was evaluated by a patient-questionnaire (N=487). 58% of the pts (70% of chemo vs 21% of SCT pts; p<0.0001) with desire to have children could realise this wish (medical intervention in 21%). This so far largest data set of medical conditions and late effects of long-term survivors after adult ALL shows favourable results. The observed diseases had only in part a potential correlation to previous ALL therapy (e.g. GVHD, sec. malignancies, osteonecrosis, neurologic diseases). Compared to childhood ALL, the rate of late effects is remarkably low. The impaired health status in pts with SCT compared to chemo underlines that indications for SCT should be made carefully in high-risk patients only. Aftercare should consider specifically the most frequent late-effects i.e. osteonecrosis, fatigue, endocrinology and fertility. These may be cumbersome in individual pts. There is a trend towards more pronounced late effects in the most recent trial with intensified therapy. Therefore the analysis will be carried on.

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Table 1. Most frequently involved organs/syndromes in long-term survivors of adult ALL.

Patients	N=402	%
No diseases	**132**	**33**
Organ systems with pathologic findings	**270**	**67**
Neurologic system	110	27
Endocrinium (male)	56	22
Endocrinium (female)	45	30
Skin and mukosa	59	15
Lung	22	5
Kidney and liver	36	9
Cardiovascular system	61	15
Stomach and gut	21	5
Eye	37	9
Specific Syndromes	**158**	**39**
Infections within last 12 months	51	13
Fatigue	45	11
Graft versus host disease / sicca syndrome	42	10
Osteonecrosis	32	9
Secondary malignancies	19	5
Hyperthyreosis	14	4
Hypothyreosis	5	1

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A RANDOMISED PHASE II STUDY OF PEGYLATED LIPOSOMAL DOXORUBICINE IN ELDERLY PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): THE GRAALL-SA1 STUDY

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The GRAALL-SA1 study aimed to randomly compare the efficacy and toxicity of liposomal pegylated doxorubicin (Peg-Dox) vs continuous infusion doxorubicin (CI-Dox) in elderly patients with Philadelphia chromosome-negative ALL. Induction chemotherapy, supported by G-CSF, comprised either CI-Dox 12 mg/m^2/d and CI-VCR 0.4 mg/d on d1-4 or Peg-Dox 40 mg/m^2 IV and standard VCR 2 on d1, in combination with dexamethasone. A second cycle, reinforced by cyclophosphamide 1 g/m^2, was started at d28. Consolidation included two additional cycles using reduced doses of CI-Dox (12 mg/m^2/d d1-3) or Peg-Dox (30 mg/m^2 d1) with VCR in both treatment arms (5 minutes infusion on d1,8,15) alternating with two cyclophosphamide (650 mg/m^2 d1) / thioguanine (60 mg/m^2 d2-d15) / cytarabine (75 mg/m^2/d4-7 and d11-14) cycles. Maintenance included 6-MP and methotrexate for 2 years. CNS prophylaxis was based on 6 triple ITs and cranial irradiation. From March 2002 to October 2006, 60 patients aged 55 years or more from 26 centers were enrolled in this multicenter study. The median age was 66 years (range 55-80), 17% had initial WBC >30x10^9/L, 2 patients had CNS leukemia and 6.6% had mediastinal involvement. Immunophenotyping showed 85% B-lineage ALL, 12% T-ALL, and 3% aberrant expression of myeloid antigens. Patient characteristics were comparable in the two randomization groups except for a small unbalance with more T-ALL and more steroids resistant leukemia in the Peg-Dox group (6/29 vs 1/31, p=0.05 and 5/29 vs 1/31, p=0.1, respectively). Tolerance was improved in the Peg-Dox arm with less Grade 3-4 infectious events during induction (27% vs 48%; p=0.04), less Gram negative bacteriemia during induction (1 vs 9, p=0.02) and consolidation courses (2 vs 7, p=0.07) and less cardiac toxicities (1/29 vs 6 /31, p=0.12). Duration of neutropenia was reduced from a median of 7 days in the CI-Dox arm to 6 days in the Peg-Dox arm (p=0.06). RBC units transfused were reduced from 7.2 to 4.5 during induction (p= .02) and from 2.9 to 1.1 during consolidation course (p= .003). Days with platelets <50x10^9/L were reduced from 13.4 to 6.4 (p=0.03). After the 2 induction cycles, CR rate was 82%, with 10% failures and 8% induction deaths. With a median follow-up of 40 months, median OS for the entire population was 10 months and 19% of the patients were alive in CR at 3 years (95% CI, 9-30). Despite the better tolerance, there was a trend for a worse 2-year OS in the Peg-Dox arm (11 vs 27%, p=0.07), due to higher induction failure (17 vs 3%) as well as relapse incidence rates (62 vs 39%). Younger age, B-lineage, and steroid sensitivity were the three factors identified for a higher CR rate in multivariate analysis. The decreased toxicity of Peg-Dox over CI-Dox was thus offset by a decreased efficacy. Further studies should evaluated higher doses of pegylated versus conventional formulations of anthracycline in patients with ALL.

Acute myeloid leukemia - Biology II

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IDENTIFICATION OF NOVEL MUTATIONS IN ACUTE MYELOID LEUKEMIA USING WHOLE TRANSCRIPTOME SEQUENCING

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Background. Acute myeloid leukemia (AML) is a genetically heterogeneous disease. While approximately half of AML patients have at least one chromosomal aberration, the other half classifies as cytogenetically normal (CN-AML). In CN-AML patients an increasing number of recurring somatic mutations have been identified during the last decade. Despite their pathological and prognostic relevance, none of these mutations are sufficient to cause AML on their own. Furthermore, in approximately one quarter of the CN-AML patients none of the known mutations can be detected. *Aims*. To identify novel mutations in AML by transcriptome sequencing using a next generation sequencing machine. *Design and Methods*. The term transcriptome summarizes all mRNA transcripts present in the cells from a certain patient sample. To identify tumor-specific somatic coding mutations, we sequenced the transcriptome of a CN-AML and a remission sample from the same patient using an Illumina GAII machine. Poly-A selected, fragmented RNA was used to synthesize double stranded cDNA, which was then sequenced. SNPs were called with the MAQ software. *Results*. We generated 20.4 and 15.6 million 32 bp paired-end reads of the CN-AML and remission sample, respectively, which mapped to exons of UCSC genes. 8.9% of reads for the AML and 5.0% reads of the remission sample mapped to intergenic regions. Of the 11,178 transcripts with a higher expression than 60 reads per gene (corresponding to approximately 1 transcript per cell), we sequenced 5,911 with an average coverage of greater than seven. By comparing the 63,159 SNPs discovered in the CN-AML sample with the remission sample, we identified 5 non-synonymous mutations not present in either the remission sample or in dbSNP. One of these point mutations affected the RUNX1 gene which forms a well known fusion gene in AML (RUNX1/RUNX1T1) and is a known mutational target in AML. The second mutation affected a gene which encodes a RUNX1 interacting protein, and a third mutation was found in the cellular homolog of a viral oncogene. The two other mutations occurrred in a phospholipase gene and a nuclear chaperon gene. The five genes identified as mutational targets are currently studied in a larger patient cohort of 200 patients with normal karyotype in order to determine the frequency of mutations in these genes. *Conclusions*. Transcriptome sequencing of AML patients is a pioneering application of the latest sequencing technology that may allow the unbiased detecting and understanding of the majority of genetic lesions that contribute to the onset and progression of AML. For mutation screening in the coding regions of expressed genes, whole transcriptome sequencing is currently 5-10 times faster and more cost effective than whole genome sequencing.

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SETBP1 OVEREXPRESSION IS A NOVEL LEUKEMOGENIC MECHANISM THAT PREDICTS ADVERSE OUTCOME IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemias (AML) are clonal malignant disorders that result from multiple genetic alterations in normal hematopoietic stem cells. In recent years, several genetic markers with prognostic impact in AML have been identified, permitting a better understanding of the biology of this disease and, in some cases, providing targets for molecular therapies. However, the outcome of older patients with AML has not improved in the last three decades, due to both patient-specific and disease-specific factors. Here, we describe a novel t(12;18)(p13;q12) involving ETV6 in a patient with AML. The translocation resulted in no functional fusion gene, indicating that a different mechanism might be acting. The SETBP1 gene (18q12), located close to the breakpoint, was overexpressed in the patient, suggesting that expression of this gene was upregulated by the translocation. Overexpression (OE) of SETBP1 through retroviral insertion has been reported to confer a growth advantage in hematopoietic progenitor cells, and SETBP1 interacts specifically with the SET protein, a potent inhibitor of PP2A; however, the role of this gene at the molecular level remained unknown. We demonstrate that SETBP1 OE leads to higher levels of SET due to formation of a SETBP1-SET heterodimer that protects SET of the protease activity, increasing the amount of full-length SET protein of 39 KDa, and decreasing the shorter SET processed forms. We also observed the formation of a SETBP1-SET-PP2A complex that, eventually, results in PP2A inhibition. Furthermore, the deregulation of PP2A activity promotes proliferation of the myeloid leukemic cells. We also analyzed the prevalence of SETBP1 OE in a series of 192 patients with AML at diagnosis. SETBP1 was overexpressed in 28% of patients with AML, and it was associated with unfavorable cytogenetic prognostic group, monosomy 7, and EVI1 OE ($p<0.01$). We found a significant shorter overall survival (OS) in patients with SETBP1 OE. The impact prognosis was especially remarkable in the group of patients older than 60 years in both OS ($p=0.015$) and event free survival ($p=0.015$). In conclusion, our results show a novel leukemogenic mechanism: SETBP1 overexpression would lead to the formation of a SETBP1-SET-PP2A complex that increases the amount of full-length SET protein, resulting in PP2A inhibition and, therefore, promoting the proliferation of the cells. Moreover, we have shown that SETBP1 overexpression is a recurrent molecular event with prognostic impact in AML, especially in the subgroup of elderly patients. 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. Systematic molecular genetic studies in AML patients not only are useful for the evaluation of biomarkers for prognostication, but also for the identification of predictive factors for response to novel therapies. Our data suggest that SETBP1 overexpression could be a predictive factor for response to PP2A activators such as FTY720, which has been proposed as a new alternative for treating blast crisis CML and Philadelphia chromosome-positive ALL.

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STUDYING THE ROLE OF PIM KINASES IN FLT3-ITD-INDUCED LEUKEMIA REVEALED PIM1 AS REGULATOR OF CXCL12/CXCR4-MEDIATED HOMING AND MIGRATION

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Background. The PIM1 and PIM2 serine/threonine kinases are overexpressed in most human hematological malignancies. By expression of siRNAs or dominant-negative mutants, we have previously shown that PIM kinases are important for proliferation and survival of hematopoietic cells transformed by oncogenic protein tyrosine kinases such as the FLT3-ITD mutant associated with acute myeloid leukemia (AML). *Aims*. To study the role of PIM kinases in FLT3-ITD mediated leukemogenesis. *Design and Methods*: Reconstitution assays with FLT3-ITD expressing PIM-/- bone marrow cells were performed. We also modulated the function of PIM kinases in cell lines by small molecule PIM inhibitors and isoform-specific siRNAs. *in vitro* kinase assays as well as masspectrometry was used to characterize putative PIM phosphorylation sites. *Results*. Unexpectedly, bone marrow cells deficient for PIM1 failed to reconstitute lethally irradiated wild-type recipients, whereas, the absence of PIM2 did not interfere with the induction of a FLT3-ITD-mediated leukemia-like disease. PIM1-/- bone marrow cells were impaired in early homing to bone marrow and spleen. PIM1-/- but not PIM2-/- bone marrow cells displayed decreased surface expression of the CXCR4 receptor and were defective in migration towards the CXCL12 ligand. By blocking PIM1 function or re-expression in PIM1-/- bone marrow cells, we found that PIM1 activity was essential for proper CXCR4 surface expression and migration towards CXCL12. By expression of wild-type and mutant GST-CXCR4-C-terminal mutants we identified Serine 339 in the CXCR4 intracellular domain as being phosphorylated by PIM1 and essential for proper recycling of the receptor to the surface. In addition, expression of S339 CXCR4 mutants in cell lines functionally

confirmed the importance of this residue. Interestingly, elevated levels of surface CXCR4 in AML blasts were associated with increased PIM1 mRNA expression, and could be significantly reduced by a small molecule PIM inhibitor in 4 out of 6 patients. *Conclusions*. Our data suggest that PIM1 activity is essential for homing and migration of hematopoietic cells through direct modification of CXCR4. Since CXCR4 is also important for homing and maintenance of leukemic stem cells, PIM inhibitors may exert their anti-leukemic effects in part by interfering with interactions with the microenvironment.

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EVIDENCE FOR CD34+ HEMATOPOIETIC PROGENITOR CELL INVOLVEMENT IN ACUTE MYELOID LEUKEMIA WITH NPM1 GENE MUTATION: IMPLICATIONS FOR THE CELL OF ORIGIN

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Acute myeloid leukemia expressing mutated NPM1 gene and cytoplasmic nucleophosmin (NPMc+ AML) [*Falini B et al, NEJM 2005;352:254-266*] is a new entity of WHO classification that shows distinctive biological and clinical features, including a unique molecular signature characterized by downregulation of CD34 and upregulation of most HOX genes [*Falini B et al, Blood 2007;109:874-885*]. Involvement of HOX genes in the maintenance of the stem-cell phenotype strongly suggest that AML with mutated NPM1 originates from a multipotent hematopoietic progenitor (HSC). This view is also supported by immunohistological findings showing that AML with mutated NPM1 frequently displays multilineage involvement [*Pasqualucci L et al, Blood 2006;108:4146-4155*]. On the other hand, the frequent negativity of NPMc+ AML for the HSC-associated antigen CD34 raises the question of whether the mutation event occurs in a CD34-negative HSC (these cells have been identified in mice) or whether a minimal pool of CD34-positive NPM1-mutated leukemic cells does exist. Currently, the hierarchical level of stem cell involvement in NPMc$^+$ AML is unknown. To address this issue, we purified CD34$^+$ cells from NPMc$^+$ AML patients and detected NPM1 mutant protein in the sorted population by Western blot with anti-NPM mutant specific antibodies [*Martelli MP et al, Leukemia 2008*] (Figure 1A).

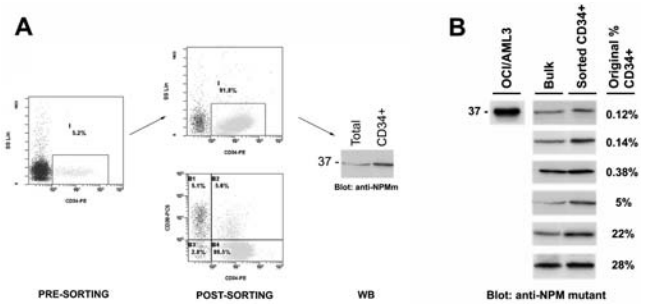

Figure 1.

We investigated 13 NPMc+ AML patients presenting at diagnosis with 0.05 to 28% CD34$^+$ cells in the peripheral blood. In all cases, CD34+ fractions (purity >90%) harboured NPM1 mutant protein, indicating they belong to the leukemic clone (Figure 1B). The percentage of most undifferentiated CD34$^+$/CD38$^-$ cells in the CD34$^+$ fractions ranged from 5 to 97%. Notably, in at least one case, all CD34$^+$ NPM1-mutated leukemic cells were CD38-negative. Moreover in all cases, CD34$^+$ NPM1-mutated leukemic cells appeared to express CD123 (IL-3 receptor), considered a marker of the leukemic stem cell and target of potential therapy. Double staining of bone marrow biopsies with anti-CD34 and anti-NPM antibodies revealed that the rare CD34$^+$ cells expressed NPM1 aberrantly in the cytoplasm. Inoculation of CD34$^+$ NPM1-mutated AML cells into sublethally irradiated NOD/SCID mice resulted into leukemia engrafment in various body sites, especially bone marrow, spleen, lung and liver. Preliminary results showed that CD34$^+$ leukemic cells reacquired the same leukemic phenotype as the original patient's, including CD34-negativity of the leukemic bulk in spite of any lack of differentiation. This finding suggests that NPM1 mutant protein may be involved in downregulation of CD34 antigen, while keeping a gene expression profile typical of the hematopoietic stem cell. These findings suggest the CD34$^+$ fraction contains the SCID-leukemia initiating cells (SL-IC) and point to CD34$^+$/CD38$^-$ HSC as the cell of origin of AML with mutated NPM1.

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PROTEIN SYNTHESIS ESCAPES MTORC1 CONTROL AND CONSTITUTES A PROMISING THERAPEUTIC TARGET IN ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) is a clonal hematological disease characterized by differentiation arrest and by the proliferation of immature myeloid progenitors, both sustained by the deregulation of multiple signaling pathways. The mammalian Target Of Rapamycin Complex 1 (mTORC1) consists of mTOR, raptor, mLST8 and PRAS40. It governs cell growth and regulates cap-dependent translation of mRNAs. Translation is tightly regulated at the initiation level. The eIF4E protein recognizes the 7-methyl-GTP cap structure of mRNA and associates with eIF4G to form the translation initiation complex (eIF4F). The mTORC1 substrate 4E-BP1 inhibits the formation of eIF4F by competitively inhibiting the recruitment of eIF4G and sequestering eIF4E in an inactive complex. Sequential phosphorylation of 4E-BP1 by mTORC1 decreases its affinity for eIF4E and facilitates the assembly of active eIF4E/eIF4G complexes. The rapalogs (e.g. RAD001, rapamycin) are specific mTORC1 inhibitors and have been developed as anti-cancer therapeutics. Despite the rationale for their use as cancer therapeutics, the results of clinical trials are heterogeneous. In AML, mTORC1 is frequently activated but rapamycin is essentially cytostatic and induces apoptosis only when associated with cell-cycle dependent chemotherapies. Thus, its clinical efficacy in monotherapy is limited in AML. *Aims and methods*. As mTORC1 is known to control protein synthesis, the moderate anti-leukemic activity of rapamycin led us to investigate the importance of translation in AML biology. We performed experiments in 24 primary AML samples obtained from patients treated in varying studies of the GOELAMs French group after informed consent according to the declaration of Helsinki. MTORC1 signaling was modulated by RAD001 and raptor siRNA and studied by immunoblot. Cap-dependent mRNA translation was studied by 7m-GTP pulldown assay, polysome analysis, [3H]leucine pulse assay and immunoblot. AML blast cells survival was assessed by methylcellulosis cultures and annexin V fixation in flow cytometry. *Results*. We show herein that directly targeting translation has a marked anti-leukemic potential in primary AML cells. Indeed, specific inhibition of cap-dependent translation was achieved using the small molecule 4EGI-1. This compound dramatically decreased the clonogenic growth of leukemic progenitors and induced massive apoptosis of AML cells, with minimal impairment of normal hematopoiesis. Moreover, we observed that mRNA translation escaped mTORC1 control in AML blasts. Indeed, P70S6K phosphorylation on T389, a typical downstream relay of mTORC1, was abrogated by RAD001 or raptor siRNA whereas 4E-BP1 phosphorylation on S65, essential for translation-initiation control, was unaffected. Therefore, RAD001 failed to inhibit translation. Unlike 4EGI-1, RAD001 did not inhibit the assembly of eIF4F complexes and did not reduce the association of c-Myc mRNA with polysomes. Moreover, we identified the Pim-2 serine/threonine kinase as mainly responsible for the 4E-BP1 phosphorylation on S65 and subsequent cap-dependent translation control in AML. Indeed, pim-2 knockdown and treatment of AML cells by 4EGI-1 strongly reduced the accumulation of oncogenic proteins regulated at the translation initiation level whereas RAD001 failed to modify their cellular levels. *Conclusions*, Our results implicate a rapamycin-insensitive deregulation of oncogenic protein expression in leukemogenesis and strongly support the development of therapeutic strategies that directly target the eIF4F translation initiating complex in AML.

Anemia, aplastic anemia - PNH, Red Blood Cells and Iron

1110

ECULIZUMAB REDUCES PULMONARY HYPERTENSION THROUGH INHIBITION OF HAEMOLYSIS-ASSOCIATED NITRIC OXIDE CONSUMPTION IN PATIENTS WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

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Background. Pulmonary hypertension (PHT) has been mechanistically and epidemiologically linked to intravascular hemolysis and NO depletion. Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by chronic hemolysis, as well as elevated cell-free plasma hemoglobin. We have previously reported that ~50% of PNH patients have PHT as measured by Doppler echocardiography. Further, the level of N-terminal pro-B-type natriuretic peptide (NT-proBNP) has been demonstrated to be a strong predictor of PHT and mortality in patients with hemolytic anemias (defined as NT-proBNP ≥160 pg/mL). Eculizumab significantly and rapidly reduces hemolysis. *Aims and Methods*. To evaluate the efficacy of eculizumab in the regulation of cell-free hemoglobin levels, NO depletion, and subsequent cardiovascular morbidities, levels of hemoglobinemia, arginasemia and nitric oxide depletion were assessed in 73 eculizumab- and placebo-treated PNH patients in the phase III randomized, placebo-controlled trial (TRIUMPH). In addition, levels of NT-proBNP as a measure of PHT and systolic and diastolic systemic arterial pressures were examined in eculizumab- and placebo-treated patients.

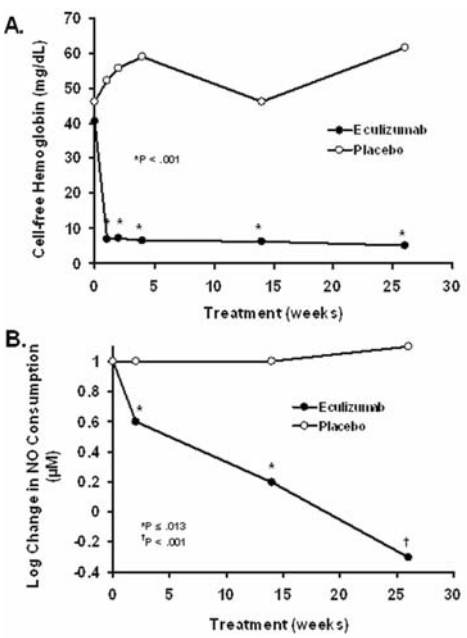

Figure. Effect eculizumab on cell-free plasma hemoglobin and NO consumption.

Results. At baseline, levels of lactate dehydrogenase (LDH), cell-free plasma hemoglobin, arginase 1 and arginase 1 enzyme activity were highly elevated compared to normal values. Levels of hemolysis and NO consumption were shown to be much greater in PNH (more than 6- and 10-fold, respectively) than in patients with other hemolytic diseases. There were significant correlations between cell-free plasma hemoglobin levels and both LDH (R = 0.5094) and plasma consumption of NO (R = 0.9529). Strong correlations between arginase 1 and both cell-free plasma hemoglobin (R = 0.9367) and arginase 1 enzyme activity (R = 0.9081) were also demonstrated. Following eculizumab therapy, measures of hemolysis were significantly reduced from baseline, including LDH (2200±158 to 327±68 U/L) and cell-free plasma hemoglobin (98.8±23.24 to 15.2±5.05 mg/dL), while levels in placebo-treated patients remained unchanged; a concomitant reduction in NO consumption was also observed (see Figure). In addition, at baseline, 46.6% (34/73) of PNH patients in the TRIUMPH study had levels of NT-proBNP ≥160 pg/mL, indicating PHT in these patients. Eculizumab-treated patients showed a 50% reduction in the incidence of PHT over the course of the 26-week treatment period from 52.5% to 26.3%, while PHT did not change with placebo (39.4% to 43.8%; p=0.057). Additionally, eculizumab significantly improved dyspnea (EORTC-QLQ-C30) compared to placebo (Effect size 0.69; p=0.0002). Similarly, eculizumab treatment was associated with a concomitant decrease in systemic arterial blood pressure as compared to patients receiving placebo. *Summary.* In patients with hemolytic anemia, the depletion of NO is associated with the development of cardiovascular morbidities, including PHT. The current data demonstrate that intravascular hemolysis in untreated patients with PNH is associated with high levels of plasma ferrous oxyhemoglobin, which stoichiometrically catabolizes NO, and high levels of plasma arginase 1, which catabolizes arginine - all leading to significant depletion of NO. We show a high prevalence of PHT in untreated patients with PNH as indicated by levels of NT-proBNP. Further, the data demonstrate that in a placebo-controlled study, the antihemolytic effect of chronic eculizumab treatment significantly increases nitric oxide bioavailability and reduces pulmonary hypertension.

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UNRELATED CORD BLOOD TRANSPLANTATION FOR ACQUIRED BONE MARROW FAILURE SYNDROMES: A RETROSPECTIVE EUROCORD AND EMBT SEVERE APLASTIC ANAEMIA WORKING PARTY ANALYSIS

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Allogeneic haematopoeitic stem cell transplantation is a treatment option for patients with acquired bone marrow failure syndromes (BMFS) who fail immunosuppressive treatment (IST). Unrelated cord blood transplantation (UCBT) offers an alternative source of hematopoietic stem cells for patients lacking a suitable sibling or matched unrelated donor. We studied 59 patients reported to Eurocord who received UCBT as their first allograft for either acquired severe aplastic anaemia (SAA) (n=53) or paroxysmal nocturnal haemoglobinuria (PNH) (n=6). Forty-eight received single and 11 received double UCBT. Median age at transplant was 11,5 years (1-68 years), 59% were children. Most (61%) patients had received more than 20 platelet transfusions by time of transplant, while half (49%) had received more than 20 red cell transfusions. Forty one patients with SAA had received at least one cycle of IST prior to transplant. Among those with sufficient data available (29), 55% had failed one cycle of IST before transplant, 28% two cycles and 7% more than two cycles. Median delay between diagnosis and transplant for patients with SAA was 13 months (2-128 months). Among the 48 single cord transplants, 5 patients (12%) were 6/6 matched, 17 (40%) had one HLA mismatch, 19 (44%) had two mismatches and 2 had three mismatches. For double UCBT, 2 patients had one HLA mismatch, 6 had two mismatches and one had three mismatches when examined according to greatest cord-recipient HLA disparity (2 missing HLA data). Median total nucleated and CD34+ cell doses infused were 3,35*107/kg (1,71 to 16*107/kg) and 1,82*105/kg (0,29 to 13,3*105/kg) respectively for single cords and 6,17*107/kg (3,21 to 9,7*107/kg) and 2,78*105/kg (1,1 to 7,1*105/kg) respectively for double cords. Thirty six patients (64%) received reduced intensity conditioning, although conditioning regimens varied. Twenty-three patients received cyclophosphamide (CY) and fludarabine (FLU) (14 of these had CY dose <100 mg/kg and FLU 120-200 mg/m^2), 12 patients received CY (5 in combination with total body irradiation), 11 patients received CY and busulphan. Forty-five patients (85%) received anti-T-cell immunoglobulins. Median follow-up was 28 months (3-71 months). Median time to neutrophil recovery was 24 days, with a cumulative incidence (CI) of 68 ±7%. No late graft failure was reported. Of those who achieved neutrophil recovery, 29 out of 35 had chimerism results; 26 (90%) had full donor chimerism. The CI of acute graft vs. host disease (GVHD) (grade II-IV) was 31±7%. Of 31 of 45 eligible patients at risk, 14 had chronic GVHD (45%) (limited in 50%). Two year overall survival (OS) estimates were 42±6%. Of 24 patients without engraftment, information on subsequent treatment was available for 14. Ten (71%) of these went on to receive a second allograft, of which 7 were UCBT, and one was alive at last follow-up. Infection remains the most common cause of death (13 patients (38%)). High incidence of graft failure is a problem in UCBT for acquired BMFS; however, the procedure remains a feasible salvage therapy for this group of very high risk patients. More studies with higher patient numbers are required to assess factors affecting transplant outcomes.

1112

COMPARISON BETWEEN LYMPHOGLOBULINE- AND THYMOGLOBULINE-BASED IMMUNOSUPPRESSIVE THERAPY AS FIRST-LINE TREATMENT FOR PATIENTS WITH APLASTIC ANEMIA

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Background. Immunosuppressive therapy (IST) is considered to be the first-line treatment in patients with severe aplastic anemia (AA) who are not eligible for hematopoietic stem cell transplantation (HSCT). Most IST schemes are based on the combination of anti-thymocyte globulin (ATG) plus cyclosporine A (CsA). Differently from other countries, two ATGs have been approved in Spain for AA from 2003 to 2007: Lymphoglobuline (LG) (raised in the sera of horses) and Thymoglobuline (TG) (produced in rabbits). So, during this period of time, the standard therapeutic protocol of the Spanish study group for AA included both options, and the physicians chose LG or TG based on their own wishes. Most published studies in AA are with LG, which is no longer manufactured. Recent limited data have confirmed therapeutic efficacy of TG, but no randomized studies have been performed comparing both products' activity. The aim of this report is to communicate the outcomes of a group of patients with AA who received either a LG- or TG-based scheme as first-line treatment. *Design and Methods*. We retrospectively investigated the outcome of 101 patients with AA treated with IST at front line between 2003 and 2008. Twenty-nine patients (28%) got LG (15 mg/kg/day/x5 days), and 72 patients (72%) got TG (2.5 mg/kg/day/x5 days). All patients also received methylprednisolone and CsA. Response rate (RR) was assessed at post-IST day +90, day +180, and day +365. If complete response (CR) was not reached, patients received a second course of IST, a second-line therapy (HSCT or androgens), or no treatment. When a second course of IST was employed, it included LG at the same dose as in the first course (15 mg/kg/day/x5 days), or TG at a higher dose (3.5 mg/kg/day/x5 days). CR was defined as a neutrophil count >1.5x10^9/L, a platelet count >100x10^9/L, and a hemoglobin level >120 g/L. Partial response (PR) was defined as a neutrophil count >0.5x10^9/L, a platelet count >20x10^9/L, and a hemoglobin level >80 g/L. Subgroup analyses were conducted and differences in response were tested using the chi-square statistic test. Results: After the first course of IST, 27 patients (27%) reached CR (group A) (LG: 38%, TG: 22%), and 20 patients (20%) reached PR (LG: 10%, TG: 24%). Thirty-one of the patients who did not reach CR after the first IST course, received a second course of IST (5 with LG and 26 with TG) (group B), and 43 patients underwent a different approach (second-line therapy or no treatment) (group C). After the second course of IST, 12 patients (38%) reached CR (LG: 40%; TG: 38%) and 11 patients (35%) reached PR (LG: 40%; TG: 34%). If we exclude patients in group C, the RR among the remaining 58 patients (who underwent 1 or 2 courses of IST) was 86% (67% CR, and 19% PR). No major drug-related toxicities were reported in the whole group of patients. *Conclusions.* ATG-based schemes with both LG and TG were well tolerated as treatment of patients with AA. RR after first course of IST was similar in the LG and in the TG group (48% versus 46%). RR after second course of IST was also similar when LG and TG were employed (80% versus 73%). After excluding those patients who, not having reached CR after the first course, underwent an approach different from a second course of IST, RR to IST was 86%, with 67% of CR. No statistical differences were found based on the type of ATG administered. The results of this study show that TG is, at least, as effective as LG for the treatment of AA patients. Based on these and other recently published data, the current standard therapeutic protocol of the Spanish study group for AA includes TG at the dose of 3.75 mg/kg/day/x5 days for both the first and, if necessary, second course of IST. To our knowledge, no reports are available in the medical literature comparing the outcome of patients with AA who received LG or TG as the first-line therapy for AA. So, in spite of the fact that our study is retrospective and not randomized, we think our data are unique and very useful for helping physicians in switching from LG to TG.

1113

CONDITIONAL INDUCTION OF MKK6-P38 MAPK SIGNALLING IN GRANULOCYTES INDUCES PHENOTYPIC CHANGES TO MONOCYTE-LIKE CELLS

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How intracellular signalling cascades downstream of cooperating cytokine signals orchestrate transcription factors (TFs) to induce defined myeloid sublineage differentiation programs is so far poorly understood. Lineage instruction by TFs can be demonstrated by over-expression or knock-down experiments. In addition, post-translational modifications such as phosphorylation of TFs by upstream protein kinases are key determinants of their function. MKK6 represents a direct upstream kinase of p38MAPK. The MKK3/6-p38 MAPK signalling cascade is induced by cytokine and immune receptor signals and is constitutively activated in monocytes (Mo) found in rheumatoid arthritis (RA) lesions. How p38MAPK is involved in orchestrating and relaying signal input to the downstream induction of gene activation programs in myeloid cells is poorly defined. We used a retroviral Tet-on system to express genes in primary human hematopoietic cells, and analyzed concomitant cellular and molecular changes. In particular, we asked for the consequences of MKK6-p38 activation in G-CSF-dependent late-stage granulocytes (G) and in immature dendritic cells. We found that conditional induction of dominant-active-MKK6 redirects G-CSF-induced G differentiation to Mo-like cells. Furthermore, conditional induction of MKK6 in immature DCs was sufficient to induce DC maturation. We further analyzed downstream mechanisms in MKK6-induced G to Mo lineage conversion. We observed a rapid p38-dependant induction of Mo promoting TFs c-Jun and concommitant reduction of G lineage associated TF C/EBPα in response to induced MKK6. These changes were followed by the upregulation of MafB and KLF4 as well as by the downregulation of Gfi-1. DOX-titration and pulse chase experiments revealed that G to Mo lineage conversion required low expression levels of MKK6 as well as only a short-term expression of MKK6 (6 h). RA-joint lesional proinflammatory cytokines similarly induced MKK6 in G associated with Mo lineage conversion. *in vivo* validation experiments revealed that Lactoferrin+ late-stage neutrophils from G-CSF-mobilized blood but not from normal blood could be redirected by proinflammatory cytokines to undergo Mo differentiation. Furthermore, these cells could be driven to acquire osteoclast features *in vitro*. Therefore, MKK6-p38 activation by cytokines, microbial products, and stress signals might alter the lineage differentiation of late stage G to monocytic cells. Furthermore, left-shifted Lactoferrin+ G might contribute to the pool of monocytic cells in inflammatory lesions.

1114

THE -509C/T POLYMORPHISM OF TRANSFORMING GROWTH FACTOR-B1 IS ASSOCIATED WITH INCREASED RISK FOR DEVELOPMENT OF CHRONIC IDIOPATHIC NEUTROPENIA

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Background. Chronic Idiopathic Neutropenia (CIN) is a disorder of granulopoiesis characterized by increased apoptosis of bone marrow (BM) granulocytic progenitor cells. This impaired granulopoiesis has been associated with an inflammatory BM microenvironment consisting of pro-inflammatory cytokines and pro-apoptotic mediators such as tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1 and Fas-Ligand (Fas-L). The origin of these molecules is not entirely known. *Aim*. Because genetic polymorphisms of TNF-α, TGF-β1 and Fas-L have been associated with changes in cytokine production in a variety of disease states, we sought to investigate the frequency of TNF-α, TGF-β1 and Fas-L gene polymorphisms in CIN patients and explore their role in excessive cytokine/mediator production and their association with the development of CIN. *Design and Methods*. Fifty-seven patients fulfilling the previously defined diagnostic criteria of CIN and 60 healthy subjects were included in the study after informed consent. All subjects originated from Crete, a well-defined area with genetically homogeneous population. Genomic DNA was extracted from peripheral blood samples and genotype analysis for the detection of TNF-α -308G/A, TGF-β1 -509C/T, +869T/C, +915G/C and Fas-L -844T/C polymorphisms were evaluated using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) assay. Cytokine levels in serum and long-term BM culture (LTMBC) supernatants of patients and controls were evaluated by means of an enzyme-linked immunosorbent assay (ELISA). *Results*. The mutant allele T of TGF-β1 -509C/T polymorphism was found in higher frequency in CIN patients compared to controls (p=0.037). This difference reflected mainly the homozygosity for the allele T which was identified in a proportion of 14.0% of the patients versus 1.7% of the controls (p=0.012) whereas no statistically significant difference was demonstrated between patients and controls in the proportion of subjects with CC homozygocity (p = 0.3916) or CT heterozygocity (p=0.423). Compared to wild type genotype, the TT genotype was associated with

increased risk for CIN development (Odds Ratio:12; 95% Confidence Intervals:1.24-115.4; p=0.020) whereas no statistically significant association was demonstrated between the presence of CT genotype and the risk for development of CIN (p=0.593). Compared to healthy controls, patients with CT and TT genotypes displayed increased TGF-β1 levels in serum (p<0.0001 and p=0.0002, respectively) and LTBMC supernatants (p<0.0001 and p=0.0002, respectively) suggesting an association of the mutant allele T with increased levels of TGF-β1 in patients with CIN. No significant difference was found between patients and controls in the frequency of TNF-α-308G/A, TGF-β1 +869T/C and +915G/C, and Fas-L -844T/C polymorphisms. *Conclusions*. The TGF-β1-509C/T polymorphism is associated with increased risk for CIN and contributes to the pathophysiology of the disorder by inducing TGF-β1 overproduction. This is the first study providing evidence that genetic factors may predispose to CIN and may have a role in the pathophysiology of the disorder.

1114a

SUCCESSFUL PREGNANCY OUTCOME IN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA ON LONG TERM ECULIZUMAB

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Background. Paroxysmal nocturnal haemoglobinuria (PNH) is a rare disorder of haemopoietic stem cells resulting in chronic haemolysis and an increased thrombotic risk. During pregnancy, PNH frequently worsens, posing additional risk of morbidity and mortality to mother and fetus. Eculizumab is a terminal complement inhibitor that has been shown to reduce haemolysis, transfusion requirements, thrombotic events, and chronic kidney disease, while improving fatigue and quality of life in patients with PNH. Since pregnancy was an exclusion criteria in the clinical trials, its use in this setting has not been evaluated. *Aim*. The safety and efficacy of eculizumab in the management of PNH during pregnancy. We sought to review the data, to-date, including physician reported adverse events, PK/PD, and distribution of eculizumab, in patients with PNH who received eculizumab. *Methods*. Reports of pregnancy during the eculizumab clinical trial were reviewed for safety, duration of drug exposure during gestation, complications during and after pregnancy, and general health of the newborn. Additionally, two patients were followed who received eculizumab treatment either during the last trimester (started eculizumab at week 27) or throughout gestation to term. Maternal blood, cord blood and breast milk data and PK/PD analyses are presented. *Results.* We present data on 6 pregnancies in patients receiving eculizumab. 1 patient elected termination of the pregnancy and 3 withdrew from eculizumab between 4-16 weeks gestation, as pregnancy was an exclusion criteria in the clinical trials, and continued their pregnancies. All 3 patients delivered healthy babies without complications. One patient received eculizumab throughout her pregnancy. At week 28 and week 30 gestation, she experienced haemoglobinuria and abdominal pain just prior to her eculizumab dose. The 900mg dose interval was adjusted from 14 to 12 days and there were no further breakthroughs. She delivered a healthy boy at term. No eculizumab was detected in the cord blood or breast milk during the first week after delivery. One patient had IVF resulting in a twin pregnancy and started eculizumab at 27 weeks gestation. She also required higher doses of eculizumab to control her haemolysis and delivered healthy babies by elective caesarian-section at 35 weeks gestation. She developed a postpartum haemorrhage needing uterine artery embolisation due to retained products of conception. Cord blood analysis suggested eculizumab was at very low background levels. Both patients on eculizumab were also treated with LMW heparin throughout their pregnancy. *Summary.* Review of these 6 cases of treatment with the complement inhibitor eculizumab during pregnancy does not indicate an apparent safety signal. Further, the first evaluation of eculizumab treatment from conception to delivery in a patient with PNH treated with eculizumab indicates that the pregnancy was successful and eculizumab does not appear to cross the placenta nor is it secreted into breast milk. There were no congenital anomaly/birth defects and no thromboses reported. Eculizumab appears to be well tolerated during pregnancy in PNH and adds to the management during this high risk time but might need to be given at higher doses during the 2nd and 3rd trimesters.

Quality of Life

1115

WHAT HAS BEEN LEARNED FROM MEASURING PATIENT-REPORTED OUTCOMES IN CLINICAL RESEARCH OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES? A SYSTEMATIC REVIEW FROM 1980 TO 2008

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Background. Patient-Reported Outcomes (PROs) are increasingly reported in hematological research and they can potentially provide valuable information to further support clinical decision-making. PROs include a number of parameters (e.g. quality of life and symptom burden) which all have in common the characteristic of being self-reported by the patient. *Aims.* Quality of life (QoL) and other key subjective health status domains, such as fatigue, are very important in the treatment of patients with myelodysplastic syndromes (MDS), but there is currently lack of data regarding the amount of research conducted in this area and the possible added value of using PROs in clinical research (including randomized controlled trials-RCTs) of patients with MDS. The purpose of this work was to systematically investigate traditional clinical and PROs in prospective studies of patients with MDS. *Design and Methods.* A systematic review was performed, broadly following the Cochrane methodology on all prospective studies (including RCTs) having PROs as an endpoint (either primary or secondary) published between January 1980 and July 2008. Candidate articles were identified mainly by PubMed and the Cochrane library. Two reviewers independently assessed all studies to consistently evaluate their methodological quality according to a previously developed protocol reviewer. This included a number of methodological and statistical quality criteria such as PRO missing data documentation, timing of assessment, discussion of outcomes in terms of clinical significance and questionnaire used. Both PROs and traditional clinical outcomes were also systematically analyzed to evaluate their relevance for supporting clinical decision-making. *Results.* Nine prospective studies were identified, four of which evaluated PROs in a RCT setting and interestingly, all the studies were published after the year 2001. Four studies used the EORTC QLQ-C30 to evaluate QoL while five used the FACT-An to also focus on fatigue related problems, in all RCT studies PROs were used as a secondary endpoint. Six out of the nine studies included less than 100 patients thus limiting the interpretation of PROs. In addition to small sample sizes, methodological drawbacks were mainly identified in terms amount of missing data over time and lack of reporting of important details about the design of the PRO assessment. Out of the four RCTs including PROs, important evidence emerged from two studies comparing azacitidine (AZA) and decitiabine versus supportive care. There is robust indication that AZA provide beneficial effects in terms of fatigue, physical functioning, dyspnea, positive affect and psychological distress. Another study also provided a preliminary indication that decitibaine could provide some benefit in terms of QoL compared to supportive care alone. *Conclusions.* The study revealed the paucity of research in this area and some methodological limitations when designing and reporting PROs. However, PROs have become more relevant only in recent years in clinical research of patients with MDS and there is strong evidence of the potential role in providing key outcomes to further support clinical decision-making. Investigators are encouraged to include PROs in future trials of patients with MDS.

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PAIN AND EMOTIONAL DISTRESS IN LEUKAEMIA PATIENTS SINCE DIAGNOSIS AND DURING ALL PHASES OF THE DISEASE

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Background. The few available studies on leukaemia patients have so far addressed only the issue of physical pain and emotional distress in terminally ill patients. *Aim*. We used visual analogue scale (VAS), Edmonton Symptoms Assessment System (ESAS) and Hospital Anxiety and Depression Scale (HADS) to investigate the frequency of physical pain and emotional distress in patients with leukaemia, at 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, as controls. *Patients and Methods*. 53 patients with acute leukaemia (40 myeloid and 13 lymphoblastic; 36 male, 17 female; median age 50 years-range 32-72-) and 76 patients with solid cancer; 29 female, 47 male; median age 65 years-range 42-83-) were enrolled. The diagnosis of depression and/or anxiety was made with a score of 8 of 21 or more in the HADS questionnaire. In the ESAS analysis, depression and anxiety were considered present with a score of 2 of 10 or more. *Results*. According to the HADS score, during the induction phase, depression was reported in 21%, 36% and 33% of the patients, at diagnosis (time 0), at +15 and at discharge, respectively, while anxiety in 30%, 40%, 36% of the cases, at the same time intervals, respectively. Depression was reported in 32% while anxiety in 38% of all questionnaires, respectively, collected from patients at all time intervals during all clinical phases. According to the VAS score, during the induction phase, mild pain was reported in 39.5%, 27%, and 16.6%, while moderate to sever pain in 11.6%, 20.4% and 5.5% of the cases, at diagnosis, at +15 and at discharge, respectively. According to the ESAS score during the induction phase, depression was reported in 45%, 38.6% and 27.7% of the patients, at diagnosis, at +15 and at discharge, while anxiety in 46.5%, 40.4%, 41.6% the cases, at the same time intervals, respectively. ESAS showed a sensitivity of 57% and 74%, and a specificity of 67% and 67% for depression and anxiety, respectively, when all questionnaires are considered. In solid cancer patients, at diagnosis, mild to moderate to severe pain was reported in 38% of the cases. HADS score was positive for depression in 25% while for anxiety in 19.7% of the cases. ESAS score was positive for depression in 28.9% while for anxiety in 40.7% of the cases, showing a sensitivity of 63% and 86.6% and a specificity of 82% and 70% for depression and anxiety, respectively. *Conclusions*. Contrary to what is generally accepted, not only solid cancer but also leukaemia patients may have physical pain and suffer from emotional distress since diagnosis and during all phases of the disease, as revealed by HADS. ESAS may be a useful tool for the screening of depression and anxiety in leukaemia patients.

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QUALITY OF RANDOMIZED CONTROLLED TRIALS (RCTS) IN HEMATOLOGICAL MALIGNANCIES: WHAT WAS REPORTED VERSUS WHAT WAS DONE

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Background. RCTs are considered the *gold standard* for providing research evidence for interventions in healthcare. However, the validity and reliability of trial results are largely dependent on the study design, analysis and trial conduct. Poorly conducted and reported research seriously compromises the integrity of the research process, especially if biased results receive false credibility. *Aims*. To establish whether reporting of methods in haematological malignancies RCTs conducted by the NCI cooperative groups (CGs), which conducts all publicly sponsored RCTs in cancer in the US, reflect the actual methodological quality. *Design and Methods*. We compared data on methodological quality reported in publications and research protocols. Data were extracted on methodological domains acknowledged as important for minimizing bias in the conduct and analysis of RCTs. We also extracted data on measures that are important to minimize play of chance namely, expected effect size, sample size calculations, α and β error. *Results*. Altogether, between 1955 and 2000, 4 CGs under the ageis of NCI conducted 120 hematological malignancies RCTs enrolling 37, 845 patients. Allocation concealment was adequate in 91% (109/120) of trials but reported only in 18% (21/120) of publications. A priori sample size calculations were performed and reported in 100% of trials. Expected difference in outcome was stated in 98% (118/120) of the trials while it was reported only in 39% (47/120) of the papers. α and β errors were defined and prespecified in 92% (110/120) and 94% (113/120) of the trials but reported in 30% (36/120) and 37% (36/120) of trials, respectively. An intention to treat analysis was done in 98% (118/120) of trials but clearly reported in 23% (27/120) of the trials. *Conclusions*. Reporting of methodological aspects of randomised controlled trials does not necessarily reflect the conduct of the trial. However, to the extent that published records affect the decision-making of physicians and policy makers, our study indicates the need for urgent measures to improve the reporting of RCTs in hematological malignancies.

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'PAINLESS' BONE MARROW ASPIRATION BY SLOW SUCTIONING DO NOT AFFECT PERIPHERAL BLOOD CONTAMINATION TO THE SPECIMEN: RANDOMIZED PROSPECTIVE CROSSOVER TRIALS

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Background. The pain during bone marrow aspiration (BMA) can be best explained by negative pressure inside the bone marrow cavity. Although most standard textbooks recommend rapid suctioning with high negative pressure to minimize peripheral blood contamination, this technique might be associated with more pain. To date, there is no evidence that rapid suctioning can reduce peripheral blood contamination. *Aims*. To find a technique that reduces pain without loss of specimen quality, we conducted two trials. *Design and Methods*. The first was a randomized, crossover trial comparing slow aspiration with low negative pressure (L method) and rapid aspiration with high negative pressure (H method). To evaluate pain during the procedure, 49 patients with hematological malignancy who required BMA more than once were randomly assigned to receive either the first aspiration by the L method and the second by the H method or vice versa. Under local anesthesia, a 16-gauge BMA needle was inserted into the BM cavity. A 1-mL syringe (L method) or a 30-mL syringe (H method) was used to aspirate 0.5 mL of BM fluid. To ensure accuracy of aspiration volume in the H method, an extension tube was connected between the 30-mL syringe and needle, and clamped by forceps when the aspirated volume reached 0.5 mL. Pain during each procedure was evaluated using a visual analog scale (VAS), verbal descriptor scale (VDS), and McGill pain questionnaire (MPQ). In the first trial, repeated measures one-way analysis of variance design was used for serial measurement of pain, and the one-tailed Wilcoxon's test was used to compare pain between the two groups. The second trial compared aspirate quality between the L and H methods. Sixteen allogeneic transplantation donors (median age, 33.5 years; range, 22 to 62 years), underwent the first puncture of the harvest procedure under general anesthesia. BMA was drawn simultaneously from the right and left posterior iliac crest with a 1-mL and a 30-mL syringe; the aspiration side and syringe size were randomized. Nucleated cell counts (NCC) of the BM specimens were determined by automated hematology analyses. The non-inferiority margin was defined as less than 25% on the basis of the absolute difference of NCC between the L and H methods. Informed consent for the procedures was obtained from all donors and patients. *Results*. In the first trial, 34 of the 49 patients (67%; 21 men and 13 women; median age, 49 years; age range, 17 to 77 years) completed the study and provided VAS, VDS and MPQ score data after two consecutive BMA. Present pain intensity (PPI) score of the MPQ was significantly decreased with L method when compared with H method (median absolute difference 0, p=0.037) (Table 1). VAS score and total pain rating index (PRI) of the MPQ were also lower with the L method (median absolute differences -0.6 and -0.5), although these results did not reach statistical significance. There was no significant ordinal interaction in repeated measurement of any scores except the

affective-PRI of the MPQ, which was significantly higher at the first aspiration, regardless of the negative pressure intensity (p=0.024). In the second trial, the geometric mean of NCC with the L method was non-inferior to the mean with the H method (p=0.025) (Table 2). *Summary and Conclusions*. These findings indicated that BMA with low negative pressure tended to be less painful, while maintaining specimen quality. There remains difficulty in evaluating the impact of psychological factors on the results. Nonetheless, on the basis of our results we recommend suctioning slowly with low negative pressure using a small syringe in order to reduce pain during BMA.

Tables 1 and 2.

Table 1

	L-method median (range)	H-method median (range)	Absolute difference (95%CI)	P-value
VDS	1 (0 to 3)	1 (0 to 3)	0 (0 to 0)	0.212
VAS	2.2 (0 to 8.4)	3.2 (0 to 9.0)	-0.6 (-1.8 to 0.2)	0.055
MPQ				
PRI-S	3 (0 to 16)	4 (0 to 30)	0 (-3 to 1)	0.080
PRI-A	0 (0 to 7)	0 (0 to 9)	0 (0 to 0)	0.192
PRI-E	0 (0 to 4)	0 (0 to 4)	0 (0 to 0)	0.414
PRI-M	2 (0 to 11)	3 (0 to 11)	0 (-2 to 0)	0.093
PRI-T	6 (0 to 32)	7.5 (0 to 53)	-0.5 (-3 to 0)	0.063
PPI	1 (0 to 4)	1 (0 to 5)	0 (-1 to 0)	0.037

Table 2

	L method	H method	P-value
Genomic mean of NCC ($x10^4/\mu L$) (95%IC)	6.6 (4.6 - 9.5)	7.8 (5.9 - 10.4)	0.025

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RISK FACTORS FOR ATTEMPTED SUICIDE AND SUICIDE FOLLOWING A DIAGNOSIS OF HEMATOLOGICAL MALIGNANCY

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Background. It has been well documented that patients with a diagnosis of cancer are at an increased risk of committing suicide. Male gender, head and neck and lung cancer have been associated with a higher risk of suicide. Psychiatric co-morbidity is also an established risk factor for suicide. However, there is a paucity of literature on the risk of suicide in patients with hematological malignancies *Aims.* To define the incidence and risk factors of suicide and suicide attempts in patients with a broad range of hematological malignancies. *Design and Methods* Through the Swedish Cancer Registry we identified 47,220 patients (54.5% men) diagnosed with a hematological malignancy between 1992-2006. For each patient, five population-based controls (n=235,868) matched by gender and year of birth were chosen randomly from the Swedish Population base. Information on suicide was obtained from the Cause of Death Registry and admissions due to suicide attempts and psychiatric illness from the Swedish Hospital Discharge Register. History of psychiatric illness was measured as having at least one admission with a psychiatric diagnosis prior to the malignancy, or the corresponding time for the controls. Cox-regression was used to analyze the risk of suicide/suicide attempt after a diagnosis of a hematological malignancy. Results are presented as hazard ratios (HR) with 95% confidence intervals (CI). *Results*. The HR for suicide/suicide attempt in patients with hematological malignancies was approximately two times elevated during the first three years after diagnosis, (HR=1.9, CI:1.5-2.3). The overall excess risk was modified by time since diagnosis and no increased risk was seen when three years had elapsed (HR=1.1, CI:0.9-1.4). An analysis only including suicide showed identical risks, HR=1.9 (CI:1.3-2.8) during the first three years after diagnosis, and HR=1.2 (CI:0.8-1.8) after 3 year of follow-up. The overall excess risk associated with malignancy was not significantly modified by age, gender, calendar year or type of hematological cancer. However, data suggests that patients diagnosed up to 1998 were at higher risk (HR=2.2, CI:1.7-2.9) compared to those diagnosed after 1998 (HR=1.6, CI:1.2-2.1). Multiple myeloma was the malignancy associated with the highest estimated risk (HR=3.4, CI:2.3-5.0). In an analysis of the joint effect of malignancy and history of psychiatric illness these factors were shown to interact multiplicatively (Figure). Compared to controls without psychiatric illness, the following risks were seen for the first three years of follow-up: cases without psychiatric illness HR=1.8 (CI:1.4-2.3); controls with psychiatric illness HR=10.8 (CI:8.8-13.2); cases with psychiatric illness HR=23.3 (CI:16.6-32.6). *Conclusions.* Hematological malignancies are associated with an increased risk of suicide and attempted suicide during the first three years after diagnosis. This risk is further increased in patients with a history of psychiatric illness. Patients with multiple myeloma carry the highest estimated risk. These findings have implications for the care and treatment of patients with hematological malignancies.

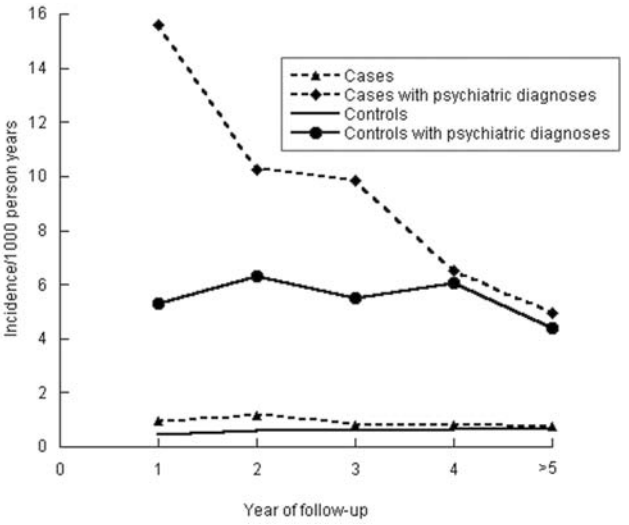

Figure.

Transfusion medicine

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ANIMAL MODELS FOR THE TWO HYPOTHESES OF TRALI PATHOLOGY

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Background. Transfusion-Related Acute Lung Injury (TRALI) is a life-threatening complication of blood transfusion. Patients with TRALI present a rapid onset of respiratory failure that typically arises within two hours of the start of blood transfusion, with full presentation of the reaction occurring within six hours. Among the possible molecules triggering TRALI are leukocyte antibodies, present in fresh frozen plasma and platelet concentrates, and neutrophil priming agents released in stored cellular blood components. However, the pathogenesis of TRALI remains controversial. Two proposed pathophysiologic mechanisms for TRALI have received the most attention: the one-event and the two-event hypotheses, both leading to a final common pathway resulting in lung edema. *Aims*. Our aim was to develop mouse models of TRALI that simulated both hypotheses. In the first model we evaluated the role of human neutrophil-specific antibodies anti-HNA-2a in NOD/SCID mice as the single event causing TRALI. The two-event model was developed through the transfusion of Platelet Activating Factor (PAF) in Balb/c mice already primed with endotoxin. *Design and Methods*. In the first model (antibody model) we evaluated the *in vitro* effect of the interaction between human neutrophils and anti-HNA-2a monoclonal antibodies through the production of nitric oxide, hydrogen peroxide, cytokines and adhesion molecules expression. To the *in vivo* assays, human neutrophils expressing HNA-2a antigen, treated or not with anti-HNA-2α antibodies, were injected in NOD/SCID immunodeficient mice. Six hours after cells injection, mice were sacrificed and TRALI was determined by Evans blue dye leak in the bronchoalveolar lavage (BAL), cell influx, cytokines and chemokines (IL-1α, KC, MIP-2 e TNF-α) concentrations in the lung homogenate and the lung histology. In the second model (two-event model), Balb/c mice were treated (primed) or not with LPS and after two hours were i.v. inoculated with PAF. The animals were sacrificed 40 min after and the parameters analyzed were the same used for the first model. *Results*. In the one-event model, we detected an *in vitro* increase of IL-8 production and CD11b, CD18, CD54 e CD66b intensity of expression compared to control. In addition, we observed an *in vivo* increase of the vascular permeability and cell influx, measured by Evans Blue dye leak and lung MPO content, respectively, compared to control. These results were confirmed by histological analysis, which revealed intense vascular congestion neutrophil infiltrates in lungs. There were no significant differences on IL-1 and KC production, but we observed an increase on MIP-2 levels. Regarding to the two-event model there was a significant increase of the cell influx and lung permeability only in animals treated with LPS plus PAF. LPS+PAF treatment induced significant increased levels of TNF-α and MIP-2. Histological analysis revealed inflammatory infiltrates in animals treated with PAF and LPS+PAF. 5. *Conclusions*. The animal models described here support both theories of TRALI, the one and two-event, and demonstrated the essential role of neutrophils to inducing this syndrome. As observed in lungs of both models, the cellular infiltration and histopathological analysis corroborate with the arise of pulmonary permeability, what is similar to human TRALI.

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AN ACTIVE HEMOVIGILANCE PROGRAM TO CHARACTERIZE THE SAFETY PROFILE OF 16,631 PLATELET COMPONENTS PREPARED WITH PHOTOCHEMICAL PATHOGEN INACTIVATION TREATMENT TRANSFUSED IN ROUTINE CLINICAL PRACTICE

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Background. Inactivation of pathogens and leukocytes in platelet components using amotosalen and UVA light (INTERCEPT') is in routine use in many European blood centers. An active hemovigilance (HV) program was implemented to characterize and extend the safety profile of INTERCEPT Platelets (I-PLT) in routine use. *Aims*. This report summarizes 16,631 I-PLT txn administered to 3,274 pts at 17 different sites in 8 different countries and provides a safety profile of I-PLT transfused in routine practice to a broad patient population. *Design and Methods*. Apheresis or buffy coats platelet components were leukoreduced, suspended in ~35% plasma and 65% InterSol', treated with the INTERCEPT and stored for up to 7 days depending on respective national regulations. INTERCEPT treatment replaced bacterial screening at all sites and γ irradiation for 97.5% (16,325/16,631) of I-PLT txn. Blood centers using INTERCEPT Platelets completed a data form after each txn regardless of whether a reaction occurred. The focus of these studies was response to txn within the first 24 hr regardless of outcome; and there was no time limit on reporting. A common INTERCEPT Hemovigilance Transfusion Report Form was utilized for all 3 studies. Investigators recorded: patient (pt) demographics, primary diagnosis and therapy, and type of I-PLT product. For each txn, the following data were collected: time of adverse event following txn, clinical description of event, objective clinical parameters (vital signs), clinical and laboratory data (radiographs, bacterial cultures), event severity (grade 0-4), serious or non-serious classification, and causal relationship to txn (unrelated, probably unrelated, possibly related, probably related, or related). *Results*. From October 2003 to the present, data from 16,631 I-PLT txn administered to 3,274 pts (60.3% males/ 39.7% females) have been collected. The majority of the recipients were hematology/oncology patients (1,643, 50.2%) many of whom received hematopoietic stem cell transplants s (n=307). The majority of I-PLT were administered in non-intensive care hospital units (13,152, 79.1%), and the remainder were transfused in intensive care units (2,496, 15%) and outpatient clinics (981, 5.9%). Transfusions associated with "related" (possibly related, probably related, or related) adverse events following I-PLT txn were infrequent (110/16,631=0.66%). Eighty-two pts (2.5%) experienced at least one related adverse event following one or more INTERCEPT txn. Most reactions were mild and of grade 1 severity and were representative of the events expected with conventional PLT txn. The most frequently reported signs/symptoms were chills, fever, and urticaria. Eleven SAE's were reported, with one having causal relationship (hypotension possibly related) to I-PLT txn. No cases of Transfusion Related Acute Lung Injury (TRALI), TA-GVHD, transfusion related sepsis or death due to an INTERCEPT txn were reported. *Conclusions*. In this program, 99.34% of I-PLT administrations were without a related txn reactions. Adverse events following I-PLT txn classified as related to txn were infrequent, mild in severity, and representative of the events expected with PLT txn. We believe that the use of an HV program to capture ongoing safety information is a valuable tool to characterize the safety of I-PLT.

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UNDERSTANDING AND MEETING CLINICAL PLATELET REQUIREMENTS: AN ANALYSIS OF INDICATIONS AND URGENCY OF PLATELET USE FROM THE PROSPECTIVE UTILISATION OF PLATELETS AND PLASMA (PUPPY) STUDY

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Background. Meeting clinical requirements for platelet products requires an understanding of usage, in order to plan for evolving demand. Similarly, ensuring availability during emergencies may require triaging transfusion by restricting supply to clinically urgent cases or deferring elective surgery. However, few data are available to inform supply and contingency planning. *Aims*. To determine indications and urgency of platelet transfusions in Victoria, Australia *Design and Methods*. A random sample survey was performed with methods adapted from the Bloodhound study of red cell utilisation. From August 2008 to February 2009, 1257 platelet units (753 pooled, 476 apheresis, and 7x4 Paediatric Apheresis) were randomly tagged with the case report form (CRF) at production and distributed as usual. When issued for transfusion CRFs were completed by the issuing hospital scientist. *Results*. Interim analysis of the first 706 (56.2%) returned CRFs is presented. 447 (63.3%) were issued for transfusion; 243 (34.4%) expired at hospitals prior to use; 16 (2.2%) recalled or other disposal. Clinical conditions requiring transfusion included haematology/oncology in 287 cases (64.2%; this comprised 118 acute leukaemias [26.4% of total], 43 lymphoma [9.6%], myeloma 25 [5.6%], benign haematology 25 [5.6%] other malignancies 74 [16.6%], platelet disorders 2 ([0.4%]), cardiothoracic surgery 46 (10.3%), urolog-

ical surgery 22 (4.9%), gastroenterology 17(3.8%), solid organ transplant 13 (2.9%), trauma 11 (2.5%) and other or unknown in 51 (11.4%). In 118 cases (26.4%) transfusion supported surgery or an interventional procedure (78 major procedures [66.1%], 20 minor [21.2%], 10 line insertion [8.5%], 10 other or unknown [8.5%]); 37 (31.4%) of these procedures were elective. Clinical urgency of transfusion was acute (required in <1 hour) in 69 (15.4%); urgent (<24 hours) in 288 (64.4%); semi-urgent (<1 week) in 77 (17.2%) and unknown in 13 (2.9%); no transfusions were deferrable for over 1 week. Median platelet count prior to transfusion was $18x10^9$/L (IQR 11 - 45); median platelet count for non-surgical patients $15x10^9$/L, and for surgical patients $67x10^9$/L where reported, and median platelet doses issued was 1 (IQR 1 - 2). The recipient was actively bleeding in 106 cases (23.7%); febrile or septic in 112 (25.1%); receiving anti-platelet agents in 12 (2.7%) and undergoing cardiac bypass or extra-corporeal membrane oxygenation in 40 (8.9%). *Conclusions*. Clinical platelet usage is highly concentrated in specialised areas, predominantly to support patients with haematological and malignant disorders, those undergoing major surgery, and the critically ill. Changes in clinical practice in these areas may have a substantial effect on supply requirements. High levels of urgent transfusion and relatively low numbers of transfusions for elective surgery seen here, demonstrate that in a shortage conventional triage strategies would have little impact on requirements. Additional strategies are required to ensure continued adequacy of supply.

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A SELF-OPERATING PRETRANSFUSION BLOOD SAMPLING DEVICE FOR ENHANCED TRANSFUSION SAFETY: PROSPECTIVE PHASE II TRIAL IN EMERGENCY CARE.

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Although blood safety with respect to infectious complications has reached very high standards, delayed or incorrect blood component administration due to human error still represents the greatest transfusion-associated risk. Pretransfusion blood sampling (PTBS) is a crucial upstream checkpoint in the process chain. In the setting of emergencies or disasters, infusion of colloidal plasma expanders and type 0 Rhesus negative red blood cell (RBC) concentrates potentially interfering with blood typing due to red cell pseudoagglutination or mixed-field agglutination, respectively, in theory ask for collection of the very first blood sample along with IV placement at the site of emergency. Vasoconstriction from hemorrhagic shock and unknown patient identity, however, are factors obviating PTBS. Retrospective analysis of 22 patients presenting with WHO grade 4 hemorrhage to a tertiary care emergency center revealed that the PTBS was performed in no instance at the site of trauma. The median time from admission to completion of blood typing was 26 minutes (range 13-61). 3 out of 7 severely traumatized and massively transfused patients suffering fatal outcome within 24 hours following admission had no pretransfusion sample taken necessitating transfusion with type 0 RBC concentrates throughout treatment. In one incidence, lack of PTBS contributed to delay in transfusion and death from hemorrhage. In order to facilitate PTBS at the scene of primary care a new IV catheter system was invented with an integrated blood reservoir containing EDTA anticoagulans. A flexible plastic band holds the reservoir attached to the patient and exerts a forcing function in a way that self-filling of the reservoir precedes intravenous catheter placement. Upon arrival at the hospital, the reservoir is labeled, disconnected from the patient and immediately sent to the laboratory for blood typing. A prototype version was tested in a randomized controlled phase II clinical trial. Outpatients with traumatic or internal hemorhage, and patients requiring emergent blood typing for other indications (n=90) were randomized to receive IV access using standard IV catheters or the new system. Handling of the self-sampling catheter was rated comparable to the standard in 90% of treatments by emergency physicians. Blood volumes collected with the emergency IV catheter were sufficient to allow for determination of red cell antigens including Rhesus subtypes, isoagglutinins, and irregular antibodies in 100%, 97%, and 60% of cases, respectively. Repeated blood typing from two truly independent venipunctures under emergency conditions occured in 5/6 patients needing immediate transfusions in the experimental arm as compared to 1/11 patients in the control group. The emergency catheter reduced the mean door-to-blood-type-time from 22.5 min (range: 13-121; n=45) to 15 min (range: 7-24; n=45; $p<0.001$).). In conclusion, the novel emergency IV catheter system has the potential to preserve pretransfusion blood sample unaffected by emergency medication at the scene. It thereby can improve transfusion safety by accelerating pretransfusion serology and by avoiding side effects from immunization and mistransfusion, in particular if targeted to areas where treatment is complex and urgent. It may help to protect the supply with valuable type 0 RBC concentrates in emergency and disaster management.

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EVALUATION OF THE CLINICAL SIGNIFICANCE AND RISK FACTORS FOR INTRA-VENOUS IMMUNE GLOBULIN INDUCED HAEMOLYTIC REACTIONS.

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Background. Hemolysis has been described as a complication of the use of Intravenous Immune Globulin (IVIG). In a report distributed in October 2005 by the IVIG manufacturer Talecris, and endorsed by health Canada, the estimated haemolytic reaction rate was 0.04%. Few other data described the average incidence and possible predisposing factors, and to date, there are no special recommendations to prevent this potentially serious complication. We describe our experience over the last 4 years and analyse the possible predictors of IVIG induced haemolytic reactions. *Patients and methods*. From January 2005 through November 2008, all patients receiving IVIG at the Sherbrooke University Hospital Center were identified, and all patients reported by our internal blood-product surveillance system to have had secondary hemolysis were analyzed. Hemolysis secondary to IVIG was defined as a reduction of haemoglobin with elevation of bilirubin and lactate deshydrogenase and appearance of a positive direct antiglobulin test (DAT) and/or a positive eluate and/or a positive screening for not previously diagnosed allo-antibodies. The hemolysis wasn't explained by other illness. Data collected included patient's demographic data, blood groups, clinical indication for IVIG, type of IVIG, total dose and administration schedule, documentation of IVIG-induced hemolysis and other possible causes that may obscure diagnosis, Hemoglobin (Hb) level changes over the period following administration of IVIG, DAT post IVIG and anti-erythrocyte antibodies specificities. Other biological parameters including serum lactate dehydrogenase, bilirubin, reticulocyte count, haptoglobin, C-reactive protein, ferritin, fibrinogen, albumin and creatinine were also analysed. *Results*. Over a period of 46 months, 359 patients received IVIG in our center. Secondary haemolytic reactions were reported for nine patients (2.5%), 2 males, 7 females, age range 0.7 - 86 years. 3 patients were treated for immune thrombocytopenia, 3 for Kawasaki syndrome, and the others were treated for guillian-barre syndrome, adult-onset still's disease and chronic inflammatory demyelinating polyneuropathy. The average total received IVIG dose was 3.4 g/kg body weight (1.67-6). The average decrease of haemoglobin level was 4.7 g/dL (1.4-6.6), and the average time to Hb nadir was 10.7 days (5-22). Six patients were of blood group A and three were of blood group AB. Eight patients presented positive DAT at the time of hemolysis, and all patients presented anti-A antibodies. One patient of blood group AB presented additional anti-D and anti-E antibodies, and another patient of the same blood group presented additional anti-B antibodies. There was no clear correlation with other analyzed biological parameters including inflammatory serologic markers. *Conclusion*. Our results underline the significance of post IVIG hemolysis in clinical practice. The reported incidence goes with two other recent studies presented late 2008, and reporting an incidence of 1.6 and 4%. This incidence is much higher than the previous data reported in 2005. In this study, patients of blood group A or AB, female patients, and patients receiving high dose IVIG seem to be at higher risk.

Publication only

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PROLIFERATIVE POTENTIAL OF HUMAN MESENCHYMAL STROMAL CELLS

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Background. Mesenchymal stromal cells (MSCs) are multipotent adherent stromal cells, initially isolated from bone marrow (BM), able to differentiate along multiple cell lineages, such as osteoblasts, adipocytes and chondrocytes. Ability of MSCs to self-renew is not proved for human. *Aims*. The aim of the study was to investigate the proliferative potential of human donors MSCs, marked by lentivector, and of MSC from patients with aplastic anemia (AA). *Design and Methods*. BM cells were obtained from donors and AA patients after their informed concern, then 10^5 nucleated BM cells were seeded per cm^2 of culture flask. In 12-15 days confluent monolayer was treated with 0.05% trypsin+0.02 mM EDTA and the cells were reseeded at the density of 4500 per cm^2. The multilineage potential of MSC was evaluated on 1-4 passages by differentiation under proper stimuli. In 2-4 hours after first passage donors MSCs were infected with concentrated (10^8 viral particles/mL) self-inactivating (SIN) HIV vector harboring EGFP under the control of PGK promoter with polybrene for 6 hours. Adherent cell layers (ACLs) of 2-week old long-term BM cultures (LTBMCs) were infected with the same viral stock overnight. To clone MSCs, 1, 2 or 10 cells were seeded per well of 96-well plate. Irradiated with 40 Gy, MSCs were used as a feeder (1000 cells/well). ACL cells were trypsinized and plated 10000 cells/well of 96-well plate 2 weeks after the infection. *Results*. Cell growth kinetics did not differ in GFP-positive and negative MSCs. The number of marked MSCs slightly decreased during passages. The same is true for stromal cells from LTBMCs. The cloning efficiency of GFP-negative MSCs did not differ from GFP-positive (13.7±6.9% versus 14.5±7.0%). All MSCs clones were able to reach the confluence in the well of 96-wells plate after the cloning procedure, and then undergo 5 mitosis, but only 1 out of 22 clones was able to divide once more. Addition of bFGF to cultures did not increase the proliferative potential of MSC. Stromal cells from ACLs showed approximately the same proliferative capacity as MSCs independent of GFP expression. MSC from AA patients and healthy donors did not differ either. Time needed to form confluent monolayer from the initially seeded nucleated BM cells considered as passage zero (Po) was approximately the same for AA and donor cultures, moreover time to each next passage did not differ significantly. Cumulative cell production was also similar in both types of cultures for the first 6 passages. At that point donor MSC almost ceased growth; while AA MSCs continued to proliferate and were able to undergo at least 6 more passages, thus doubling total cell production. This phenomenon could be a consequence of increased bFGF expression in AA MSC when compared with donors (relative expression level in cultures from AA patients was 0.78±0.15, in cultures from donors 0.55±0.09). *Conclusions*. The data suggest that human MSCs have high but limited proliferative potential and are not able to self-renew in culture. MSC of AA patients proliferated longer than donor ones.

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SELF-RENEWAL EMBRYONIC GENES EXPRESSION IN ADULT HUMAN MESENCHYMAL STEM CELLS

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Background. Adult human mesenchymal stem cells characterised by the expression of CD29, CD44, CD73 (SH3), CD90 (Thy-1), CD105 (SH2) and CD166 (ALCAM) were identified in 8 distinct adult tissues including bone marrow, mobilised peripheral blood, arterial endothelium, menstrual blood stroma, adipose tissue, eye conjunctiva and limbal stroma, lung bronchial fibroblasts and exocrine pancreas ductal epithelium. The human mesenchymal stem cells from these adult tissue sources undergo 40-70 doubling populations and differentiate into chondrocytes, osteoblasts and adipocytes in defined culture conditions. Recently, human mesenchymal stem cells from foetal (liver, haematopoietic tissues and amniotic fluid) and neonatal (amniotic epithelium and fluid, placenta decidua, umbilical cord blood and vien) tissues were shown to express key embryonic genes including Oct-4, Nanog-3, Rex-1, Sox-2, SSEA-4 and Bmi-1. *Design and Methods*. Therefore, the expression of self-renewal embryonic genes: Oct-4, Nanog-3, c-Kit and Rex-1 in human mesenchymal stem cells from the 8 adult tissue sources: bone marrow, mobilised peripheral blood, arterial endothelium, menstrual blood stroma, adipose tissue, eye conjunctiva and limbal stroma, lung bronchial fibroblasts and exocrine pancreas ductal epithelium was evaluated using immunoflourescence and immunocytochemistry. *Results*. Immunoflourescence and/or immunocytochemistry staining techniques in the presence of both positive and negative controls revealed the expression of Oct-4 and Nanog-3 in human mesenchymal stem cells from all 8 adult tissue sources, The c-Kit expression was found mainly in in bone marrow-, mobilised blood- and menstrual blood stroma-derived mesenchymal stem cells whilst Rex-1 in bone marrow- and eye conjunctiva stroma-derived mesenchymal stem cells. *Conclusions*. Adult human mesenchymal stem cells possess key self-renewal embryonic genes including Oct-4, Nanog-3, c-Kit and Rex-1 that play crucial role in maintaining their stemness to provide for cell regeneration and repair throughout the years of adult human life.

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BONE MARROW ENDOTHELIAL CELLS IN CHILDHOOD MALIGNANCIES AND BLOOD DISEASES

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Background. Endothelial progenitors cells (EPCs) are a heterogeneous subpopulation of bone marrow mononuclear cells, can differentiate into mature endothelial cells, circulate in the bloodstream, participate in endothelial homeostasis and stimulate the formation of new blood vessels. An increasing interest for their role in angiogenesis of ischemic tissues, tumor microenviroments and the course of hematologic malignancies is recently present. *Aims*. The study of bone marrow progenitors and mature endothelial cells (ECs) of children with blood diseases and cancer as well as the detection and characterization of the immunophenotype of their subpopulations. *Design and Methods*. Bone marrow cells from children with acute lymphoblastic leukemia (ALL) at diagnosis (ALL d, n=8), ALL high risk group under consolidation therapy (ALL HR, n=14), ALL during maintenance (ALL M, n=9), solid tumors at diagnosis without bone marrow involvement (ST, n=13) and children with idiopathic thrombocytopenic purpura (ITP, n=15) were studied. The determination of the putative antigenic phenotypes of EPCs and mature endothelial cells was performed by flow cytometry (Epics Elite Coulter) with the following combinations used: $CD133^+/VEGFR\text{-}2^+$, $CD34^+/VEGFR\text{-}2^+$, $CD34^+$ /VEGFR-2^+ /CD133-, $CD34^+/CD31^+/CD133$, $CD34^+/CD31^+$/ VEGFR-2^+, $CD34^+/CD146^+/CD31^+$, $CD34^+$/ $CD146^+$, $CD146^+/CD31^+$. The differences between groups were evaluated with the non-parametric Mann-Whitney test using the SPSS 16.0 program. *Results*. Endothelial progenitors and mature cells are adequately detected in the bone marrow by flow cytometry. The lower levels of $CD133^+/VEGFR\text{-}2^+$ endothelial progenitors were estimated at ALL diagnosis with statistical significant differences with the ALL -R (0.067±0.016 vs 0.37±0.1, p=0.030), ALL M (0.067±0.016 vs 0.39±0.17, p=0.029) and the solid tumors' group (0.067±0.016 vs 0.64±0.24, p=0.011) in which the highest value was determined. The solid tumors without bone marrow involvement has also the highest levels of CD34+/VEGFR-2+ endothelial cells with statistical difference comparing with the ALL HR (0.33±0.08 vs 0.2±0.08, p=0.026)and ITP (0.33±0.08 vs 0.14±0.037, p=0.007) patients in which group the lowest levels were estimated. The comparison between the ST and ALL HR revealed higher levels in the ST group of the $CD34^+/VEGFR\text{-}2+$ (0.33±0.08 vs 0.2±0.08, p=0.026), $CD34^+/VEGFR\text{-}2^+/CD133-$ (0.16±0.038 vs 0.07±0.019, p=0.047) and $CD34^+/CD31^+/CD133-$ cells (5.02±0.71 vs 1.69±0.34, p=0.001). Between the ST and the ALL M there was no statistical correlation for any endothelial cell subpopulation. The levels of $CD34^+/CD31^+/CD133-$

mature ECs were higher in ITP comparing with the ALL HR(4.36±0.7 vs 1.69±0.34, p=0.003). In the ITP group the lowest levels of the CD34+/VEGFR-2+/CD31+ (0.092±0.034 vs 0.28±0.096, p=0.003) and CD146+/CD31+ (0.45±0.096 vs 0.94±0.28, p=0.046) ECs were estimated and showed significant difference compared with the ALL M. *Conclusions*. A significant decrease of EPCs is evident at diagnosis of ALL, which is reversed in remission. In the ST group without bone marrow involvement the highest values of progenitors and mature endothelial cells are detected without statistical difference compared with ALL M. The role of these findings in the pathogenesis and course of the diseases has to be further investigated.

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THE WASHOUTS OF DISCARDED BONE MARROW COLLECTION BAGS AND FILTERS ARE A VERY ABUNDANT SOURCE OF HMSCS EXPANDED IN GMP CONDITIONS

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Background. Human Multipotent Mesenchymal Stromal Cells (hMSCs) are considered good candidates for a growing spectrum of cell therapies. We have validated, in two different Cell Factories which operate in strict GMP compliance, a protocol for the production of very large numbers of hMSCs for therapeutic use which makes use of the washouts of normally discarded collection sets left over at the end of the filtration of the bone marrow explants performed for hematopoietic stem cell (HSC) transplantation. This protocol reduces manipulation to a minimum and limits the use of animal and non clinical-grade reagents. *Design and Methods*. Total nucleated cells (TNC) were isolated both from unmanipulated bone marrow aspirates and from washouts of discarded bags and filters, left over after the filtration of whole bone marrow explants which is routinely performed before infusion to patients. Cells were seeded without any manipulation in 5% human platelet lysate (hPL) supplemented α-MEM. After 48 hours nonadherent cells were removed and the adherent cells were expanded for 7-14 days with periodic feeding. The cells were then harvested and seeded at low density (100-200 cells/cm^2) and allowed to expand for additional 10-20 days. Finally the cells were harvested and frozen. *Results*. In a median of 26 days, 14 bags for adult patients and 9 bags for pediatric patients for the standard dose of 1x10^6 hMSCs/kg body weight could be prepared from the expansion of a fraction of the cells recovered from 7 independent washouts. Moreover, 151 vials could be frozen from the remaining cells. The theoretical full expansion of all the frozen vials (validated by the expansion of 2 independent vials) could have allowed the production of 173 bags for adults and 348 bags for pediatric patients. Clinical scale expanded hMSCs identity and purity were assessed by testing viability and expression of CD14, CD34, CD45, CD73, CD90 and CD105. Cytogenetic analysis and clonogenic assay in methylcellulose were performed and no chromosomal alteration or tumorigenic trasformation has been revealed. Bacterial, fungal, mycoplasma and endotoxin contamination were tested by validated tests according to European Pharmacopea guidelines and always found negative. *Conclusions*. The washouts of discarded bags and filters left over at the end of the routine bone marrow explants filtration are a very abundant source of hMSCs precursors which can be easily utilised to produce large numbers of hMSCs clinical bags ready to use under strict adherence to GMP procedures.

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MAY THE C-FOS TO BECOME A NEW COMMON MARKER FOR MESENCHYMAL STEM CELLS ?

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Background. The characteristics of mesenchymal stem cell (MSC) populations obtained from different organs are very similar, suggesting a closer relationship among them. However, a definitive molecular marker of MSCs remains obscure. The surface proteins such as CD29, CD44, CD90, CD105, CD106 and CD166 in particular, have been used to characterize MSCs. None of these markers are solely specific for MSCs. Transcriptor factor AP-1 (activator protein-1) consists of Fos proteins (c-Fos, Fos B and Fra 2), which are nuclear protein products of c-fos genes belonging to intermediate early genes (IEGs). AP-1 components are specific for some biological processes including cellular proliferation, differentiation, apoptosis and oncogenic transformation. Particularly, c-fos gene has been associated with the coordinated regulation of gene expression during cellular proliferation and differentiation. Therefore, we hypothesized that c-fos may be a good candidate to become a new common marker for MSCs. *Aims*. The objective of this study was to look for a new marker that might be used to characterize the mesencymal stem cells. To reach this objective, the expression of c-fos was analyzed in MSCs from human-derived bone marrow (hBM), cord blood (hCB), dental pulp (from impacted third molars and exfoliated deciduous teeth) (hDP), periodontal ligament (hPDL), adipose tissue (hAT), endometrium (hE), amniotic fluid (hAF) and placenta (hPL) by using RT-PCR and immunocytochemistry. *Design and Methods*. The expressions of CD10, CD11b, CD13, CD14, CD15, CD19, CD33, CD34, CD44, CD45, CD73, CD90, CD117, CD146, CD166, and HLA-DR were analyzed in isolated MSCs from various human derived- organ or tissues by using flow cytometry. Similarly, these cells were analyzed for CD31, CD34, CD44, CD71, CD105, vimentin, fibronectin, β-tubulin, α smooth muscle actin (α-SMA), actin, desmin, and c-fos by using immunohistochemical/immunofluorecence techniques. Total cellular RNA isolated from human derived- MSC cultures (from passage 3) was reverse transcribed the resultant cDNA was used in PCR with the following primers: c-fos forward: AGAATCCGAAGGGAAAGGAA; c-fos reverse: CTTCTCCTTCAGCAGGTTGG. GAPDH was used as an internal control. Potential for multilineage differentiation were determined. *Results*. The expression of surface antigens (hBM, hCB, hDP, hPDL, hAT, hE, hAF and hPL expressed CD10, CD13, CD44, CD73, CD90, CD146 and CD166, but not, CD11b, CD14, CD15, CD19, CD33, CD34, CD45,CD117, HLA-DR) and human tissues expressed specific markers including vimentin, fibronectin, β-tubulin, α-SMA, actin, desmin in MSCs used in this study indicated their undifferentiated state. In addition the cells possessed morphological characteristics and multilineage differentiation potential. The same cells strongly expressed c-Fos as shown by immunohistochemical staining and RT-PCR which were not reported elsewhere. *Conclusions*. In our study, high expression of c-fos antigen in human derived MSC lines when examined with immunohistochemical and molecular methods provided clues that c-fos may be used as a new stem cell marker.

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ISOLATION, CHARACTERIZATION AND DIFFERENTATION POTENTIAL OF HUMAN AMNIOTIC FLUID DERIVED MULTIPOTENT MESENCHYMAL STEM CELLS

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Background. Up to now, some reports exist regarding the characterization of human amniotic fluid cells (hAFCs) that should be considered as an interesting new source of prenatal stem cells (SCs) devoid of ethical issues involved in embryonic SC research. Recent studies reported that hAF obtained by amniocentesis, during treatment of polyhydramnios, and at caesarean section contain fetal MSCs with a multilineage differentiation potential and derived embryonic and extra-embryonic tissue. hAF is significant due to routinely obtain from spare source after birth utilizing minimally invasive or noninvasive tecnique and rich source of SC. Further studies of hAF derived MSCs of classification, characterization, and biology are necessary. *Aims*. To establish a new source of therapeutic cells, we aimed to isolate hAF-MSCs from AF obtained during treatment of polyhydramnios at 27-32 weeks of pregnancy, and to further characterize these cells by investigating their immunophenotypes, the *in vitro* growth kinetics, replicative lifespan, morphology and differentiation potential throughout their existence. *Design and Methods*. Ten millilitres of hAF samples were obtained from polyhydroamnios patients during removal excessive amniotic fluid for routine therapy at 27-32 weeks of pregnancy. hAF samples were centrifuged at 300g for 15 min, and the resulting pellets washed twice with MEM-Earle to remove blood and cell debris. All of the cells isolated from samples were plated in a 25 cm^2 culture flask containing MEM supplemented with 100U/mL penicillin, 0.1 mg/mL streptomycin, and 15% FBS. Viability and proliferation index of the cells were determined using conventional hemocytometric method and MTT Cell Growth Assay. The ability of ex vivo expansion was investigated until senescence, and stem cell-like characteristics were analyzed by examining immunophenotypic features, differentiation potential and messenger

RNA expression (RT-PCR) of the cells at each passage 1-10. Lastly, we analyzed ultrastructural and differentiation characteristics of MSCs. *Results*. Our data indicated that MSCs from hAF expressed CD13, CD44, CD90, CD166 and Oct-3/4, but not, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117, HLA-DR, TR-1-60, TR-1-81, SSEA-4 and consistent with their undifferentiated state. In the IHC examination; hAF-MSC's showed negative immunoreactivity for CD34, CD31, CD71, and showed positive immunoreactivity for CD105, nestin, fibronectin, vimentin, c-fos, tubulin-β, α-smooth muscle actin, desmin, osteocalcin, osteonectin, and γ-enolase. Gene expression studies confirmed this immunophenotypic data. These cells were successfully differentiated into adipogenic, osteogenic, chondrogenic, neurogenic and myogenic lineages. *Conclusions.* Our study has provided evidence that: (i) hAF-MSCs are a promising source due to their high proliferation ability; (ii) hAF-MSCs could differentiate to many kinds of cells including osteocyte, chondrocyte, adipocyte, neuro/glial cell and myocyte, and could be obtained and duplicated easily; (iii) the expression of embryonic stem cell marker such as oct3/4 is required for the maintenance of somatic stem cell properties; (iv) they could be easily used as a convenient resource for future medical trials.

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HAEMATOPOIETIC PROGENITOR CELLS (HPC) FROM HUMAN PRENATAL AND POSTNATAL CORD BLOOD

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Background and Aims. Human haematopoietic progenitor cells (HPC) obtained from adult peripheral blood and from postnatal cord blood have been used to treat several hematological conditions. In this study, the presence of HPCs were evaluated in prenatal (intra uterus) and in postnatal cord blood (obtained immediately after delivery) using hematological and immunological tests. *Design and Methods.* Prenatal cord blood (32 samples) was obtained by cordocentesis through intrahepatic vein puncture guided by ultrasound, from pregnancies of 16-32 weeks of gestational age. In all cases, conditions such as congenital fetal malformation, sensitization due to Rh incompatibility, maternal age over 35 years in the first pregnancy or congenital rubella were suspected. 2. Postnatal cord blood (35 samples) was obtained immediately after delivery from placentas of normal pregnancies. The following tests or parameters were performed or detected on all samples: Hb, hematocrit, Hb electrophoresis, FACS immunophenotyping studies with the following monoclonal antibodies: HLA-Dr, CD2, CD4, CD5, CD8, CD10, CD11b, CD13, CD,14, CD 19, CD20, CD33, CD34, and CD35. To differentiate subtypes of CD34+ cells the following reagents were used: CD34PE / CD33-FITC and CD34PE / HLA-Dr-FITC. Some of the cord blood cell samples were cultured in semisolid medium in the presence of erythropoietin and IL-3. *Results*. Prenatal cord blood (16-32 weeks of gestation) showed higher values of Hb and haematocrit as compared to postnatal cord blood, associated with extensive macrocytosis (mean VCM in 25 samples: 132 fl at 16-20 weeks of age). Immuno-phenotyping of prenatal cord blood cells allowed the identification of leukocytes (CD45+ and HLA-Dr+ cells), T-lymphocytes (CD2+, CD4+ and CD8+ cells), B-lymphocytes (CD10+, CD19+ and CD20+ cells), cells expressing myeloid markers (CD13+, CD14+ and CD33+ cells), and haematopoietic progenitor cells ((CD34+ cells). The number of CD34+ cells showed a maximal value of 4.35% in a sample of 16 weeks of age of gestation, with a tendency to decrease as the pregnancy progressed. In postnatal cord blood the value of CD34+ cells was 0.85%±0.28, most of them of the CD34+ / CD33- phenotype. Both, prenatal and postnatal cord blood, when cultured in semisolid medium, generated erythroid and granulocytic-macrophagic colonies after 7 days of incubation. The earliest samples of cord blood showed more colonies than the older samples. *Conclusions*. Haematopoietic progenitor (CD34+) cells and various types of mature leukocytes can be easily identified in fetal blood obtained from 16-32 week-old pregnancies. The higher value of CD34+ progenitor cells was detected in samples from the 17th week, and the lowest in samples of postnatal blood.

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COMPARATIVE ANALYSIS OF MESENCHYMAL STEM CELLS FROM BONE MARROW, DENTAL PULP, ADIPOSE TISSUE AND ENDOMETRIUM

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Background. Several studies have reported the *in vitro* expansion of mesenchymal stem cells (MSCs) from human bone marrow (hBM), dental pulp (from impacted third molars-hDP- and exfoliated deciduous teeth-hSHED), adipose tissue (hAT) and endometrium (hE) and differentiation of these MSCs into multilineage cells. However, MSCs from these different sources have different characteristics. *Aims.* The present study aimed at providing a detailed morphological, immunophenotypical, and growth kinetics of MSCs from five sources using flow cytometry, immunocytochemical techniques and MTT test. *Design and Methods.* MSCs from various human derived- organ or tissues were isolated using appropriate methods. Isolated MSCs analyzed for CD10, CD11b, CD13, CD14, CD15, CD19, CD33, CD34, CD44, CD45, CD73, CD90, CD117, CD146, CD166, and HLA-DR by using flow cytometry. Similarly, these cells were analyzed for CD31, CD34, CD44, CD71, CD105, vimentin, fibronectin, β-tubulin, α smooth muscle actin (SMA), actin, desmin, myogenin, myosinIIa, myoD, osteonectin, osteocalcin, osteopontin, MEPE, BMP-2, BMP-4, collagen type I and II, γ-enolase, βIII-tubulin, neurofilament, GFAP, MAP2a,b, HNK-1ST, nestin and c-fos by using immunohistochemical techniques. Analysis of MTT was made using MTT Cell Growth Kit on passage 3 cultures of MSCs for 1, 4, 7, 11, 14, 17 and 21 days. *Results*. Our data indicated that MSCs from various human derived tissues including hBM, hDP, hSHED, hAT, and hE expressed CD10, CD13, CD44, CD73, CD90, CD146 and CD166, but not, CD11b, CD14, CD15, CD19, CD33, CD34, CD45,CD117, and HLA-DR, consistent with their undifferentiated state. The MCSs of this study isolated from human tissues expressed specific markers including vimentin, fibronectin, β-tubulin, α-SMA, actin, desmin, and possessed morphological characteristics and multilineage differentiation potential. The expression of surface antigens in MSCs agreed with the previous reports regarding MSCs, and indicated that the cells used in our study had the characteristics of MSCs reported elsewhere. In the present study, MSCs expressed some antigens which are indicators of glial and neuronal (γ-enolase, MAP2a,b, βIII- tubulin and GFAP), myogenic (myogenin, myosinIIa, desmin, actin and α-SMA), osteogenic (osteonectin, osteocalcin, osteopontin, type I collagen, BMP-2 and BMP-4) and chondrogenic (type II collagen) differentiations without a need for induction. According to the results of MTT test, MSCs derived from hSHED posses the highest proliferation potential, followed by MSCs derived from hDP, hAT, hBM and hE. *Conclusions*. In our study , MSCs have been conventionally isolated from various tissues and organs including bone marrow, dental pulp, adipose tissue, and endometrium, and these cells had similar morphology and, to a certain extent, immunophenotipic characteristics. Similar findings suggested that these cells were basically the same, although small differences that probably reflect a specialized gene program related to their anatomic localization were observed. When proliferation index was considered, an order of hSHED >hDP>hAT>hBM>hE was observed. Both dental pulp and adipose tissue are attractive to bone marrow as sources for isolating MSCs.

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FOXP1 IS PRESENT IN NON-LYMPHOID HEMATOPOIESIS

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Background. FOXP1 gene is located on chromosome 3, region p14.1. Immunoenzymatic staining of normal tonsil shows FOXP1 in nucleus of mantle zone cells, scatered B-cells inside some germinal centers and few B-cells in T-cell area. After the activation of B lymphocytes higher amounts of its mRNA were observed. It was also suggested that FOXP1 has an essential role in B-cell development and that it is one of the most important transcription regulators in those cells. There are no data about FOXP1 protein presence and/or function in myeloid and erythroid cell linages. *Aims*. To investigate FOXP1 presence in non-lymphoid hematopoiesis. *Design and Methods*. 15 bone marow trephine samples were taken from patients with no hematopoietic malignancies. Immunohistochemical double staining was performed on 4 mm tick, formalin fixed paraffin embedded bone marrow trephine sections according to the protocol provided by the manufacturer of the visualization LSAB/HRP

and LSAB/AP kits (Dako, Glostrup, Denmark). Anti-FOXP1 antibody (JC12) was used in combination with anti-glycophorin C or anti-myeloperoxidase antibodies. *Results*. All 15 samples showed similar results. 60-90% of all cells that had glycophorin C expression showed JC12 immunostaing. 80-90% of all cells that had myeloperoxidase expression showed JC12 immunostaing. All samples with both staining combinations had cells of lymphocyte morphology that were JC12 positive and glycophorin C/myeloperoxidase negative. The reliability of both double staining was confirmed by repeated double blind reading. *Summary and Conclusions*. Presented data suggest presence of FOXP1 protein and its possible function in developmental stages of myelopoiesis and erythropoiesis.

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STEM CELL - PLATELET COMPLEXES IN STEM CELL PRODUCTS

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Background. Activated platelets express P-selectin (CD62P). The binding of this P-selectin to its ligand PSGL-1 , results in complex formation between activated platelets with most white blood cells. Monocytes-platelet complexes(PMCs) in this respect are most known and shown to have a pro-adhesive phenotype by the platelet expressed P-selectin but additionally by increased β1 and 2 -integrin-expression on the monocyte. Stem cell products contain blood platelets in huge quantities; therefore platelet- stem cell complexes (PSCs) may also be formed. We hypothesize that such a platelet binding may similarly influence the adhesion (and homing) capacity of stem cells and possibly their differential behavior. *Aims*. To study:a. the extent of PSC complex formation in different stem cell products and the role of the CD62P-PSGL-1-axis in this respect; and b. the influence of anticoagulation, storage time and the extent of platelet activation on this complex formation. *Design and Methods*. PSC occurrence was measured in citrate anticoagulated mobilized peripheral stem cell transplants (MPSCT) and umbilical cord blood (UCB), as well as in heparin anticoagulated bone marrow (BM). Flowcytometry measured particles that were double positive for CD45, CD34 on one hand and CD61 (platelet glycoprotein IIbIIIa) and CD62P on the other hand were considered to reflect PSC. CD14- CD61 positive events were considered as platelet/ monocyte complexes. Complexes were measured at t=1 and 24 hours. Addition of anti-CD62P or EDTA (15 minutes resp 1 hour incubations) were used to investigate if PSC formation could be blocked or disrupted .

Table 1. PSCs and PMCs in different sources.

	MPSCT	BM	UCB
% PSC ± S.E.M	18,4 ± 6,4	9,4 ± 3,1	7,5 ± 1,6
+ 24 hours	37,4 ± 15,0 *	11,5 ± 3,5	8,5 ± 1,8
+EDTA	16,6 ± 5,2	5,1 ± 1,0	7,8 ± 2,8
+Activation	27,8 ± 13,5 *	16,4 ± 4,4	6,9 ± 0,8
% PMC ± S.E.M	89,4 ± 5,4	71,8 ± 29,4	98,0 ± 1,5
+ 24 hours	97,3 ± 1,4 *	83,4 ± 24,0	99,5 ± 0,6 *
+EDTA	14,3 ± 3,0 *	4,5 ± 2,0*	4,4 ± 2,0 *
+Activation	94,8 ± 5,7 *	73,0 ± 29,3	98,1 ± 1,5

Additional PSC formation was attempted by addition of 10E-4M ADP, a naturally occurring platelet activator. *Results*. All products contained PSCs and PMCs (Table 1). PSC numbers increase about 50% in time (24 hrs) in MPSCT (p=0.002) and to a lesser extent in BM and UCB (both p=0.1). PMCs showed a vast increase in time in MPSCT and UCB (p=0.004 res$p$=0.03) but not in BM (p=0.24). ADP-mediated platelet activation resulted in an increase in both PSC and PMC in MPSCT (p<0.006 resp.p=0.03). No additional effect of platelet activation on PSC or PMC was observed in BM and UCB (p=0.25 resp p=0.7 for PSCs; p=0.3 resp p=0.97 for PMC). PSC-increase in time and after platelet activation was successfully inhibited by anti-CD62P in MPSCT and to a lesser extent in BM and UCB (not shown). In contrast, addition of anti-CD62P effectively inhibited increase in PMC in time as well as after platelet activation in all sources as compared to control samples. No EDTA-effect was found for PSCs in all sources while in all products samples PMCs dissociated after adding EDTA (MPSCT p<0.0001, BM p=0.02 and UCB p<0.001). *Conclusions*. Both PSCs and PMCs are present in different stem cell products. Our results confirm that PSC- formation is CD62P-dependent. Additional platelet activation and longer storage time can increase PSC upto 30-40% in MPSCT products. Further studies are necessary to study the differences between CD34+ stem cells in the different products for their complex forming capacity and the role of PSCs in the efficacy of stem cell transplantation.

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COMPARATIVE ANALYSIS OF RAT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS CULTURED UNDER DIFFERENT CONDITIONS

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Background. Mesencymal stem cells (MSCs) are multipotent cells and can differentia into multiple lineages. These properties allow for an autologous cell source, which in some cases circumvents host immune response issues, and make them a good candidate for potential therapeutic applications such as cellular and gene therapies. In general, MSCs have been successfully isolated by two different culturing techniques: (i) gradient density centrifugation and (ii) direct flushing into culture flask with plastic adherence from the BM of many mammals from human to laboratory rodents. However, culture medium compositions showed differences from one study to another and media like IMDM, DMEM/F12, DMEM, L-DMEM, H-DMEM, α-MEM, and RPMI 1640 were used with different percentages of fetal bovine serum (2, 5, 10, 15 and 20 %). From these studies, contradictory results about the need for the number of passages to obtain the highest immunophenoypically-pure MSC came out. *Aims*. In this study, we cultured MCSs isolated from bone marrows of rats in three different media (RPMI 1640+10% FBS, MEM-Earle+15%FBS and L-DMEM+10% FBS) to determine the number of passages necessary to obtain the highest immunophenoypically-pure MSCs. *Design and Methods*. MSCs have been successfully isolated from femurs and tibias bone marrow of adult Wistar rats by direct flushing method with adherent properties. After each passage, cell suspensions were analyzed by using flow cytometry. Similarly, the cells were analyzed for CD31, CD34, CD44, CD71, CD105, vimentin, fibronectin, β-tubulin, α-smooth muscle actin (SMA), actin, desmin, nestin and c-fos by using immunohistochemical techniques. Viability of MCSs and their proliferation ability were determined with MTT and pulsed BrdU incorporation. Lastly, we analyzed ultrastructural and differentiation characteristics of MSCs. *Results*. When proliferation index was considered, an order of MEM> L-DMEM > RPMI-1640 was observed in MTT analysis. The expressions of the cell surface proteins such as CD29 (positive), CD45 (negative) and CD90 (positive) did not show differences under three different culture conditions throughout passages 1 to 20. However, CD45 expression of MSCs increased significantly in MEM medium after passage 20 and both in RPMI and LDMEM media after passages 30. This increase might be due to *in vitro* maturation of MSCs. In addition, our immunohistochemical studies and differentiation experiments showed that the cells expressed the other markers that were specific for MSCs. These cells were successfully differentiated into adipogenic, osteogenic, neurogenic and myogenic lineages. There was no difference observed in ultrastructural properties when cells were grown in three different media. *Conclusions*. This study clearly demonstrated that cells from early passages should be used in stem cell research due to heterogeneity problem of the cells in late passages. MEM as a culture medium is a good candidate for two reasons; (i) cultured MSCs have high proliferation capacity in this medium (ii) it is much more economical. However, other media (L-DMEM and RPMI) should also be considered when immunophenotypically more stabile passages were aimed at.

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BONE MARROW METASTASES OF NEUROBLASTOMA DETECTED BY IMMUNOHISTOCHEMICAL STAINS: CHROMOGRANIN A, CD56, AND SYNAPTOPHYSIN

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Background. Neuroblastoma is one of the most frequently occurring malignant solid tumors in childhood. Evaluation of the bone marrow

(BM) is an important part of clinical staging in neuroblastoma patients because with the presence of BM metastasis, the patient is classified into stage IV or IV-S neuroblastoma. Due to the difficulty in detecting neuroblastoma cells with conventional bone marrow aspirate smears and biopsy specimens, especially in cases where metastases is not prominent, we proposed immunohistochemistry (IHC) as a potential diagnostic tool. *Aims.* The purpose of our study was to evaluate the application of three neuroendocrine markers (chromogranin A, CD56, and synaptophysin) for detecting minimal metastatic neuroblastoma in bone marrow specimes. *Design and Methods*. We studied 59 patients that were newly diagnosed with neuroblastoma between January 1998 and March 2008. A total of 102 BM biopsy and clot sections that consisted of 16 unilateral and 42 bilateral samples were evaluated. Immunohistochemical stains using chromogranin A, CD56, and synaptophysin monoclonal antibodies were done on the Benchmark XT auto-stainer (VENTANA, Tucson, USA). *Results*. Fifteen out of 59 patients (25.4%) had been diagnosed as having bone marrow metastasis. Immunohistochemical staining of clot sections were generally more sensitive than biopsy or aspirate smears for detecting tumor cells. Clot sections stained with chromogranin A identified tumor cells in all H&E-positive specimens as well as 8 additional H&E-negative specimens. Chromogranin also had the lowest false-negative result (H&E positive, IHC negative; n=1) among the three IHC methods. Adding the H&E-/IHC+ cases to the detection rate showed that using a combination of chromogranin A and CD56 was superior in detecting bone marrow metastasis. *Conclusions*. Immunohistochemical analysis of bone marrow specimens is more sensitive than conventional analysis and using a combination of chromogranin A and CD56 appears to be the most sensitive method for detecting neuroblastoma cells. To overcome the discrepancies between routine smears and immunohistochemial stains (H&E negative, IHC positive), immunohistochemical analysis of the bone marrow biopsy and clot sections should be warranted as a routine component in the diagnostic work up of neuroblastoma.

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IN VITRO INDUCTION OF FETAL HEMOGLOBIN BY TRANSFORMING GROWTH FACTOR-B AND STEM CELL FACTOR IN ERYTHROID CELLS DERIVED FROM CD133+ CELLS

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Background. Increased fetal hemoglobin (HbF) in β-globin gene disorders, ameliorates the clinical symptoms of the underlying disease. 5-azacytidine, butyrate and Hydroxyurea, have been shown to activate β-globin gene expression. Also, It has been shown that hematopoietic growth factors can influence expression of β-globin in erythroid culture and in animal models. *Design and Methods*. This study was designed for *in vitro* evaluation of stem cell factor (SCF) and transforming growth factor-β (TGF-β) on β-globin gene reactivation of erythroid precursors derived from CD133+ cells *in vitro*. *Results*. The gene expression analysis showed increased expression of β-globin transcript in all groups (the cell culture groups containing TGF- β or SCF or both) as compared with control (2.2, 2.7 and 5.5 folds, respectively) (p<0.01). Also, HbF production in differentiated population was demonstrated using flow cytometry. *Conclusions*. In conclusion, the results of this report suggests that SCF and TGF-β warrant further evaluation as potential therapeutic drugs for treatment of β-globin gene disorders.

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DYNAMICS OF DONOR T CELL ACTIVATION DURING EARLY PHASE OF GRAFT VERSUS HOST DISEASE (GVHD): DONOR ANTIGEN PRESENTING CELLS CAN ACTIVATE ALLOGENEIC T CELLS

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Background. Bone marrow transplantation (BMT) is increasing rapidly as a curative treatment; however, graft versus host disease (GVHD) is one of the major limiting factors. GVHD is basically an uncontrolled immune response of donor T-cells against host alloantigen. Pre-transplant conditioning is important factor in GVHD initiation; however its occurrence depends mainly on the immune compartments regardless the type of conditioning. The role of host antigen presenting cells in the activation of donor T cells has been reported previously. *Aim*. In the present investigation we tracked host versus donor immune cells in a chorological approach to determine the phenotypical and biological activation pattern at early stage of GVHD. *Design and Methods*. Female and male BALB/c and C57BL/6 mice were used as recipient and donor. Recipient mice were conditioned using busulfan and cyclophosphamide, and were transplanted using allogeneic or syngeneic donors. Recipients were killed at early time points post transplantation (day 0, +1,+3,+5,+7 and +21); immune cells phenotype and function were evaluated in spleen and bone marrow. Cytokine levels in serum were measured using multiplex fluorescence assay. To confirm the emergence of GVHD and T-lymphocyte infiltration, various tissues including spleen were examined utilizing histopathology and immunohistochemistry. *Results*. GVHD occurred 7 days after allogeneic BMT. Donor dendritic cells (DCs) actively expanded and matured early after allo-BMT (day+3) followed by a short period of host DCs expansion. Expansion of donor DCs was significantly higher (p<0.05) compared to host DCs three days after BMT. T-cells repopulation was similar in both allogeneic and syngeneic transplanted groups until day+3. However, five days after BMT donor CD8+ cell expansion in GVHD group was as high as 230% compared to that observed in control untreated group and 700% higher compared to CD8+ cells found in syngeneic transplanted mice. Dramatic expansion of donor T-cells in GVHD mice followed the peak expansion and maturation of donor DCs. IL2, IFN-γ and TNF-α were found to be at the highest level 5 days after BMT which was in agreement with T-cell activation. Following this activation most of naïve T-cells gained effector-memory phenotype and migrated from the spleen to periphery. *Conclusions*. Tracking of host versus donor immune cell at early phase of GVHD have shown that donor DCs has essential role in the activation of alloreactive donor T-lymphocyte. Moreover, during the activation phase of T-cells in the spleen donor naïve cells gain effector-memory phenotype and initiate GVHD.

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FACTORS ASSOCIATED WITH SEVERITY OF MUCOSITIS AFTER REDUCED-INTENSITY-CONDITIONING FOR ALLOGENEIC STEM CELL TRANSPLANTATION

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Chemotherapy-induced mucositis is associated with considerable morbidity. Furthermore, breakdown of the mucosal barrier allows bacteria easy penetration into tissue and bloodstream. Severity of mucositis in allogeneic stem cell transplant (SCT) recipients is less intensive after reduced-intense-conditioning (RIC) protocols compared to classic myeloablative conditioning, however, even after RIC severe mucositis may occur. *Aims*. The aim of the present investigation was to identify factors influencing the severity of oral mucositis after reduced-intense-conditioning for allogeneic stem cell transplantation. *Design and Methods*. 52 patients [male: n=35 (67%), female: n=17 (33%)] with a median age of 62 years (35-73) underwent allogeneic SCT between 2005 and 2008. Conditioning was either TBI(2Gy)/Fludarabine (n=33, 63.5%) or chemotherapy based (treosulfan/fludarabine, melphalan/fludarabine, cyclophosphamide/fludarabine). ATG was given in cases of transplantations from mismatched or unrealated donors (n=44, 85%). GvHD-prophylaxis was carried out with cyclosporine-A and - depending on the protocol - with MMF. Additionally, 45 patients (87%) received short-course MTX. Supportive therapy, anti-infectious therapy and prophylaxis followed standard protocols. Palifermin was given in five cases (10%). Mucositis was graded according the Bearman- and WHO-scale. A variety of clinical and laboratory parameters was correlated with severity of mucositis. *Results*. Both mucositis scales showed an excellent correlation (cc=0,95, p<10e-20, Spearman-rank correlation). The mucositis was significantly more severe after chemotherapy-based conditioning compared to conditiong with TBI(2Gy)/Fludarabin (p<0.002, Mann-Whitney U-test) and when the serum-creatinin was above the upper normal value on day +3 after SCT (p<0.05). Furthermore, the severity of mucositis correlated with time to engraftment of leucocytes (cc=0,26, p<0.02) and thrombocytes (cc=0.38, p<0.001). Other parameters such as the number of preceding therapy lines, the interval from last therapy to conditioning, the use of ATG, voriconazole or TMP-SMZ, a weight gain after SCT, pathological liver parameters or patients age were not associated with the grade of mucositis. *Conclusion*. Mucositis contributes to morbidity after SCT, even after reduced-intensity conditioning. The condition regimen was identified as the sole factor predicting severity of mucositis after RIC and allogeneic SCT. An influence of cumulative toxicity of preceding chemotherapy on mucositis was not observed. Creatinine on day +3 after SCT may help to identify patients at higher risk for severe mucositis in the further course of transplantation.

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OPTIMAL TIMING OF G-CSF INJECTION FOR EFFECTIVE PERIPHERAL BLOOD PROGENITOR CELL COLLECTION IN PATIENTS WITH MULTIPLE MYELOMA OR LYMPHOMA

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Background. There have been few attempts to define optimal timing of G-CSF administration for Peripheral blood progenitor cell (PBPC) collection and little is known about short-term kinetics of G-CSF. *Aim*. The objective of this study was to identify better timing of G-CSF administration which improves the efficacy of PBPC collection for autologous stem cell transplantation (ASCT). *Design and Methods*. A total of 262 patients who underwent PBPC collection from January 2000 to March 2008 were included. The patients were diagnosed with Hodgkin's lymphoma, non-Hodgkin's lymphoma and multiple myeloma and eligible for ASCT. PBPCs were mobilized with lenograstim following chemotherapy. Before November 2004, patients received lenograstim injection at a dose of 10 μg/kg subcutaneously at 8 p.m., 13 hours before apheresis (PM group), after then patients received lenograstim at 6 a.m. (AM group), 3 hours before apheresis. *Results*. Injection of lenograstim at 6 a.m. was superior to injection of lenograstim at 8 p.m. with greater numbers of total collected $CD34^+$ cells/kg ($13.29x10^6$/kg versus $8.51 x10^6$/kg, AM and PM group, respectively, p=0.001) in shorter duration of leukapheresis procedures (2 days vs 3 days, p=0.035). The median number of CD34+ cells/kg collected at first leukapheresis was also greater in AM group ($3.94x10^6$/kg vs 2.47 X 106/kg, p=0.001). Stem cell collection efficacy defined as ratio of total collected CD34+ cells per days of leukpaheresis were 5.34 and 2.96, respectively. It was significantly better in the AM group (p=0.001). The median number of patients who achieved a total collected $CD34^+$ cells > $5x10^6$/kg was also greater in the AM group (113 vs 96, p=0.002). *Conclusions*. The present study shows that injection of lenograstim 3 hours before apheresis (AM group) improves the efficacy of stem cell collection with greater number of collected CD34+ cells/kg in shorter duration of leukapheresis procedures compared to that of PM group.

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KINETICS OF JAK2 V617F MUTATION IN FIVE JAK2 POSITIVE MDS PATIENTS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. JAK2 V617F is a gain of function mutation in the Janus kinase 2 (JAK2) that was described in several myeloproliferative disorders. This mutation was found to exist also in nearly 5% of myelodysplastic syndromes. Recently several groups have further defined a subgroup of myelodysplastic patients who show simultaneously some clinical and pathological characteristics of myeloprolifertive disorders. This group is now referring to the new WHO classification of 2008 diagnosed as myelodysplastic/ myeloproliferative neoplasm, unclassified (MDS/MPN). Another group of patients exhibits features of essential therombocythemia combined with ringed sideroblasts in bone marrow and is now classified according to same system as Refractory anemia with ringed sideroblasts with marked thrombocytosis (RARS-T). JAK2 V617F mutation was detected in the majority of patients from the both previously mentioned groups. *Aims and methods*. We have treated five patients with myelodysplastic syndrome who harbored this mutation with allogeneic stem cell transplantation (ASCT) and monitored the kinetic of JAK2 mutation in peripheral blood after ASCT using a highly sensitive Taqman Polymerase chain reaction method. *Results*. The mean age of the patients was 51y (42-68 y). One of them had an acute myeloid leukemia AML-M1 probably after MDS, 2 had refractory cytopenia with multilineage dysplasia RCMD, 1 had refractory anemia with excess blasts RAEB II, and 1 had a refractory anemia RA which has evolved into RAEB II. Two of these patients showed marked fibrosis in the bone marrow histology. Two patient were conditioned using a standard conditioning regimen, the others received reduced conditioning. JAK2 V617F mutation copies decreased dramatically after transplantation in all patients. In three patients the mutation became negative after a median of 31 (16-46) days after transplantation .The 4th patient hat fluctuating very low values of JAK2 V617F compatible with a chimerism also ranging between 97-99,9 after ASCT, he is alive in a hematologic remission and doing well. In the 5th patient JAK2 V617F burden is decreased steeply but is not negative 4 months after transplantation. *Conclusions*. We conclude that JAK2 V617F mutation status decreases after stem cell transplantation and might be used as a marker of minimal residual disease after ASCT.

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CARDIAC STEM CELLS : A NEW POTENTIAL TOOL FOR CARDIAC REPAIR?

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Background. The current therapy for myocardial infarction (MI) is not adequate enough, since mortality after MI remains very high. Cardiac repair by injection of stem cells is a promising therapy. Previously, several clinical studies using bone marrow stem cells were undertaken to explore the possibilities of stem cell therapy. Meanwhile, more evidence emerges that cardiac stem cells (CSCs) are present in the adult human heart. These CSCs are claimed to be actual stem cells residing in the heart and not just bone marrow stem cells mobilized to the heart. Therefore, CSCs are probably better suited for cardiac repair, since they are most likely already "pre-programmed" to become cardiomyocytes. *Aim*. To support this hypothesis, the aim of our study was to isolate human CSCs and compare them phenotypically and functionally with human mesenchymal stem cells (MSCs). *Design and Methods*. For the isolation of CSCs the two main methods described by Messina *et al*. and by Anversa *et al*. were followed. Messina *et al*. defines CSCs as cardiosphere derived cells (CDCs), which can be obtained by culturing phase-bright cells that appear after two weeks in a culture of cardiac explants. On the contrary, Anversa et al. consider c-kit+ cells isolated from cell outgrowth from a cardiac explant as CSCs. Upon isolation, CDCs, c-kit+ cells and MSCs appeared to be morphologically very similar in culture. Therefore, different panels of antibodies were used to phenotypically characterize these three cell types. *Results*. CDCs, c-kit+ cells and MSCs were found to be positive for CD13, CD29, CD44, CD55, CD90, CD49c, CD73, CD105 but negative for CD34, CD45 and CD133. The main difference between cells isolated from heart tissue and MSCs was that only MSCs stained positive for CD140b For the functional analysis, cells were subjected to a stem cell differentiation assay. While MSCs could easily differentiate to adipocytes, osteocytes and chondrocytes, CDCs could not. In addition, the possibility of differentiation into cardiomyocytes was examined. Therefore, cells of all three populations were cultured in differentiation medium containing 2% FCS and 5-aza, DMSO or TGF-β respectively. To investigate differentiation, cells were harvested and analyzed for expression of cardiomyocyte specific genes by RT-PCR. Preliminary results from these monocultures indicated that cells isolated from heart tissue had more potential to differentiate into cardiomyocytes than MSCs, because the former cell populations expressed cardiac troponin T and β-actinin in these conditions while the MSCs did not. *Conclusions*. These results indeed suggest that there is a phenotypical and functional difference between CSCs and MSCs, but further investigations have to be performed to show that these CSCs are able to differentiate into cardiomyocytes *in vitro* and *in vivo*.

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BORTEZOMIB AND BEAM CONDITIONING REGIMEN FOR AUTOLOGOUS STEM CELL TRANSPLANTATION IN MANTLE CELL LYMPHOMA

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Background. Mantle cell lymphoma (MCL) is an agressive non Hodgkin lymphoma with median overall survival (OS) in most series of 3-4 years. Although frontline therapies with autologous stem cell transplantation (ASCT) induce high rates of complete remission (CR), relapse is usual. Bortezomib (VEL) has demonstrated clinical efficacy in relapsed and refractory MCL, a synergistic effect with Cytarabine and a lack of sustained haematological toxicity when combined to high dose Melphalan (in multiple myeloma). The combination of VEL to BEAM could be a logical approach to improve the OS in MCL. *Aims*. The purpose of this monocentric prospective study was to evaluate the feasibility and safety profile of this association. *Design and Methods*. Between 05/06 and 11/08, 13 consecutive patients with MCL were enrolled to receive intensification with VEL-BEAM. VEL (1.3 mg/m^2) was delivered on days - 6,

- 3, + 1, + 4 and BEAM (300 mg/m^2 BCNU, 4x200 mg/m^2 Etoposide, 4x200 mg/m^2 Cytarabine and 140 mg/m^2 Melphalan) on day -7 to -2. Peripheral blood stem cells (median 6.6x10^6 CD34/kg) were infused on day 0. We secondary conducted a retrospective comparison with 8 frontline MCL patients who received "classical" BEAM followed by ASCT. *Results*. Main characteristics of the patients in both groups are resumed in table 1. All patients except 1 were in CR after induction therapy with 3 RCHOP/ alternating RDHAP chemotherapy. VEL did not increase the haematological toxicity observed with BEAM (Table 2). Median duration of neutropenia (<0.5x10^9/L) and thrombocytopenia (<50x10^9/L) was 9 and 13 days respectively. No grade 3/4 extra-haematological toxicity was observed. Two patients were transferred to USIC for septic choc with pneumoniae during neutropenic period. At time of reporting (02/09), only 1 patient relapsed at 18 months (mo). Two patients died: 1 at 3.5 mo of intracerebral hemorragia and 1 because of disease progression at 22 mo. In the classical BEAM group, 2 patients relapsed at 18 and 20 mo respectively with 1 subsequent death. Median follow-up (19 mo) is currently too limited to comment on whether the conditioning regimen with VEL-BEAM will translate into an improved progression free survival and therefore OS. *Conclusions*. These results suggest that VEL (1.3 mg/m^2) and BEAM is a feasible and safe conditioning regimen with promising PFS and OS results in MCL.

Table 1. Baseline characteristics of patients.

	BOR-BEAM	BEAM
Patients characteristics	**n=13**	**n=8**
Sex: M/F, n	12/1	6/2
Median age, y (range)	51 (33–61)	56 (43–61)
Ann Harbor stage IV/II, n	11/2	8
Bone marrow involvement, n	11	8
Leukemic phase at diagnosis, n	4	3
Elevated LDH, n	5	4
PS 3-4	0	0
Frontline treatment		
RCHOP/ alternating RDHAP, n	13	8
Response at time of ASCT		
CR (complete response), n	12	8
PR (partial response), n	1	

Table 2. Engraftment and transplantation related toxicity

	BOR-BEAM	BEAM
	n=13	**n=8**
No. of CD34 infused, median (range)	6,6 (3,8-19,3)	6,1 (2,7-10)
GCSF injection, yes/no, n	5/8	1/7
Duration of neutropenia (ANC < 500/mm3), median (range)	9 days (7-11)	11,5 days (6-25)
Duration of thrombocytopenia <50 G/l, median (range)	13 days (8-22)	8,5 days (6-19)
No. of platelet transfusions, median (range)	3 (1-5)	2 (1-5)
No. of packed red blood cells transfusions, median (range)	0,5 (0-3)	1,5 (0-2)
Mucositis grade 1-2/3-4	13/0	8/0
Renal dysfunction, n (%)	2(15,5)	3 (37,5)
Liver dysfunction, n (%)	3 (23)	1 (12,5)
Cardiac arrhythmia grade 1-2 (%)	1 (7,5)	0
Dermatologic /Allergic reactions grade 1-2 (%)	1 (7,5)	1 (12,5)
Septic choc / infectious complication,n (%)	3 (23)	2 (25)
Toxic death	0 (1 at 4 months post ASCT)	0

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IMMUNOPATHOLOGY OF ATG PRIOR TO BONE MARROW TRANSPLANTATION IN MOUSE MODEL

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Background. Polyclonal anti-thymocyte globulin (ATG) is mainly used in hematopoietic stem cell transplantation, as a prevention for graft rejection as well as for GVHD. Immunosuppressive properties of ATG have been considered primarily from the depletion of peripheral lymphocytes. However direct or indirect effects of ATG on other immune components still is controversial. In the present study we evaluated the immunopathologic effects of ATG on immune system in a mouse model. *Design and Methods*. Ten to fourteen weeks old BALB/c and C57BL/6 mice, were injected intra- peritoneally or intravenously with different doses of ATG (0.2 mg/kg -25mg/kg) at three dosage schedules. Considering bone marrow transplantation as day 0, ATG were given at days -10, -7 and -5 or -7, -5 and -1 or -5, -3 and -1. At day 0, mice were killed; spleen (SP), bone marrow (BM), lymph nodes (LN) and thymus were removed and analyzed for T-, B-, DC, NK-, T-reg and myeloid cells. To evaluate the efficacy of immunosuppressive effect of ATG, a group of BALB/c mice were conditioned using busulfan (Bu) and ATG and compared to a control group of BALB/c conditioned with Bu (80 mg/kg) followed by cyclophosphamide (200 mg/kg). Both groups transplanted with BM and spleen cells from C57BL/6 and followed for engraftment and/or GVHD. *Results*. The administration of ATG (0.2- 4.5 mg/kg) has resulted in an increase in spleen cellularity while in lymph node and thymus a decreased cellularity was observed. We have found that injecting 4.5 mg/kg of ATG at day -7, -5 and -3 significantly decreased T-cell population in spleen and LN compare to Bu-Cy conditioning. The ratio CD4/CD8 increased after ATG treatment showing that CD8 cells are six-fold more sensitive to ATG treatment compared to CD4 lymphocyte. Interestingly T-reg cell population increase after ATG at day 0, however, the increment was negatively correlated with the administration time and positively correlated with the dose. ATG treatment has resulted in an increase in B-cell population by two- and three- fold in spleen and lymph nodes, respectively. Moreover, 1.2- to 3.5-fold increase in DCs, NK and myeloid cells was observed in SP and LN. Thymus cellularity and cell phenotype was less affected while BM cellularity was not affected by ATG treatment. No differences in the ATG effect on cellularity or cell phenotype between IP and IV route was observed. Despite the high immunosuppressive effect observed in T-cell population compared to that seen in Bu-Cy conditioning, no chimerism was observed when Cy was substituted with ATG (4.5-10 mg/kg). *Conclusions*. No donor chimerism could be obtained using ATG as a single agent up to 25mg/kg. ATG dose and the administration time are important factors affecting the repopulation of residual T-cells in spleen and lymph node that have to be considered in bone marrow transplantation setting.

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APOPTOSIS-INDEPENDENT ACTIVATION OF CASPASE-3 IN CD34-POSITIVE (CD34+) MOBILIZED CELLS

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Background. G-CSF mobilized peripheral blood CD34$^+$ cells are now the preferred and major source of hematopoietic stem and progenitor cells harvested for both autologous and allogeneic transplantation. Several mechanisms, like SDF-1/CXCR4 interactions or degradation of adhesion molecules by proteolytic environnement, are involved in the mobilization process. However this phenomenon is still partially understood. Gene expression analysis has identified an overexpression of the caspase-3 gene in CD34$^+$ mobilized cells, compared to CD34$^+$ from normal bone marrow. Caspase-3 is the main effector of the terminal phase of apoptosis. However recent studies have provided evidence of its implication in non apoptotic cellular processes, such as differentiation, migration and cytoskeleton modelling. AIM: The objective was the evaluation of caspase 3 activity compared to apoptosis in peripheral blood mobilized CD34+ cells by flow cytometry, and the determination of an anti apoptotic phenotype in these cells. *Design and Methods*: We evaluated by multicolour flow cytometry the expression of activated caspase-3 in G-CSF mobilized CD34$^+$/CD45$^+$ cells from blood (n=18). CD34$^+$/CD45$^+$ cells from normal bone marrow (n=11) served as control. Caspase-3 activity on fluorescent substrate (PhiPhiLux method) and apoptosis (Annexin V assay) were also evaluated. Finally we analysed the expression of anti apoptotic proteins Bcl-2, Bcl-Xl, and of Heat Shock Proteins HSP27, HSP70 and HSP90 in the same cell population. *Results*. There was significant difference for apoptosis between mobilized and bone marrow CD34$^+$ cells (19,8% versus 32,4% apoptotic cells respectively). Conversely activated caspase-3 levels were higher in mobilized CD34$^+$ cells. This was consistent with cleavage of caspase-3 substrate observed in mobilized cells. A significant increased expression of HSP90 (of which caspase-3 is a client protein), HSP70 and Bcl-Xl was observed in peripheral CD34$^+$ cells, but there was no variation of BCl-2 and HSP27 expression. *Conclusions*. Our results show an activation of caspase-3 in the mobilized peripheral blood CD34$^+$ cells, which appears to be independent of apoptosis induction. The role of this activation and possible control by HSPs warrants further analysis to establish its relationship with mobilization mechanisms.

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COMPARISON BETWEEN SPONTANEOUSLY OCCURRING AND POST-TRANSPLANT METABOLIC SYNDROME

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Background. Central obesity and insulin resistance, which are linked by low adiponectin levels, play a primary role in the pathogenesis of metabolic syndrome (MS), and obesity-driven subclinical inflammation may contribute to determining the clinical pattern. We have previously observed 29 cases of metabolic syndrome (MS) in 85 adult long-term hematopoietic stem cell transplantation (HSCT) survivors. Aims, To compare common metabolic parameters and adipohormone profiles in post-transplant and "classical" MS patients *Design and Methods* The 29 post-transplant MS patients (15 women and 14 men; mean age 49.8±9.3 years; 17 autologous and 12 allogeneic transplant recipients) were compared with 29 "classical" MS patients (15 women and 14 men; mean age of 52.9±8.0 years) and 39 healthy subjects (19 women and 20 men; mean age 40.1±16.9 years). A record was made for each patient of conventional clinical and metabolic parameters, serum leptin, insulin, quantitative C-reactive protein (CRP), α-TNF and adiponectin Results Serum leptin, CRP, α-TNF and adiponectin levels were all significantly different in the three groups and every post-hoc test was also significant: in comparison with "classical" MS, the patients with post-HSCT MS had significantly higher levels of leptin (22.18 vs 15.3 ng/mL; $p<0.001$), CRP (0.32 vs 0.13 mg/dL; $p<0.05$), α-TNF (7.7 vs 2.8 pg/mL; $p<0.001$) and adiponectin (15.82 vs 9.44 ng/mL; $p<0.001$). There was no significant difference in insulin levels. Serum leptin levels and BMI were related in the patients with "classical" MS but not in those with post-HSCT MS. There were no significant differences between the allogeneic and autologous HSCT recipients. *Conclusions.* Unlike *classical* MS patients, patients with post-transplant MS do not show any physiological relationship between obesity and leptin or adiponectin levels, and so central obesity does not seem to play a primary role in this subset of patients. Conversely, their significantly higher leptin, CRP and α-TNF levels indicate chronic inflammation as an obesity-independent event possibly leading to insulin resistance and MS. The absence of any relationship between MS and allogeneic HSCT or GVHD suggests that chronic inflammation may be a common pathway due to multiple and heterogeneous transplant-related events affecting cytokine expression and immune response. This strengthens the view that post-HSCT and spontaneously occurring MS are separate entities. The former possibly shares some pathogenetic markers with the similar metabolic disorders observed in patients undergoing solid organ transplantation and those with HIV infection.

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QUANTITATIVE ANALYSIS OF CHIMERISM BY REAL-TIME POLYMERASE CHAIN REACTION OF INSERTION/DELETION POLYMORPHISM IS A GOOD PREDICTOR OF RELAPSE AFTER ALLOGENIC STEM CELL TRANSPLANTATION FOR ACUTE LEUKEMIA

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Background. Quantitative analysis of chimerism after allogeneic stem cell transplantation (allo-SCT) for acute leukemia is helpful to monitor the kinetic of engrafment. Usually, chimerism is assesed by variable number tandem repeat (VNTR) or short tandem repeat (STR) amplification by polymerase chain reaction (PCR), which have a sensitivity of 1 to 5 %. Insertion/Deletion (InDel) polymorphism analysis by real-time quantitative PCR (InDel-QPCR) is a recent and more sensitive method. Aims. As InDel-QPCR is a sensitive method for quantitative analysis of chimerism, it should be able to predict relapse earlier. *Design and Methods*. We conducted a unicentric retrospective study and we evaluated the files of all consecutive patients transplanted from May 2004 to April 2008 which relapsed of an acute leukemia after allo-SCT. Sixteen patients (12 myeloblastic and 4 lymphoblastic acute leukemia) were included in the study. Median age was 45 years (22-60). Conditionning regimen was myeloablative in 12 patients, the source of stem cell was bone marrow in 8 patients. Quantitative analysis of chimerism by InDel-QPCR method was usually performed every month post transplant. *Results*. The median delay between transplant and cytological relapse was 8.5 months (2-32). In three patients the quantification of recipient DNA rate was always superior to 1% at each point post transplant and they presented an early relapse (at 2, 3 and 4 months after transplant). The 13 remaining patients achieved a recipient DNA rate inferior to 1% at a median time of 35 days, in 11 patients recipient DNA rate was less than 0.1% at 56 days and in 7 patients recipient DNA rate was less than 0.01% at a median time of 189 days. The first 13 patients presented an increased chimerism preceding cytological relapse, at a median interval of 52 days (5-154). At the time when an increased chimerism was detected, the peripheral blood count was normal in 12 patients, one had persistent thrombocytopenia since the allo-SCT. *Conclusions*. The 0.01% to 1% range was found to be valuable for an early relapse prediction. Thus, chimerism evaluation by InDel-QPCR is a necessary method for detection of post transplant relapse before cytological abnormalities in acute leukemia, allowing early therapeutic measures.

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B CELL RECOVERY AND PLASMATIC BAFF LEVELS IN GRAFT VERSUS HOST DISEASE

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Background. B-lymphocyte activating factor (BAFF), belongs to the tumour necrosis factor (TNF) ligand super family and is expressed by macrophages, dendritic cells and neutrophils especially when there is a proinflamatory stimulation. Its effect is mediated mainly by BAFF receptor (BAFF-R). High levels of this protein are associated with B-cell function and development, but have also seen in B-cell neoplasm, autoimmune diseases and related with chronic Graft versus Host Disease (GvHD). *Aims*. Analyze differences of plasmatic BAFF levels and CD 19 and BAFF-R expression during first 6 months after allogeneic stem cell transplantation (SCT) between patients with and without GvHD. *Design and Methods.* Prospective study of 44 patients who underwent allogeneic stem cell transplantation (SCT) between 2006 and 2008 in our center. Patients characteristics were: 24 male /22 female; Median age: 48 yrs (16-64); Primary disease (AML:39%,ALL:14%,MM:14%, CLL:9%, NHL:7%, HL:5%, MDS: 5%, Aplastic Anemia: 5%, Other: 7%); Donor (MRD:48%; UD: 30%;MmRD:5%; MmUD:18%); Stem Cell source (BM:84%; PB:16%); Conditioning (BUCY: 16%; ICT-CY:2%; Flu-Mel: 32%; FluBu:46%;Other: 5%); GvHD prophylaxis (Csa-MTX:55%; Csa-MMF:43%; Csa alone:2%); ATG infusion: 14%; BAFF plasma levels were analyzed by ELISA test and CD19 and BAFF-R were determined by flow cytometry. Determinations were done before conditioning, in day of stem cell infusion (day 0 of transplant), weekly during first 4 weeks, and then monthly until six months. *Results*. Patients who developed some form of GvHD (acute or chronic) showed significantly higher levels of BAFF after transplantation (378 pg/mL vs 1314 pg/mL, $p= 0,003$) and during the first month post transplant (524 pg/mL vs 1790 pg/mL, $p<0.001$). Plasma BAFF levels were matched between both groups after the fourth month after transplantation. The recovery of CD19+ B cells tends to be significantly slower ($p=0.05$) in patients who develop GvHD during the first six months after transplantation (1m: 0,14% vs 0,31%; 2m: 0.59% vs 1.3%; 3 m: 0,72% vs 3,8%; 4m: 1% vs 3.2%; 6m: 1% vs 4.4%). We have not observed significant differences in the expression of BAFF-R between both groups. *Conclusions*. In our study, we have observed that plasma levels of BAFF are elevated from the beginning of transplantation and in some patients even before receiving conditioning regimen. The presence of high plasma levels of BAFF in the first month post trasplant is related to the subsequent development of acute and/or chronic GvHD. Like the previously published, CD19+ B cells recovery tends to be significantly slower during first six months after transplant. However and despite the differences observed between both groups in plasma levels of BAFF, we did not find differences in expression of BAFF-R.

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IMPLEMENTATION OF NEW METHODS INTO INVESTIGATIVE PROTOCOL FOR STANDARDIZATION AND OPTIMIZATION OF LEUKAPHERESIS PROCEDURES

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Background. The quality of peripheral blood stem cells (PBSC) depends on the $CD34^+$ cells yield in grafts. Timing of PBSC harvest varies - in patients on the 4th-10th day, in donors mostly on the 4th day after the beginning of growth factor mobilization. The minimal necessary yield for later autolougus or allogenic transplantations is 2 x 10^6 $CD34^+$ cells/kg. At present, the determination of $CD34^+$ cells is per-

formed by flow cytometry, which is very expensive and time consuming. Therefore the separation centers try to find less expensive and more effective leukapheresis procedures that would mean fast laboratory results, minimization of excessive administration of growth factors, and reduction of ineffective harvests. *Aims*. We introduced new methods of PBSC determination. One of them was the detection of hematopoietic progenitor cells (HPC) by hematology analyzer Sysmex. The principle of this method is exposure of the cells to high-frequency current penetrating into them and direct current flowing around them. The cells are separated according to their inner density and size. This method is fast, simple and inexpensive. Another enzymatic method is the determination of aldehyddehydrogenase (ALDH) in PBSC by flow cytometry. The third method applied is the determination of $CD133^+$ cells by flow cytometry. *Design and Methods*. We examined a group of 54 persons (M30, F24, aged 20-67, median 56 yrs) suffering from multiple myeloma (n=28), lymphoma (n=19), acute leukemia (n=1), solid tumor (n=2), and healthy donors (n=3). In the period from August 2007 to December 2008, we performed 159 leukaphereses (the mean 3 at one person). We determined the PBSC count in the peripheral blood (PB) before, and in the cell concentrate (CC) after the collection, making use of all the methods. We correlated new methods with the CD34+ cells method using Spearman rank correlation coefficient. Further, we evaluated the sensitivity and specificity of the HPC method. *Results*. The correlation of CD34+cells vs. HPC (R=0.592) and $CD34^+$cells vs. ALDH (R=0.574) in PB was good, and the correlation of $CD34^+$ vs. CD133+cells (R=0.907) and $CD34^+$ vs. $Cd34^+/CD133^+$ cells (R=0.915) in PB was very good. The correlation after separation in CC was very good in $CD34^+$cells vs. ALDH (R=0.865), $CD34^+$ vs. CD133+cells (R=0.971) and $CD34^+$ vs. $Cd34^+/CD133^+$cells (R=0.972), the correlation $CD34^+$cells vs. HPC (R=0.244) was, however, very low. We found that with increasing HPC count in specimens the sensitivity of HPC method decreases and the specificity increases. *Conclusions*. The HPC method appears acceptable for the improvement of leukapheresis procedures. The HPC count 30/mL (sensitivity 92.3%, specificity 60.3%) in PB may be considered the minimum count to start the PBSC collection. The HPC count 10/mL (high sensitivity 96.9%) in PB may be the limit to exclude the collection and investigation of $CD34^+$ cells. The HPC method cannot substitute the determination of $CD34^+$ cells in CC. Flow cytometry methods are comparable with the $CD34^+$cells method (correlation, time, costs) but disadvantageous for investigation of PB. The ALDH method may be, however, suitable for determination of the PBSC vitality in CC.

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CLINICAL RESULTS OF DONOR LYMPHOCYTE INFUSIONS (DLI) AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: ROLE IN THE TREATMENT OF MIXED CHIMERISM AND RELAPSE

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Background. DLI have proven to be effective in different settings after allogeneic stem cell transplantation (SCT) such us treatment of leukemic relapse, especially in CML, and management of persistent mixed chimerism (MC). Few reports have evaluated its usefulness in the setting of graft rejection. Aim. To evaluate the role of DLI in the treatment of persistent MC, MC with incipient graft rejection, and the treatment of disease relapse after allogeneic SCT. Design and Methods. 32 patients (pts) who underwent allogeneic SCT between 1996 and 2008 were retrospectively studied. Pts characteristics are shown in Table 1 and 2. Thirteen pts received a myeloablative SCT from an HLA-matched sibling donor, 2 from an unrelated donor, and 2 from a haploidentical donor. Six patients underwent a RIC SCT. Seven patients received CD3 depleted grafts. *Results*. A total of 12 DLI were used to treat MC in 9 pts: in 5 pts due to persistent MC and in 4 pts with MC in the context of incipient graft rejection. Five pts of this group had received a manipulated graft (CD3 depleted). CD3 doses and time of infusion are shown in Table 1. Seven out of 9 pts (77%) showed response (return to CC in PB and/or BM with normal blood count) after a median of 100 days (61-228). Two patients did not respond: an AML pt who relapsed shortly after DLI and a pt with hamophagocytic syndrome as underlying disease. GVHD rate was 77%, mostly acute grade II-III. After a median follow-up of 34 months (1.8-106), 4 pts are alive with an OS of 44%. On the other hand, 30 DLI were used to treat relapse in 14 pts, 9 of which received an escalating dose scheme (Table 2). Interestingly, a high proportion of the underlying disease of this group of pts were lymphoid malignancies (9/14). Only two pts had received a manipulated graft for SCT. Six patients received chemo/radiotherapy for the relapse before DLI. Only 5 out of 14 pts (35%) showed response, among them 2 were CML-CP pts. GVHD rate was 50% (acute II-IV in 4 pts and chronic in 2). This lower incidence could be associated to shorter survival in this group after DLI. After a median follow-up in this group of 21 months (2.9-99), only 3 pts are alive, two of them in remission. *Conclusions*. Although these results arise from a heterogeneous and small group of patients, in our experience, the clinical benefit of DLI after allogeneic SCT is limited to the management of MC, both persistent MC and MC with incipient graft rejection. DLI was not successful in the management of relapse, even in pts receiving chemo/radiotherapy before. These data suggest that novel strategies such us prophylactic DLI should be considered in high risk pts instead of DLI in visible phase.

Table 1. DLI in mixed chimerism. Total = 9 patients.

N	Disease	Status pre-DLI	N° DLI - CD3 dose/kg	Day of DLI post SCT	Response	GVHD	Time to R (days)	Status post-DLI
MA HLA-Id Sib 6	3 AML 1 NHL 1 Histio.hemophag 1 HPN/MDS	4 Persistent MC 2 MC with IGR	9 DLI: 4 pts 1 DLI (1x10e6 - 1x10e7; 2 pts ED (1x10e6 - 10x10e6)	65 (54-215)	4R 2NR	1 cG-HD cL 3 aG-HD II-III 2 NA GVHD	100 (61-228)	2 alive 4 dead
HLA-Haploid 1	AML	MC with IGR	1 DLI 2x10e4	63	R	aG-HD II-III		alive
RIC HLA-Id Sib 2	AML MDS	MC with IGR Persistent MC	1 DLI 1x10e7 1 DLI 1x10e6	35 128	2R	2 aG-HD II-III		dead alive

MA: myeloablative; RIC: reduced intensity conditioning; MC: mixed chimerism; IGR: incipient graft rejection; ED: Escalating doses; R: response; NR: non response

Table 2. DLI in relapse. Total = 14 patients.

N	Disease	Therapy before DLI	N° DLI - CD3 dose/kg	GVHD	Response	Time to R (days)	Status post-DLI
MA HLA-Id Sib 7	1 NHL 2 CML 4 ALL (1 Ph+)	6	21 DLI: 3 pts 1 DLI (1x10e6-5x10e7; 5 pts ED (1x10e7-1x10e8)	3 NA GVHD 2 cGVHD 2 aG-HD II-III 1 NA GVHD 1 aGVHD IV	7 NR 2 R	181 (137-225)	8 dead 1 alive
MUD 2	1 HD 1 ALL						
HLA-Haploid 1	CML	-	ED (2)	-	R	250	1 alive
RIC HLA-Id Sib 4	2 NHL 2 AML	-	7 DLI: 2 pts 1 DLI (1x10e7-1x10e8; 2 pts ED (1x10e7-1x10e8)	2 aG-HD II-III 2 NA GVHD	2 R 2 NR	31 (23-40)	3 dead 1 alive

MA: myeloablative; MUD: matched unrelated donor; RIC: reduced intensity conditioning; ED: Escalating doses; R: response; NR: non response

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HIGH CD4/CD8 RATIO IN ALLOGRAFTS PREDICTS ADVERSE OUTCOMES IN UNMANIPULATED HLA-MISMATCHED/HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR CHRONIC MYELOID LEUKEMIA

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Background. HLA-mismatched/haploidentical hematopoietic stem cell transplantation (haplo-mismatched HSCT) has improved the outcome of chronic myeloid leukemia (CML) in patients without an HLA-matched donor [*Ann Medi 2008,40:444*].

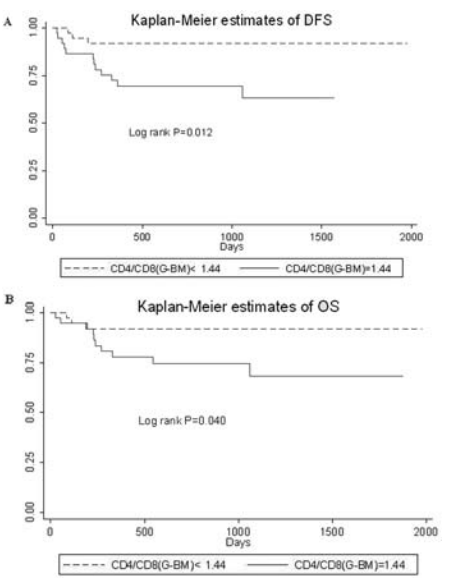

Figure 1. Kaplan-Meier estimate of disease free survival.

Aims. To further improve the treatment outcome of haplo-mismatched HSCT in CML, a modiable prognostic factor needs to be found. *Design and Methods*. The cellular composition of grafts obtained from 75 HLA-mismatched/haploidentical related donors was prospectively correlated with the outcome of patients with CML undergoing haplo-mismatched HSCT following a modified regimen of BU/CY 2 plus antithymocyte globulin. *Results*. The concentration of T-cell subsets, $CD14^+$, and $CD34^+$ cells and their relative proportions were analyzed. In univariate analyses, disease free survival (DFS) and overall survival (OS) conversely correlated with the CD4/CD8 ratio in primed bone marrow graft (G-BM) (p=0.012 and p=0.040); similarly, CD4/CD8 ratio in total grafts was also negatively associated with DFS and OS (p=0.018 and p=0.020). In multivariate analyses, a CD4/CD8 ratio in G-BM higher than the median value remained the only factor negatively affecting DFS (p=0.030; 95% confidence interval [CI]:1.166-19.341) (Figure 1A and 1B). Expectedly, high CD34+ cell dose was associated with accelerated platelet engraftment (p=0.009; 95% CI=1.181-3.271), after controlling for a high risk of disease. No other clinical parameter was influenced by graft composition. *Conclusions*. Our results suggest that a high CD4/CD8 ratio in allografts may predict adverse survival in patients undergoing an haplo-mismatched HSCT.

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FACTORS PREDICTING ALLOGENEIC PERIPHERAL BLOOD STEM CELL (PBSC) MOBILIZATION AFTER G-CSF TREATMENT IN HEALTHY DONORS

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G-CSF mobilized PBSCs are increasingly used in allogeneic stem cell transplantation (allo-SCT) due to their relative ease of collection and because higher $CD34^+$ cell doses may be associated with improved transplant outcomes in some settings. However, some healthy donors may show poor mobilization response to G-CSF and poor subsequent CD34+ apheresis yields. Therefore, identifying donors at risk for poor mobilization and a reliable estimate of a healthy donor's $CD34^+$ cell mobilization response to G-CSF and subsequent $CD34^+$ cells yield could be of value in optimising transplantation approaches. The aim of this single centre report was to analyze factors associated with PBSC mobilization and yield in an ethnically homogeneous caucasian population (n=95; 53% males) of healthy allogeneic adult donors. All donors received G-CSF dosed at 10 μg/kg/d for 5 days followed by large volume leukapheresis. Complete blood counts (total WBC, Hb, Platelets) were measured for all donors at baseline before G-CSF administration, before and after PBSC collection. Peripheral blood $CD34^+$ cell counts were also measured prior to apheresis. 69 donors (73%) were healthy sibling donors, while 26 (27%) were healthy volunteer donors for HLA-identical unrelated transplants. All donors were undergoing their first PBSC mobilization. In this cohort, donors' demographic characteristics were as follow (median; range): age, 47 (18-81) years, weight, 69 (43-106) kg, height, 170(150-187) cm, body-mass index (BMI), 23.9 (15.2-35.7) kg/m^2. As per institutional policy, the targeted total number of $CD34^+$ stem cells was between 4 and 8x10^6/kg recipient body weight to be collected in a maximum of 3 apheresis sessions. Overall, the median number of collected $CD34^+$ cells was 6.25x10^6/kg (range, 1.7-16.6), with 16 donors (17%) yielding less than 4x10^6/kg $CD34^+$ cells. In univariate analysis, female gender, lower weight, height, pre-G-CSF and post G-CSF Hb levels, and low CD34+ cell counts prior to first apheresis, were associated with significantly lower total $CD34^+$ stem cells yields (<6.25x10^6/kg). In multivariate analysis, male donor gender and higher post-G-CSF CD34+ cell counts prior to the first apheresis were most strongly associated with a higher total number of collected $CD34^+$ stem cells (OR=6.17, 95%CI (2.39-15.93), p=0.0001; and OR=3.95, 95%CI (1.53-10.19), p=0.004 respectively). Also, when considering the group of 16 "poor" mobilising donors ($CD34^+$ stem cells yield <4x10^6/kg), in multivariate analysis, we found that a higher post-G-CSF CD34+ cell count prior to the first apheresis was the strongest parameter significantly associated with a higher total number of collected CD34+ stem cells (OR=6.36, 95%CI (1.68-24.15), p=0.006). *Results*. From our study indicate that a quick assessment of risk for poor mobilization response in healthy donors can be achieved through simple demographic and routine parameters. Indeed, knowledge of predictive factors for mobilization to G-CSF may be of high interest, with the development of newer mobilizing agents like CXCR4 antagonists, and may result in overall benefit both to donors and patients

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HAPLOIDENTICAL STEM CELL TRANSPLANTATION: A SINGLE CENTER EXPERIENCE

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Introduction. Over the last years haploidentical stem cell transplantation (SCT) has become a treatment option for patients without a full matched donor. The availability of a partially mismatched related donor in most cases has potential advantages in this setting. *Aims and Methods.* We performed a retrospective analysis of all haploidentical allogeneic SCT performed in our center between 1999 and 2008. *Results*. In this period 468 allogeneic SCT were performed in 402 patients. Of these, 16 procedures (3,4%) were haploidentical and performed in 15 patients (8 males and 7 females), with a median age of 6 years (range: 6 months to 40 years old). Diagnosis were: primary immunodeficiency: 2 patients; congenital amegakaryocytic thrombocytopenic purpura: 1; acute myeloid leukaemia: 7; myelodisplastic/ myeloproliferative syndrome: 1; myeloid metaplasia: 1; Ph^+ chronic myeloid leukemia: 1; and acute lymphoid leukaemia: 2. Haploidentical SCT was performed as the first allotransplantation option in 11 patients, and in 5 as salvage therapy after graft failure of a previous transplant. All the patients had one of their parents as donor (mother in 6, and father in 9). Graft consisted of mobilized peripheral-blood progenitor cells in all cases. Patients received a median of 10.5x10^6 $CD34^+$ cells per kilogram (range: 2,86 to 15,2x10^6), after positive CD 34^+ *in vitro* selection. A median of 1.5x10^5 (range: 0.5 to 11.4x10^5) of $CD3^+$ cells was infused. Nine conditioning regimens were nonmyeloablative and 5 were myeloablative. In 9 patients bussulfan based regimens have been used. TBI was not performed. Alentuzumab as part of the conditioning was used in 6 transplants and ATG in 7. No conditioning was made in 2 cases: one primary immunodeficiency; the other a second transplant performed in the same patient. Graft-versus-host disease (GVHD) prophylaxis with calcineurin inhibitor was done in 10 patients. Grade II to IV acute GVHD occurred in 3 patients, 2 of them with evolution to chronic GVHD. At 1 year post transplantation, evaluable patients presented the following median peripheral blood cell counts: 228,5/μL (range: 9 to 936/μL) of CD4+, 420/μL (range: 0 to 3888/μL) of CD8+ and 574/μL (range: 0 to 1148/μL) of CD19+. One year TRM was 27,8% (+11.9%). Death due to disease progression occurred in 3 patients (18.75%). Eleven patients (73,3%) established successful engraftment at a median of 14 days (range: 9 to 22) post transplantation. Of those who failed to engraft, 1 received a second transplant and died of the disease. Median overall survival has not yet been attained. Median follow-up of the alive patients is 2,7 years (range: 9 months to 8 years), with a 4 year overall survival of 52% (+13%). *Conclusions*. In selected clinical situations, haploidentical SCT may be considered given the facts that the probability of finding a sibling HLA-matched donor is low, that identifying an unrelated one in the acceptable time frame can be a challenge, and in some patients with rare haplotypes or of an ethnic minority this probability can be remote.

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KINETICS OF CD34 POSITIVE CELLS MOBILIZATION IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA TREATED WITH THE DHAP REGIMEN PLUS FILGRASTIM OR PEGFILGRASTIM

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High-dose chemotherapy and autologous stem cell transplantation play an important role in achieving long-term remission in certain groups of patients with non-Hodgkin's lymphoma (NHL). A potential advantage of using mobilized peripheral blood stem cells (PBSC) is the possibility of harvesting a great number of hematopoietic stem cells and committed precursors, capable of more rapid engraftment. The DHAP regimen has thus been integrated into various treatment plans tailored for NHL patients as a salvage chemotherapy and mobilizing regimen. Fil-

grastim is commonly used to mobilize PBSC. A single injection of pegfilgrastim has been shown to be equivalent to daily filgrastim in enhancing neutrophil recovery after chemotherapy, whereas the experiences with pegfilgrastim in PBSC mobilization are limited The study included 72 patients (mean age 37 years, range 17-60) with NHL (42 follicular and 30 large cells). Sixty-four patients (88.9%) had stage III-IV disease; 48 patients (66.7%) had bone marrow involvement. Systemic B symptoms were present in 42 patients (58.3%). Mobilization chemotherapy regimens were DHAP plus filgrastim in 38 patients (52.7%) or pegfilgrastim in 34 (47.2%). Pegfilgrastim 6 mg was given subcutaneously on day +6; filgrastim 5 microg/kg/day from day +6. In 65 of 72 patients a median of 12.3x10^6/CD34+ cells (range 2.5-28.9) was harvested after a median of 13 days (range 8-16 days), with a single apheresis procedure in 58 (80.6%) cases. Failure to mobilize, defined as failure to reach a circulating CD34+ cell count of 10/mcl, occurred in 5 patients (13.2%) in the Filgrastim group and 8 (23.5%) in the pegfilgrastim group (p=n.s.). No difference was found in terms of pre-apheresis CD34$^+$ cell count. The mean number of CD34+ cells collected was 13.2x10^6/kg in the pegfilgrastim group and 12.3x10^6/kg in the filgrastim group (p=n.s.). A median of CD34+ > 10 cells/microl in peripheral blood was reached in 12 days (range 8-14) in the filgrastim group and in 15 days (range 12-18) in the pegfilgrastim group (p=n.s.) The median duration of CD34+ >10 cells/microl in peripheral blood was 4 days (1-5) in the filgrastim group and 7 days (3-12) in the pegfilgrastim group (p<0,05). All patients had grade 3-4 neutropenia, median duration was 4 days. The only adverse event was mild bone pain. To date, 65 patients have been autografted with a median of 5.4x10^6 CD34+ cells/kg (range 2.5-12.9), achieving rapid and sustained engraftment. *Conclusions*. The results confirm the efficacy and feasibility of PBSC mobilization with chemotherapy and single-dose pegfilgrastim in patients with non Hodgkin lymphomas. In less heavily pretreated patients, 6 mg of pegfilgrastim after chemotherapy induced adequate mobilization, whereas after numerous cytotoxic regimens dose and schedule need further investigation.

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DOES EXTENSIVE CHRONIC GRAFT VERSUS HOST DISEASE REPRESENTS FREQUENT COMPLICATION AFTER PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN COMPARISSON TO BONE MARROW TRANSPLANT- RESULTS OF LONG TERM FOLLOW UP

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During previous years, many studies have compared efficacy of allogeneic stem cell transplantation (SCT) from peripheral blood (PB) with bone marrow (BM), but final conclusion concerning this treatment modality is still not well defined. AIM: to compare efficacy of PBSCT with BMT in the treatment of hematological malignancies with respect to engraftment, transfusion need, frequency and severity of acute (mukositis, acute graft versus host disease- aGvHD, transplant related mortality- TRM) and late complications (chronic- cGvHD, relapses) and overall survival (OS). *Design and Methods*. We have analyzed 132 patients (pts), median age 27 years (9-52), male/female 84/48, with various hematological diseases (severe aplastic anaemia- 18, chronic myelod leukemia- 31, acute myeloid leukoemia- 29, acute lymphoblastic leukemia- 38, myelodisplastic syndrome- 8, myeloma multiplex- 2, Morbus Hodgkin- 2, Granulocytic sarcoma- 2) in whom we perfomed allogeneic SCT from 1989 till 2008. In 15 pts we perfomed secondary allogeneic SCT in due to graft rejection (2) or relapses (13 pts). Pts were divided into two groups concerning SC origin- 69 pts in BM group and 63 pts in PB group. All pts had HLA-DR sibling transplant (5 singeneic, 121 fully matched, 4 mismatched and 2 haploidentical). SC were collected from BM up to standard method and from PB with one apheresis after five days aplication of granulocytic growth factor. All pts have received unmanipulated suspension of SC. Conditioning were adjusted to primary diseases and GvHD prophylaxis was mostly combination of Cyclospirine A and Metothrexate. Prevention of infections were standard. *Results*. Pts with SC originate from PB have received significantly more mononuclear cells (10.07±7.31 vs 2,33± 0.79, p<0.001) in comparisson with BM. Engraftment was more rapid (p<0.001) in the PB group approximately for 6 days. Transfusion requirements were much higher in BM group (p<0,01). Those pts had more frequent oropharingeal mukositis grade 3-4 (33,33% vs 9,5%, p<0.05). There were no difference in the incidence of acute (44,4% vs 49,2%, ns) or chronic GvHD (38,6% vs 54,5%, ns). Pts with PBSCT had significantly more frequent extensive cGvHD (29.5% vs 12,4%, p<0.05). There were no difference considering TRM (10.1% vs 15.1%, ns) or relapses (21.7% vs 22.2%, ns). Pts with BMT had better overall survival but with no statistical significancy. *Conclusions.* Results of this retrospective analysis mostly corresponds with other studies showing that PBSCT have rapid engraftment and less acute complications. PBSCT is connected with more frequent extensive chronic GvHD that is potentialy fatal, making results of this particular treatment option less better. Definite estimation of PBSCT efficacy in comparisson to BMT should be done in homologous groups.

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HIV+ AND HIV- LYMPHOMA/MYELOMA PATIENTS SHOW SIMILAR IMMUNE RECONSTITUTION (IR) AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT)

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Introduction. Short to medium term IR after ASCT in HIV+ patients with lymphoma and myeloma (HIV+pt.), remains poorly known due to the low frequency of this procedure. However, its knowledge may constitute an important factor in preventing infections and disease relapses. Aims: To analyze and compare, prospectively, the IR of HIV+ and HIV- pt. with lymphoma or myeloma after ASCT and its influence on their clinical evolution. *Design and Methods*. IR was analyzed by flow cytometry quantifying naive, memory, memory-activated, activated and effector lymphocytes at time of transplantation as well as 3 and 6 months after ASCT. Neutrophil and platelet engraftment (>0.5x10^9/L and >20x10^9/L respectively) and infectious complications were recorded. Patients in both groups had received a median number of 3 (1-3) treatment lines prior to transplant, including local involved field radiotherapy in 2 cases. All received transplantation in complete remission. HIV+pt. cohort: 3 patients (4 ASCT) with a median age of 44 years old (42-47) (1 non-Hodgkin lymphoma-NHL; 1 Hodgkin lymphoma-HL; 1 myeloma-MM). All pt. received BEAM conditioning regimen, except for Melfalan 200 in the case of MM. The HL case received a second ASCT as consolidation treatment with BuxCy conditioning regimen. All pt. underwent ASCT with a median CD34$^+$ cells of 3.75x10^6/Kg (2.45x10^6-5.8x10^6) infused. All cases were on highly active antiretroviral therapy (HAART) during ASCT, but it was discontinued (day+9 to day+19) due to liver toxicity in NHL pt. They presented undetectable viral load, except for the NHL patient who had >50 copies due to a poor adherence to treatment. All patients had more than 400 CD4$^+$ cells/mm3. HIV-pt. cohort: 4 patients (3 NHL; 1 HL) with a median age of 29 years old (22-57). All received BEAM conditioning regimen and ASCT with a median CD34$^+$ cells of 4.3x10^6/Kg (4x106-6.4x10^6) infused. *Results*. Quantification of the different lymphoid subpopulations in the two study cohorts is shown in table 1. Both cohorts reached neutrophil engraftment at a median time of 13 days (11-15). Platelet engraftment was reached at a median time of 36 days (20-78) and 15 days (13-27) in HIV+pt. and HIV-pt. respectively. In HIV+pt., MM patient suffered a pulmonary aspergillosis 36 days after ASCT treated with Liposomal amphotericin B and there were 2 cytomegalovirus antigenemia positive tests in NHL and MM pts. Both were successfully treated. At 3 months the HIV+ group had already reached the pre-ASCT levels of the analyzed lymphoid subpopulations. Lymphocyte counts of the HIV- group at the same time point were better than pre-ASCT levels. An increase in activated lymphocytes has been described in the literature in HIV+pt. significantly associated with a worse prognosis. On the contrary, in the present study, both HIV+ and HIV-pts. showed similar values of activated lymphocytes. *Conclusions*. In our experience, we do not observe any significant differences in IR after ASTC between HIV+ and HIV-, although the only pts. who had serious infectious complications were in the HIV+ group. However, a larger number of cases is required to obtain more significant results.

Table

		Before ATPB				3 months				6 months			
		HIV-		HIV+		HIV-		HIV+		HIV-		HIV+	
		%	n°/mm³	%	n°/mm³	%	n°/mm³	%	n°/mm³	%	n°/mm³	%	n°/mm³
Naive	CD4:Rak:62L+	0.29 (0.01-0.6)	7 (0-13)	0.9 (0.04-3.09)	21.5 (2-126)	0.02 (0.01-0.23)	0.5 (0.1)	0.07 (0.01-0.12)	1.5 (0-3)	0.06 (0.02-0.54)	1.5 (0.28)	0.28 (0.01-0.55)	6 (0-14)
	CD8:Rak:62L+	0.28 (0.23-0.7)	7 (5-14)	1.26 (0.2-2.3)	47 (13-101)	0.9 (0.27-1.69)	20.5 (10-48)	0.2 (0.2-1.38)	17.5 (6-28)	1.18 (1.06-2.7)	30 (41-50)	0.59 (0.34-0.55)	16.5 (14-19)
Memory	CD4:45Ro+	4.08 (3.2-7.5)	139 (125-148)	2.68 (1.2-6.37)	130.5 (99-279)	9.25 (5-11.4)	323 (186-425)	3.9 (3-7.6)	181.5 (63-214)	14.1 (5.1-17.3)	212 (200-745)	3.94 (2.28-5.6)	117.5 (52-153)
	CD8:45Ro+	3.9 (1.09-7.25)	90 (21-73)	3.24 (0.3-22.8)	182 (54-1006)	12.45 (3.9-53)	477 (213-2299)	12.8 (7.9-37.6)	310 (262-1277)	9.4 (6.8-41.9)	142 (142-1802)	8.7 (7.5-9.9)	270.5 (269-272)
Activated memory	CD4:45Ro:DR+	0.75 (0.36-0.77)	17 (11-18)	0.29 (0.04-0.47)	12 (3-15)	1.3 (0.68-2.6)	56.5 (16-129)	0.3 (0.19-1.96)	8 (5-35)	0.47 (0.4-0.55)	13.5 (6-21)	0.18 (0.09-0.23)	4.5 (3-6)
	CD8:45Ro:38+	1.25 (0.48-2.9)	28 (9-69)	1.95 (0.11-10.3)	68 (1-463)	3.94 (2.5-50.5)	162.5 (60-2478)	4.95 (1.7-29.2)	113 (49-994)	4.9 (0.73-29.4)	73 (28-1267)	1.4 (1.2-1.6)	42 (39-43)
Activated	CD4:DR:38+	0.24 (0.39-0.6)	12 (9-13)	1.21 (0.03-0.5)	27 (1-20)	0.84 (0.37-2.6)	23.5 (8-120)	0.2 (0.11-0.76)	4.5 (2-25)	0.72 (0.12-5.4)	22 (4-234)	0.11 (0.06-0.16)	3 (2-4)
	CD8:DR:38+	0.63 (0.33-2.1)	14 (6-31)	0.73 (0.03-2.04)	27.5 (1-83)	2.45 (0.65-13.4)	91.5 (15-438)	1.15 (0.18-5.3)	30 (4-181)	0.3 (0.3-4.45)	12 (12-277)	0.245 (0.23-0.46)	10 (8-12)
Effectors	CD8:57+	3.94 (0.85-4.97)	90 (17-119)	6.56 (0.3-9.7)	255 (57-427)	12.35 (3.8-29.2)	469 (139-1400)	9 (7.5-33.4)	224 (249-1137)	7.4 (3.4-35.8)	138 (133-1539)	6.8 (5.3-8.1)	206 (198-210)
	Total N° Leucocytes	2250		2250		3900		3900		3900		3150	

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IS BEAM JUST NOT GOOD ENOUGH? A SINGLE CENTER EXPERIENCE OF DIFFERENT TRANSPLANTATION STRATEGIES IN TRANSFORMED NON-HODGKIN'S LYMPHOMA

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Background. Transformed non-Hodgkin's lymphoma has a poor outcome with conventional therapy, with median survival of 11-22 months. Therefore, consolidation treatment with more intensive regimens is commonly used to improve overall survival (OS). Autologous transplantation (AuSCT) is reported to result in 5 year OS ranging from 30-70%. Myeloablative allogeneic transplantation results in 2 year OS of 39-64%, with high treatment related mortality (TRM) and relapse rates. Results are best for upfront transplantation. However, since transformed lymphoma is usually diagnosed at higher age, myeloablative allografting is not an option for most patients. Non-myeloablative allogeneic transplantation is only reported in a small series, with a disappointing OS of <20% at 3 years. Aims: Evaluation of clinical outcome of patients with transformed NHL treated with AuSCT preceded by BEAM or Zevalin-BEAM (Z-BEAM) conditioning or treated with non-myeloablative allogeneic transplantation. *Design and Methods*. In this single center retrospective analysis we reviewed clinical records of all patients with transformed NHL treated with stem cell transplantation between January 1992 and January 2009. We defined three groups: patients treated with AuSCT preceded by BEAM, treated with AuSCT preceded by Z-BEAM, and treated with allogeneic non-myeloablative transplantation after fludarabine-cyclophosphamide conditioning. EFS and OS were estimated using the Kaplan-Meier method and compared using the Breslow test. EFS is defined as follow-up until relapse, disease progression or death of any cause. *Results*. Mean age at transplantation was 53 years (range 32-67 years). The BEAM group consisted of 32 patients, the Z-BEAM group of 6 patients and 8 patients were treated with allogeneic transplantation. Mean follow up was 40 months in the BEAM and allogeneic transplant group and 6 months in the Z-BEAM group. The groups were not significantly different with respect to age, disease stage at diagnosis, lines of chemotherapy and remission status before transplantation. In the BEAM group 13 patients were in CR and 17 in PR before transplantation, with 4 patients in CR and 4 in PR in the allogeneic group. A mean of 2 lines chemotherapy were given before transplantation (range 0-6). EFS proved to be significantly better in the group after allogeneic transplantation compared to the BEAM group. (median EFS 14 vs 58 months, p=0.049, Figure 1).

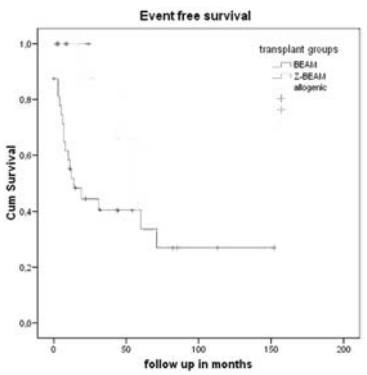

Figure 1.

Moreover, 2 year OS was also better in the allogeneic transplant group: 87% compared to 63% in the BEAM group, although this is not yet statistically significant different (p=0.09). OS and EFS were 100% in the Z-BEAM group, but follow up of this group is to short to reach statistical significance. *Conclusions*. In patients with transformed lymphoma long term survival can be reached with various transplantation strategies. In this retrospective analysis non-myeloablative allogeneic transplantation accomplished a better EFS and showed a trend towards better OS compared to AuSCT after BEAM conditioning. Since radioimmunotherapy has been introduced recently, the promising survival curve of the Z-BEAM group can only be compared after longer follow up.

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A RADIATION-FREE, TREOSULFAN CONTAINING, CONDITIONING REGIMEN IS EFFECTIVE IN HAEMOPOIETIC STEM CELL TRANSPLANT (HSCT) FOR HIGH RISK INFANT ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

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Introduction. A combination of age < 6 months, re-arrangement of the MLL gene and WCC >300x10μ/L or a poor response to the prednisone prophase identifies a very poor risk sub-set of infant ALL with a 2 year EFS of < 20%. The efficacy of allogeneic HSCT using standard myeloablative conditioning is uncertain in this sub-group. Additionally it is associated with significant toxicity including severe mucositis and veno-occlusive disease (VOD) of the liver. Treosulfan (l-threitol-1,4-bis-methanesulfonate) is a conditioning agent that promises a favourable toxicity profile compared with busulfan or TBI. *Design and Methods.* Between July 2004 and July 2008, 6 infants with ALL and high risk features (MLL gene rearrangement present in 5/6 patients, white cell count >300x10 m/L in 3/6 patients, 4 patients were <6 months of age, 2 had primary refractory disease, including the patient with germ line MLL, and one patient was transplanted in CR2) underwent allogeneic HSCT at our centre using treosulfan in place of busulfan. Donor sources included; one identical sibling and five unrelated cord blood. Of the unrelated cord transplants, 2 patients were 6/6 match, 2 patients were 1/6 loci mismatches and 1 patient was 2/6 loci mismatch. All patients received treosulfan 12 g/m^2 IV days '6, '5 and '4, and cyclophosphamide 60 mg/kg IV days '3 and '2. Four patients received additional immunosuppression (antithymocyte globulin 1-2.5 mg/kg). All patients received ciclosporin (5 mg/kg), with one patient also receiving a short methotrexate course (10 mg/m^2 days +3 and +6) as graft-versus-host disease (GvHD) prophylaxis. *Results*. Primary engraftment occurred in all patients with 100% donor chimerism at day +100. Median discharge was day +22.5 (17-57 days) with median platelet recovery >20-10^9/L at day +27.5 (18-66 days), >50 - 10m/L at day +32 (27-83 days) and neutrophil count >1-10^9/L at day +29.5 (17-53 days). Toxicity was minimal, no patients developed mucositis or VOD of the liver (despite one having recently recovered from thioguanine-related VOD). All six patients developed transient mild skin aGvHD, grade I-II. One patient relapsed and died at 10 weeks post-transplant. The remaining patients are alive in remission at a median follow-up of 25 months (range 8-56 months). *Conclusions*. Treosulfan/Cyclophosphamide is associated with minimal toxicity and provides sufficient anti-neoplastic activity in high risk infant ALL.

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INTERMEDIATE DOSE ARA-C IS A SAFE AND EFFECTIVE REGIMEN TO COLLECT PERIPHERAL BLOOD PROGENITORS CELLS IN PREVIOUS POOR MOBILIZER NON HODGKIN'S LIMPHOMA AND MULTIPLE MYELOMA PATIENTS

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Background. Autologous peripheral blood progenitor cells (PBPCs) transplantation is the best therapy for patients with relapsed/refractory hematologic malignancies. 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. *Design and Methods* Twenty patients (12 M; 8 F; median age 60 yrs; range 26-69 yrs) with hematologic malignancies: 13 non-Hodgkin's lymphoma (NHL) and 7 multiple myeloma (MM), who have failed a previous attempt between January 2007 and April 2008, were scheduled to receive intermediate dose ARA-C to mobilize PBSCs.. 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 2 chemotherapeutic regimens (range

2-6) were previously given and all patients failed prior harvesting of PBSCs. Two patients with MM were given two consecutive autotransplants with Melphalan 200 mg/mq as conditioning regimen. Collection of PBSCs was successfull in 18 out of 20 patients (90%) (13 NHL and 5 MM). Two patients whit 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 9 days (range 8-12 days) after ARA-C administration. The median number of subcutaneous injections of rhG-CSF was 7 (range 7-14). The median number of WBC count was 4550/mmc (range 2300-14800) at the time of collection with CD34+ median number of 1.53 % (range 0.3-6). In all mobilizer patients the required number of CD34 + cells were harvested after a single leukapheresis. The median number of CD34 + cells collected was $7x10^6$/Kg (range 2.41-25) with 4 as a median number of cryopreserved bags (range 2-8). All patients experienced neutropenia < 500/microL, and 7 out of 18 had febbrile neutropenia (1 to 4 days). Thirteen patients received a median of 1 packed red cell transfusions (range 1-3) and 16 patients a median of 1 apheretic platelet products (range 1-3). No patients experienced WHO grade III-IV mucositis and diarrhoea. *Results*. Our experience, showed that PBSC collection using intermediate-dose of ARA-C + rhG-CSF is safe and effective in poor mobilize patients with NHL and MM. Mobilization and collection of PBPCs were found independent from the number and type of previous chemotherapies.

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IV BUSULFAN AND FLUDARABINE (BUXFLU) AS TRANSPLANT CONDITIONING REGIMEN IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS: ANALYSIS OF A SINGLE CENTRE EXPERIENCE

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Background. Fludarabine (Flu) inhibits lymphocyte proliferation, thus causing potent immunosuppression enhancing engraftment and hematologic recovery and it is known to have a synergistic tumor-killing effect with Busulfan (Bux). Furthermore, as it has shown a safe toxicity profile, lower treatment related mortality (TRM) would be anticipated in the allogeneic transplantation setting when replacing cyclophosphamide for conditioning. Due to these properties, Flu has been successfully used in reduced intensity conditioning (RIC) regimens especially with Bux, and more recently in combination with myeloablative doses of busulfan. Aims: To analyze our experience regarding the feasibility, efficacy, and security of intravenous (IV) BuxFlu-based conditioning regimen in patients with AML undergoing stem cell transplantation.

Table.

Table: Patient features and	Auto	Allo	
		RIC	Myeloablative
n	4	5	5
Gender (M:F)	1/3	1/4	3/2
Donor source: related / unrelated	-	3/5	4/1
Disease status at SCT(n)	CR1(4)	Aplasia without blasts (2) / Progression (2) / CR1(1)	CR1(3) / MRD+ (2)
Median age in years (range)	57 (45-63)	43 (37-62)	40 (19-45)
Median time (in days) to neutrophil enfraftment (range)	14 (13-16)	18.5(11-20)	17 (15-21)
Median time (in days) to platelet enfraftment (range)	19(17-21)	12(8-14)	14 (11-15)
Mucositis: n (grade)	1(1)/3 (2)	1(1)/3 (2)	2 (1)/3 (2)
Liver toxicity: n (grade)	3 (0)/1 (1)	3 (0)/1 (1)	3 (0)/1(1)
Median follow-up in months (range)	8 (1-17)	8.5 (2-23)	12 (4-48)
Status at last follow-up (n)	Alive in CR(4)	Alive in CR(3) / Alive in progression (1) / Dead in progression (1)	Alive in CR(4) / Alive in progression (1)
Estimated OS (%) at 9 months after SCT	100%	80%	100%
Estimated PFS (%) at 9 months after SCT	100%	60%	80%

SCT: Stem cell transplantation; CR: Complete remission, MRD: minimal residual disease; PFS: progression-free survival

Design and Methods. Patients with high or intermediate-risk AML were transplanted at our center using IV BuxFlu as a conditioning regimen. The myeloablative regimen consisted of IV Flu 40 mg/m2/day and Bux 3.2 mg/kg/day given on days -6 to -3 (Total dose: Flu 160 mg/m2 and Bux 12.8 mg/Kg). RIC included: Flu 30 mg/m2/day on days -7 to -3 and Bux 3.2 mg/Kg/day on days -6 to -5 and 1.6 mg/Kg on day -4 (Total dose: Flu 150 mg/m2 and Bux 8 mg/Kg). Cyclosporine and methotrexate were used as graft-versus-host disease (GvHD) prophylaxis in HLA-identical allogeneic transplants. Thymoglobulin (ATG) (2.5 mg/kg x 3 days) was administered to those who received unrelated donor grafts. *Results*. Since March 2005 to January 2009 14 patients were transplanted using BuxFlu based-conditioning regimen. Main results are summarized in Table, according to graft type and conditioning intensity. Neutrophil and platelet engraftment occurred in all patients but 1 RIC patient who died due to disease progression on day +23. The most common conditioning-related toxicity was mucositis, present in all patients. Grade-1 liver toxicity was observed in 25% of autologous and 20% of allogeneic transplants. Three allo-grafted patients relapsed or progressed (1, 3 and 9 months after transplant). Two of them underwent rescue treatment and are alive 9 and 12 months after transplant. With a median follow-up of 9.5 (1-48) months, progression-free survival (PFS) was 100% for autologous and 70% for allogeneic transplant patients. No cases of TRM were observed. *Conclusions*. These preliminary results regarding the use of BuxFlu-based conditioning regimen in intermediate-high risk AML patients show that this approach is safe in terms of toxicity and TRM, feasible in terms of engraftment and effective in terms of disease control. These results are at least similar to those observed in patients conditioned with BuCy. Nevertheless a longer follow-up and a comparative formal analysis are needed to confirm these results.

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LARGE PERICARDIAL EFFUSION AS A MANIFESTATION OF CHRONIC GRAFT-VERSUS-HOST DISEASE AFTER ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Background. Chronic graft-versus-host disease (cGvHD) is main determination of long-term outcome after allogeneic hematopoietic stem cell transplantation (HSCT). The clinical features of cGvHD resemble those of several autoimmune disorders. Pericardial effusion has rarely been reported as a manifestation of cGvHD. We describe a patient with large pericardial effusion developing one year after HSCT due to cGvHD. *Design and Methods*. A 35-year-old woman with myelofibrosis (with extramedullary haematopoiesis and unfavourable cytogenetic abnormalities without JAK2 mutation) underwent allogeneic HSCT in July 2007 from her HLA-identical sister following myeloablative conditioning. GvHD prophylaxis consisted of mycophenolate mofetil and short-term methotrexate. The early post-transplantation phase was complicated by aGvHD treated with methylprednisolone and tacrolimus. Besides she developed a CMV reactivation requiring foscavir and cidofovir therapy. 4 months after transplantation she followed up as an out-patient with well controlled cGvHD-the dose of steroid was reduced until discontinuation in July 2008; patient remained on tacrolimus and typical antibacterial, antiviral and antifungal prophylaxis . Three weeks after steroid discontinuation she was presented to our clinic with general weakness, cough, keratitis sicca and subfebrile body temperature. Chest X-ray revealed cardiomegaly, echocardiography confirmed the presence of a large pericardial effusion with imminent cardiac tamponade.The 600 mL of haemorrhagic exudative pericardial fluid was drained. This fluid contained mononuclear cells and granulocytes without malignant cells; there were no laboratory findings suggestive of viral, bacterial or fungal infection. Our patient received 1 mg/kg mehylprednisolone intravenously and repeated echocardiography confirmed resolution of pericardial effusion. After three months after this treatment she remained in a good clinical status, without features of exudative pericarditis on small dose of oral methylprednisolone and tacrolimus. *Conclusions.* CGvHD remains the most significant complication after allogeneic HSCT although its pathophysiology is still poorly understood. Our report indicates that pericardial effusion might be a manifestation of cGvHD in the late period after transplantation. The diagnosis and distinguishing from infection could be difficult. It is worth remembering this possibility in diagnostic procedures in patients with cGvHD and atypical signs and symptoms.

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CHIMERISM STATUS ANALYSIS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION ON GRANULOCYTES AND MONONUCLEAR CELLS OF PERIPHERAL BLOOD

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Background. Chimerism Status (CS) analysis is employed to evaluate

donor cells engraftment after allogeneic haematopoietic stem cell transplantation (HSCT). The largest performed method is based on the evaluation of the DNA sequences "Short Tandem Repeats" (STR) and Variable Number Tandem Repeats" on marrow or peripheral blood derived cells. Their high polymorphism permits to differentiate donor or recipient origin of the analysed cells. PCR amplification and DNA sequencing permit their quantification. Complete CS (CCS) correlates with a favourable outcome of HSCT, conversely the mixed CS usually predicts disease relapse or transplant rejection, particularly when recipient amount rises along the time (increasing mixed CS-IMCS). AIMS: we investigated CS in granulocytes and mononuclear cells at +30, +60, +90, +120 days after HSCT. Data obtained were correlated with the different blood population at the time of analysis. *Design and Methods*. Granulocytes and mononuclear cells were obtained after double gradient centrifugation. A semi-quantitative method, based on multiplex PCR amplification of 16 STR markers using a commercial kit (PowerPlex® 16 System-Promega), was applied to define the difference between donor and recipient. We assumed the CS in granulocyte population as expression of myeloid engraftment. We also correlated the CS in mononuclear population, with the different leukocyte subpopulations (lymphocytes, monocytes) in peripheral blood count and with the reconstitution of lymphocyte subpopulations (B, T cells and NK cells) at the same time point of analysis, in order to compare the reciprocal course. We evaluated 22 mieloablative HSCT performed on 21 patients (1 was double transplanted for a rejection) affected by onco-hematologic disease in first (14) or next (6) remission. 14 HSCT were from sibling related donor (SRD) and 8 from matched unrelated donor (MUD). All the MUD-HSCT patient and one of the SRD-HSCT received Anti-Thymocyte-Globulin (ATG), 13/14 SRD-HSCT did not received ATG nor other T-depletion procedures. *Results*. 20/22 HSCT were evaluable in every four timepoints. In granulocytes CCS was observed in 19/22 HSCT since the first control, 3/22 evolved to CCS in the successive times, 1 evolved to IMCS. In mononuclear cells 13/22 HSCT presented stable CCS since the first control, 3/22 evolved to CCS in the successive times, 2/22 presented stable mixed chimerism. 4/22 HSCT presented IMCS with 2 relapse and 1 rejection,. Considering the different leucocyte populations we observed that the mononuclear cells amount was constituted for only 50% from lymphocytes, the remaining 50% was represented from monocytes. Therefore the analysis of lymphocyte subpopulations showed that T-lymphocytes were virtually absent (either T4 or T8) till to +120 in patients who received ATG. In these ones CS analysis in mononuclear cells was virtually performed only in monocytes, B and NK cells. Conversely patients who did not received ATG presented complete and more rapid reconstitution of all the lymphocyte subpopulations. *Conclusions*. our method is simple and rapid in analyzing CS. Granulocytes CS reflect the engraftment of myeloid lineage. Instead CS in mononuclear cells must be considered in the context of the type of transplantation (MUD or SRD, T-depletion or T repletion) and correlated with the single subpopulations of peripheral blood.

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UNCONTROLLED-RATE FREEZING BY IMMERSION IN A METHANOL BATH OF PERIPHERAL BLOOD STEM CELLS (PBSC) FOR TRANSPLANTATION AT HIGH CELL CONCENTRATION IN 5-PERCENT DMSO. SINGLE-CENTRE EXPERIENCE

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Background. In last years, new and simpler methods for cryopreservation of PBSC and freezing techniques are still being developed. In particular, non-programmed cell freezing at -80°C after addition of autologous fresh plasma with DMSO in a methanol bath permit to obtain constant cooling rates (mean velocity of 1.2°C/min) and clinical results similar to programmed cryopreservation (F. Hernández-Navarro et al., 1998). On the other hand, previous studies have demonstrated that PBPC can be cryopreserved at higher cell concentrations than classically recommended without loss of engraftment. *Design and Methods.* We retrospectively analyzed the dates of 45 PBSC collections from 25 valuable patients (pts) autotrasplanted between September 2007 and February 2009, as well as their haematological recovery (1 out of the 26 pts was discarded from the analysis for death before engraftment due to a lung infection). 14/25 pts were male. All the pts, except one (solid tumor), have haematological diseases. Median age was 52 years (22-69). Mobilization was performed with G-CSF (10-20 mg/Kg/d) alone or in combination with chemotherapy. Harvesting was performed with a COBE-Spectra cell separator. Before freezing, the cell count was adjusted with autologous plasma to a concentration of 250-300 x 106/ml. Thereafter PBSC were mixed with equivalent volumes of a DMSO/plasma solution to obtain a 5% final DMSO concentration. Finally, cryopreservation was carried out by freezing cells to -80° in a methanol bath and non-programmed freezer. After 24 h, the pack was placed in liquid N2 container and stored. The stem cells CD34+ were analyzed according to a method based on the ISHAGE guidelines. *Results*. The mean number of procedures needed to harvest an appropriated number of PBSC was 1.92±0,9 (1-4). The cell concentration was 283.65±50.3x10^6/mL (159-380.5; median 291.5). Patients received a mean of 6.59±3.8x10^8 MNC/kg (0.76-12.9; median 6.17) and 4.71±2.6x10^6 CD34+/kg (1.8-11.3; media 3.88). Viability after thawing was 90.7% (74-99; median 90%). All patients showed rapid and sustained engraftment. The mean time to granulocyte engraftment >0.5x10^9/L and platelet engraftment >20x10^9/L was 12.08±2.5 (9-20; median 11) and 14.04 (7-25; median 13) days, respectively. Most of these pts are alive and in remission. *Conclusions.* Our results confirm this procedure as very simple, safe and cost-effective for PBSC cryopreservation and later autologous transplant. Moreover, it diminishes the amount of DMSO infused to pts, as well as its toxicity.

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THE EFFECT OF TOTAL PARENTERAL NUTRITION ON ENGRAFTMENT IN ADULT HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Mucositis caused by chemo- and radiotherapy as a part of conditioning regimens of hematopoietic stem cell transplantation (HSCT) frequently lead to oral nutritional problems. The patients often need to take total parenteral nutrition (TPN) during the course of mucositis. Aim: We investigated the possible effect of TPN on engraftment time based on our clinical observations. *Design and Methods*. Engraftment time of patients who received TPN (TPN+) during HSCT (n=18) were retrospectively compared with those did not (TPN-) (n=20). Oliclinomel® N7 emulsion for infusion (Baxter) was used as a TPN solution at a dose of 1000-2000 mL/day with various durations. *Results.* There was no significant difference between TPN+ and TPN- groups according to transplantation type, conditioning regimen, and initial diagnosis. The engraftment time of granulocytes and platelets were found to be longer in TPN+ patients (median 16.5 vs 11 days, p=0.016 and 19 vs 14 days, p=0.004, respectively) (Figure 1).

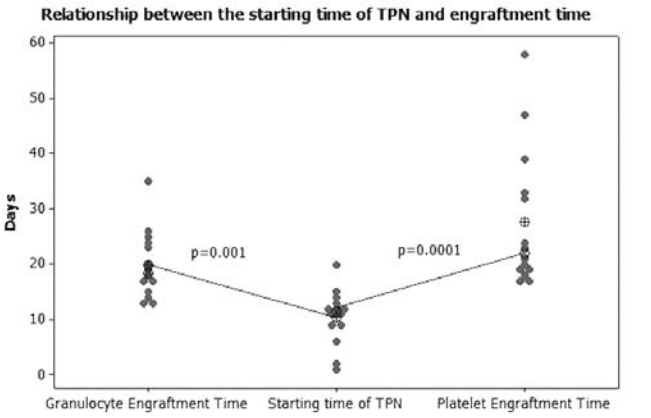

Figure 1.

Severe mucositis, the use of morpine and anaerobic antibiotics were also more frequent in TPN+ group (100% vs 60%, p=0.004, 56% vs 10%, p=0.008, 33% vs 0%, p=0.007, respectively). Survivors in TPN+ group were significantly lower than those in TPN- group (66% vs 100%, p=0.001) . *Conclusions*. The use of TPN was observed to prolong time-to-engraftment in patients after the HSCT. The patients who needed TPN had frequently more severe mucositis, hence it was not surprising that these patients had more nutritional problems and were prone to risk of infection. Thus these factors could have influenced the engraftment time and survival. But because TPN solutions are not fully replacible with oral nutrition, ingredients of TPN solutions and proportions probably have an effect on engratment time in HSCT patients.

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USE OF GLUTAMINE IN ALLOGENEIC STEM CELL TRANSPLANTATION - EFFECT ON RELAPSE RISK AND OVERALL SURVIVAL

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Background. Veno occlusive disease (VOD) of the liver arises following damage to the sinusoids and hepatic venules. One proposed mechanism leading to VOD post allogeneic transplant is depletion of glutathione (GSH). GSH and GSH-dependent enzymes are important antioxidants that play a role in conversion of drugs to non toxic metabolites in the liver and glutamine is rate limiting for the supply of GSH. Studies have suggested a possible protective role for glutamine supplementation in prevention of VOD. However, there are theoretical concerns that glutamine supplementation may increase tumour growth and this may limit its use clinically Aims: This study assessed whether use of glutamine as prophylaxis for VOD increased the risk of relapse post allogeneic transplant. *Design and Methods.* We analyzed the outcome of 261 patients who underwent allogeneic stem cell transplant within our centre from January 2000 until July 2007. Patients felt to be at high risk for development of VOD received glutamine prophylaxis, administered at a dose of 20g IV until time of engraftment. 114 (44%) patients received glutamine and 147 (56%) patients did not. *Results*. The median follow up of all patients was 1580 days. Follow up was less in the glutamine arm as it was not introduced into use until 2002. The median overall survival of the patients who received glutamine was 537 days compared to 878 days for those patients who did not receive glutamine (p=0.075, log rank test). There was only one death secondary to VOD in the glutamine arm. There was no increase in risk of relapse between the two treatment arms - 25% of the patients treated with glutamine relapsed compared to 26% of patients who did not receive glutamine (p=0.646). Subgroup analysis according to transplant type (sibling or unrelated) or conditioning regimen (myeloabalative or reduced intensity) has no increase in relapse risk between the two treatment arms. Subgroup analysis according to stem cell source has shown that there was an increase in relapse risk in the glutamine arm in patients who received bone marrow as the stem cell source (p=0.045). While not significant, the difference appeared more marked in those that received myeloablative conditioning and glutamine supplementation. *Conclusions*. This study has demonstrated that overall, there has been no increase in relapse risk seen and no significant decrease in overall survival in patients who received glutamine prophylaxis for prevention of VOD. There was an increased risk of relapse in patients who received glutamine who had bone marrow as a stem cell source. It is possible this could relate to a less marked GvL effect and more reliance in the efficacy of the conditioning regimen in this group although further studies are required to determine the true significance of this. Glutamine administration appears to be effective at preventing VOD and there is no increase in the risk of relapse in patients receiving PBSC transplants. However, glutamine should be used with caution in patients receiving bone marrow as the stem cell source.

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OVERNIGHT STORAGE OF CORD BLOOD CELLS BEFORE CRYOPRESERVATION DOSE NOT AFFECT SHORT & LONG-TERM REPOPULATING CAPACITY

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Background. We need to know whether overnight storage of cord blood cells affect short and long-term repopulating capacity or not because delivery may happened at night and holiday. We used UCB mononuclear cells for this study because mononuclear cells, not $CD34^+$ cells, usually were stored in clinical cord blood banks. *Design and Methods*. Five individual UCB were analysis by colony assay, apoptotic cell counts, and long term bone marrow culutre under four different conditions (fresh, overnight, immediate cryopreserved, overnight cryopreserved). *Results*. The numbers of colony forming unit-granulocyte macrophage(CFU-GM) were 116.2±20.1 in the fresh and 90.8±15.8 in the overnight storage group. There is no staistically difference. After cryopreservation, the numbers of CFU-GM were similar between immediate and overnight storage group, 86.9±31.2 in the initial and 83.8±37.2, respectively. Immediate cryopreservation group had significantly lower numbers of CFU-GM compared with fresh group(p<0.05). The apoptic cells were detected 21±6.8 % in the fresh and 24.2±2.4 % in the overnight storage group. There is no staistically difference. After cryopreservation, the apoptic cells were similar between immediate and overnight storage group, 32.6±11.5% and 31.7±8.2 %, respectively. Fresh group had significantly lower numbers of apoptic cells compared with immediate cryopreservation group(p<0.05). After long term stromal based culture, the mean production of CFU-GM colonies were the similar in all groups (P>0.05). *Conclusions*. This results shows that cryopreserved cord blood mononuclear cells affect short term repopulating capacity, but, does not affect long term repopulating capacity. Overnight storage before cryopreservation does not affect short and long term repopulating capacity. These results support the continue use of overnight storage of UCB before cryopreservation as a convenient and cost reduction measure.

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HYPOCALCEMIA-DERIVED COMPLICATIONS ARE AVOIDED WITH A MODIFIED TECHNIQUE FOR PERIPHERAL BLOOD STEM CELLS COLLECTION: A SINGLE CENTER EXPERIENCE

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Background. Peripheral Blood Stem Cells (PBSC) collections might be complicated by symptomatic hypocalcemia or secondary effects due to calcium-supplements. In December 2006, we decided to modify our technique for PBSC collections after observing several cases of severe symptomatic hypocalcemia or calcium-supplements secondary effects. Aims Analyze the efficacy and safety of a modified technique to reduce the total amount of the sodium citrate dextrose (ACDA) anticoagulant administered during the lymphocytoapheresis to collect PBSC in candidates for autologous PBSC transplantation. *Patients and Methods*. Consecutive PBSC collections performed at our department with a modified-technique to avoid symptomatic hypocalcemia or calcium- supplements secondary effects. Briefly the modified-technique consisted of the addition of 3,000 units of sodium heparin at 1% inside the bag containing 500 milliliters (mls) of ACDA, modifying the fixed ACDA ratio from 16 to 24 (1 mL of ACDA per 23 mls of blood) and reducing the total amount of ACDA administered during the lymphocytoapheresis. We did not perform calcium monitoring during the procedure and oral or intravenous replacements of calcium were avoided. *Results*. From December 2006 to February 2009, 31 consecutive PBSC collections were performed in 20 patients (11 males and 9 females), median age of 49 (23-65) years at collection, by using with the described modified-technique. Hematological diseases were as follows: 12 multiple myeloma, 4 non-hodgkin's lymphoma, 3 hodgkin´s lymphoma and 1 acute myelogenous leukemia. In 17 patients, PBSC mobilization consisted of only filgrastim at a median dose of 12 mg/Kg/day for 4 consecutive days, starting apheresis on the 5 th day. A semirigide Sheldom catheter was placed on a femoral vein (18 patients) or subclavian vein (2 patients). A median of 1 (range 1 to 3) lymphocytoapheresis procedure was performed per patient. The median blood volume processed per patient was 12,06 liters (6,60-15,67). Median ACDA volume administered per apheresis was 538 milliliters (227- 924). As a result of PBSC collections we obtained a median MNC of 7,84 x108/Kg (1,65- 18,72) and CD34+ of 3,55x106/Kg (1,03- 7,41). No cases of symptomatic hypocalcemia or calcium-supplements secondary effects were observed. The major secondary effects observed were 2 cases of femoral catheter thrombosis on the second day of apheresis. No clinically significant haemorragic complications were observed in our series. *Conclusions*. We conclude that this modification of the classical technique for PBSC collection is safe, effective and avoid calcium monitoring and hypocalcemia-derived complications.

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THE EFFECT OF OMEGA-3 AS A DIETARY SUPPLEMENT IN A MOUSE MODEL OF STEM CELL TRANSPLANTATION (SCT)

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Background. Several studies reported the importance of nutrition on SCT outcome. Many food components have been shown to affect the immune system and to interact with a number of drugs used in conditioning and in prophylactic treatment during SCT. Omega-3 fatty acids have immuno-modulatory effects via improving endothelial function, protection against oxidative DNA damage and by suppressing $CD4^+$ T-cell function and proliferation. In the present study we examined the effects of omega-3 on the immune system in mice undergoing SCT. *Aims*. To evaluate the effect of omega-3 on conditioning regimen To evaluate the effect of omega-3 on bone marrow transplantation *Method*. Female BALB/c mice, 8 - 12 weeks, were randomised to receive either diet enriched with menhaden fish oil (omega-3) or, corn oil (rich in omega-6). Both groups were fed for two weeks prior to conditioning (Day 0 was set to be the day of transplantation). Both groups injected intraperitoneally with 80mg/kg busulfan (Bu) for four days (day -7 to day -4) followed by 200 mg/kg cyclophosphamide (Cy) for two days (days -3 & -2). At day 0, half of the mice of both groups were killed and bone marrow, spleen and thymus were harvested and analysed with FACS to evaluate the lymphoid and myeloid cell phenotype. Serum was collected for analysis of cytokines. The remaining mice in both groups were transplanted with allogeneic bone marrow from 8-12 weeks male C57BL/6 mice ($2.0x10^7$ bone marrow cells and $3.0x10^7$ spleen cells). *Results*. Mice fed an omega 3-rich diet showed significant decrease in bone marrow and spleen cellularity compared to that observed in the corn oil group. In particular, the splenic B-cell population was severely affected, showing a 50% decrease in the omega-3 group compared to that seen in corn oil group. The T-cell populations of omega-3 fed mice were also affected, with a significant decrease by 10 to 20 % in the number of $CD4^+$ cells. In contrast, no difference was observed for CD8+ cells, compared to corn oil-fed mice. In the thymus, the number of activated T-cells was decreased by 50% in the omega-3 compared to that observed in corn oil group. NK cells were decreased by 50% in the spleens of omega-3 fed mice. In bone marrow, the myeloid cells ($CD11b^+$) were significantly lower in omega-3 fed mice compared to corn oil-fed controls. All transplanted mice that had received an omega-3 enriched diet died within the first week. These data can be compared to our previous results where more than 60% of mice receiving standard diet survive during the first week and of which at least 20% reach day+21. *Conclusion.* Omega-3 administration as a dietary supplement for two weeks prior to conditioning regimen showed significant effects on the immune system in terms of a pronounced alteration in the balance of different cells lineages in both bone marrow and spleen. Rapid death of omega-3 fed animals after allogeneic transplantation was observed. Further studies are warranted to investigate the role of omega-3 on the outcome of stem cell transplantation

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INFLUENCE OF STEM CELL SUBSETS ON MARROW REPOPULATION CAPACITY OR SOLID ORGAN REGENERATIVE POTENTIAL

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Background. Stem cells (SCs) could be defined as cells having 'unlimited' self-renewal and multilineage differentiation capacity, as well as extensive proliferative potential which guarantee the homeostasis of the hematopoietic or other tissue-generating systems. The clinical use of SCs represents an unique and well-working regenerative therapeutic maneuver. Aims: to determine SC-harvesting protocol with optimized cell source, collection time-point, CD34+ threshold-dose, as well as to evaluate the effects of immature vs. mature SC (CD34+ cell) subset ratio on the SC-therapy outcome. *Design and Methods*. Patients with hematological malignancies and myocardial setting (myocardial infarct, coronary-bypass) as well as liver failure, underwent to allogeneic (120 pts) or autologous (120 pts) SC-transplants and cell-based regenerative therapy (50 pts) were included in this study. SCs were mobilized by rHuG-CSF 5-10 microg/kgbm (allogeneic) or chemotherapy plus rHuG-CSF 8-16 microg/kgbm (autologous) and rHuG-CSF 10 microg/kgbm (cell-based therapy). SCs were harvested from peripheral blood using Cobe-Spectra (SC-transplants and liver cell-therapy) or collected by aspirations from marrow (myocardial setting). Total nucleated and mononuclear cell (TNC and MNC) count was determined by flow cytometry. Cell viability was measured by AAD-viability-dye test. The SC surface antigens (CD34-subsets markers) by the EPICS XL-MCL device were investigated. For cryopreservation, our own controlled-rate freezing protocol (with optimized DMSO) using Planer 560-16 equipment was applied. *Results*. In SC-transplant setting, the CD45+/CD34+ yields were $12.3\pm6.4x10^6$/kgbm vs. $10.8\pm3.4x10^6$/kgbm (allogeneic vs. autologous), respectively. In myocardial cell-therapy setting, the quantity of TNCs and $CD45^+/CD34^+$ cells applied was $24\pm21x10^7$ and $8.4\pm5.2x10^6$, respectively. The count of collected $CD45^+/CD34^+$ cells for liver cell-therapy was $10.3\pm4.5x10^6$. The use of large volume vs. conventional (standard) cell harvesting resulted with higher immature vs. mature CD34-subset ratio. The $CD45^+/CD34^+$ percentage was elevated in marrow vs. peripheral blood harvest. Quicker hematopoietic reconstitution was obtained when SCs from peripheral blood were applied (for leukocytes on the 9.4th and for platelets on the 12.9th day). Cell-therapy resulted in a improved myocardial perfusion and systolic activity, as well as liver function repair. All patients tolerated the use of intensive cell-therapy well, without any adverse effects. *Conclusions*. The use of large volume vs. standard apheresis resulted in improved $CD34^+$ cell yield and better immature vs. mature CD34-subsets ratio, which following superior marrow repopulation capacity. Marrow-derived SCs assured better organ repair due to higher tissue-colonizing and lineage-plasticity potential, although the ideal source and type of cells in the field of regenerative medicine (in myocardial or liver setting) have not been completely defined yet.

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SUCCESSFUL MOBILITATION OF PERIPHERAL BLOOD STEM CELLS USING IFE/G-CSF IN PATIENTS WITH MALIGNANT LYMPHOMA

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Background. High-dose chemotherapy (HDT) and haematopoietic stem cell transplantation (SCT) are effective in patients with relapse, refractory and chemotherapy-sensitive PR non-Hodgkin lymphoma (NHL). Several studies suggest also a benefit for patients in first CR with the presence of poor prognostic factors. Collection of sufficient numbers of stem cells is a prerequisite for such a therapy. IFE regimen has been integrated into various treatment plans tailored for NHL patients as a second line in PR patients or as intensification and mobilizing regimen in CR high-risk patients. *Aim.* We evaluate the efficacy for the mobilization of peripheral blood stem cells (PBSC) and treatment outcome of IFE (ifosphamide, etoposide) in combination with G-CSF in patients with NHL in our centre. *Patients and methods* We report mobilisation details and treatment outcome of 21 patients, median age 49 years (range 14 - 66) with NHL treated with 1-3 cycles of IFE (ifosphamide 10 g/m^2, etoposide 150 mg/m^2/12 h on days 1-3) with G-CSF 5 (n=1) or 10 (n=18) mg/kg/d, two patients received 6 mg of PEG G-CSF, between September 2002 and December 2008. 8 were female (38.1%) and 13 male (61.9%). Patients were treated with the intent to proceed to auto-SCT. All patients have written informed consent. The indication for IFE was failure to achieve CR with four previous escalated CHOP therapy plus rituximab (R-MegaCHOP or R-CHOP-14) (n=19) and front-line therapy in high-risk T-cell NHL (n=1) and relapse disease (n=1). 10 patients (47.6%) had bone marrow involvement at diagnose but neither of them at collection. At the time of PBSC mobilization 19 patients (90.4%) were in partial remission (PR), 1 (4.8%) in complete remission (CR), and 1 (4.8%) with sensitive response minor than PR. *Results*. The median time between IFE and collection was 15 days (range 13 - 22). The median total number of mobilised $CD34^+$ cells was $8.42x10^6$/kg (range 1.94 - 34.5) and the median CFU-GM colonies $12.12x10^4$/kg (range 2.37 - 22.34). Cells were collected with a median of 1 (range 1 - 5) leukapheresis per patient processing a median of 4.5 volumes (range 1.5-22). Only one patient failed a mobilization attempt (CD34 cell count $<2x10^6$/kg). 17 of the 21 (80.9%) patients achieved CR after IFE, 3 (14.3%) PR and one patient (4.8%) progressed. *Conclusions*. IFE combined with G-CSF is an effective mobilising regimen and has a satisfactory response rate in chemotherapy-sensitive patients. The patients who are successfully mobilized with this regimen and proceed to auto-SCT can achieve a sutained remission.

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HOW THE POST-THAWED CELL CONTROLS IN TEST-TUBE REPRODUCE THE QUALITY OF THE CRYOPRESERVED PBPC IN THE BAG TO BE INFUSED

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Cryopreservation can alter the quality of the cells for autologous PBPC transplantation. In most laboratories a test tube is frozen with the cells bags in order to test the effect of cryopreservation in cells. The objective of our study is to examine if the results obtained in the test tube are the same as those of the bag infused, that is to say, if the measures of the test tube indicate the real state of the cells to be infused. A total of 397 PBSC apheresis products harvested from 167 patients, 81 male and 86 female, aged 49 (2 to 71) years, were studied. A total of 119 PBSC products were harvested from 61 patients with MM, 115 from 43 patients with NHL, 54 from 26 patients with breast cancer (BC), 35 from 12 patients with HD, 28 from 9 patients with AML, and 46 from 16 patients with other diagnoses. PBPCs were obtained using a continuous blood cell separator (Fenwal CS3000; Baxter, Deerfield, IL) and were cryopreserved in special bags (Cryocyte; Nexell Therapeutics, Irvine, CA), mixed with DMSO at a final concentration of 9-10%. After DMSO mixture, 0.5 mL of the PBPC concentrates were transferred to small freezing test tubes (Cryovial; Simport, Quebec, Canada) and put into the freezing machine together with the collection bag. Cryopreservation was performed by a controlled rate freezing equipment (CM-25; Carburos Metálicos, Barcelona), lowering temperature at a rate of 1-2°C/min. CD34+ cells were measured by flow cytometry on a FACS Calibur (Becton-Dickinson, -BD- San José, CA). All PBSC products were tested for: viability (Tripan-blue exclusion), CD34+x10^6/L, lymphocytes, monocytes, granulocytes, and CFU-GM, before criopreservation and post-thawing (in tube and bag). Student-t-test for paired samples was used for statistical analysis. The study was performed in the total group of patients and in MM, HD, NHL, AML and BC groups. Freezing decreases cell viability and CFU-GM in tube and bag respect to pre-freezing samples (p<0.001); but, in MM group, viability was lower in bag than in tube (p=0.35) and CFU-GM were lower in tube than in bag (p=0.01). Granulocytes were lower post-freezing in both samples, significantly lower in tube than bag in total group (p=0.02) and MM (p=0.01). Monocytes and lymphocytes were higher post-thawing both in the cryotube and in the bag than in the pre-freezing amount in all samples, but monocytes higher in bag than in tube in MM and in NHL(p<0.01); lymphocytes were higher in bag in MM (p=0.02). The CD34+x10^6/L count was not different post-thawing compared with pre-freezing values except in he tube of MM (p<0.01), there were no differences between cryotube and bag. According to our study, the tube seems to be a good control for the most important parameters in most diseases, although it does not reflect exactly the effect of freezing in MM, even though we do not know if these findings have clinical relevance.

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THE CLINIC EFFECTIVENESS OF USING HEMATOPOIETIC STEM CELL TRANSPLANTATION TO CURE 156 CASES OF MALIGNANT HEMATOLOGIC DISEASES

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Objective. To retrospective analysis and explore different conditioning regimen and different transplantation methods to treat malignant hematological diseases and to evaluate the effectiveness and the risk associated with transplantation. *Design and Methods*. Altogether156 patients accepted transplantation, auto-HSCT without cleaning (auto-HSCT) (n=33), peripheral blood stem cells plus auto- activated bone marrow transplantation (PBSCT+ABM) (n=37), myeloablative allogenic bone marrow transplantation (allo-HSCT) (n=36), nonmyeloablative allogenic bone marrow or peripheral blood stem cell transplantation (allo-NSCT) (n=48) and cord blood stem cell transplantation (allo-CBT) (n=4). PBSCT+ABM, Auto-HSCT and allo-HSCT all applied with MAC or BuCY regimen chemotherapy. PSCT+ABM were infused with autologous peripheral blood stem cells and activated bone marrow about 750 mL .Those who accepted allo-BMT were infused on the zero day of transplantation with donor's bone marrow stem cells .The allo-NSCT was applied with two kind of low dose conditioning regimen FAAC and MAAC, after transplantation, according to the formation of chimerism to infuse donor lymphocytes as increasing depressively. Among the patients who accepted cord transplantation, two adopted stem cells from cord bank and more than four sites coincided as HLA zygosity reported , and two cases adopted sibling's cord stem cells, all of them accepted preconditioning of FAAC and procedures were the same with above mentioned. Those who accepted allogenic transplantation used Cyclosporin A and methotrexate or mycophenolic acid routinely. According to the recovery of leukocytes, platelets and bone marrow, the analysis of blood type and the formation of chimerism as the evidence of implantation successfully and aGVHD was analyzed by luckshezg standard and cGVHD were analyzed by Shulman standard. *Results.* Among the 156 patients, except one patient with CML died of bone marrow failure on the fortieth day after nonmyeloablative transplantation and Two AML patients died of cerebral hemorrhage before the recovery of haematogenesis, the other 153 cases all recovered haematogenesis as expectancy. The incidence of aGVHD and cGVHD of allogenic transplantation was 18.10% and 26.10% respectively. Until the time of collected materials, the survival statistics for those patients were 30.33% for ABMT(n=33), 64.49% for PBSCT+ABM(n=37), 69.51% for allo-BMT (n=27), 45.83% for NSCT (n=48) and 50.00% for CBT(n=4) respectively. *Conclusions.* At present, it was thought that hemopoietic stem cell transplantation was one curable methods for malignant diseases. With multiple forms of transplantation application generally, more and more patients profited from them especially the emergence of nonmyeloablative transplantation, which cased essential alteration in the domain of transplantation, it not only enlarged application and indication but also more safety and inexpensive. But relapse and GVHD remain the mainly causes of transplantation failue.

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TORQUETENOVIRUS VIREMIA LEVELS AFTER HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION CORRELATE WITH IMMUNOPHENOTYPIC MARKERS OF IMMUNE FUNCTION

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It is common experience that retreating patients too early after a course of intensive chemotherapy predisposes to opportunistic infections despite apparently normal lymphocyte levels. The extent of replication of persistent viruses that cause no obvious disease (and hence need no treatment) might serve as a marker to better define when a patient has recovered from this functional immune deficiency.

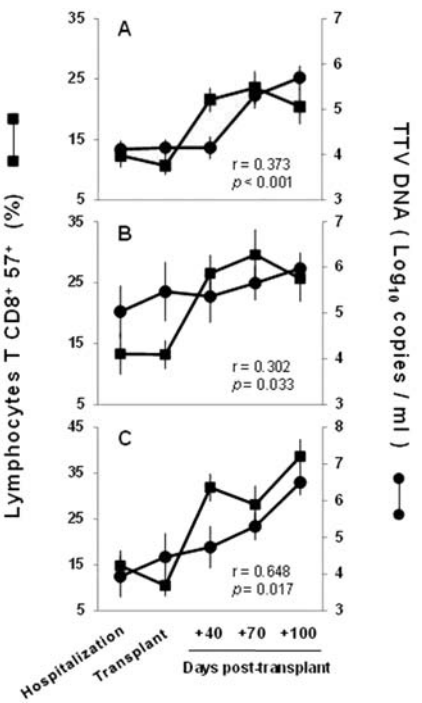

Figure.

We used real-time polymerase chain reaction to monitor the kinetics of plasma torquetenovirus (TTV) viremia in hematological patients undergoing autologous hematopoietic stem cell transplantation as support to high-dose chemotherapy (HSCT). Independently from underlying hematological disease and therapeutic regimen, TTV viremia increased post HSCT, and this increase paralleled the increase of circulating CD8+CD57+ T lymphocytes, known to represent an indirect marker of functional immune deficiency. Subsequently, within a matter of months, TTV levels returned to baseline values, at a pace that appeared to be constant over time. Collectively the findings indicate that monitoring of TTV viremia represents a unique opportunity to follow functional immune reconstitution in immunosuppressed patients. Also, the size of the TTV viremia increases resulting from immunosuppressive treatments might be of guidance in determining the appropriate time interval before delivery of a next course of therapy.

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IMMUNOLOGIC RECOVERY AFTER AUTOLOGOUS TRANSPLANT IN MULTIPLE MYELOMA AND PROGRESSION FREE SURVIVAL

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Immunologic abnormalities, such as suppression of polyclonal immunoglobulin production and decreased CD4/CD8 ratio, have been widely documented in patients with Multiple Myeloma (MM), after autologous stem cell transplant (ASCT). These abnormalities when persistent appear to be associated with disease progression. We retrospectively analyzed a series of 50 consecutive patients with MM who underwent ASCT in our centre, from 2000 to 2008, regarding recovery of IgM levels and CD4/CD8 ratio by day +100 post-transplant. Progression-free survival (PFS) was assessed from the day of the transplant to disease progression or death. The median age of patients was 57 years (range - 37 to 67), 25 were females. Median follow-up was 12 months (range - 1 to 40 months). Twenty-one (42%) patients recovered normal IgM levels by day +100 and 29 (58%) did not. Comparison of Kaplan-Meyer curves for the two groups did not show any statistically significant difference (log rank test, p=0.08) but it appears to be a difference between median PFS of the non-recovered immunoglobulin group (14 months) and the recovered immunoglobulin group (29 months). CD4/CD8 ratio by day +100 was significantly reduced (median 0.3, range - 0.12 to 1.0), essentially due to a decrease in absolute number of CD4 cells (median 262x10^6/L, range - 53 to 767x10^6/L). No association was found between CD4/CD8 ratio and PFS. In conclusion, we could not find a definite relationship between immune function recovery and PFS although there is a trend to a better outlook of patients who recover normal IgM levels shortly after ASCT. One can speculate that prompt recovery of diversity of antibody production and other immune functions may have an impact in disease outcome.

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ALEMTUZUMAB ONLY NON CHEMOTHERAPY BASED HAEMATOPOIETIC ALLOGENIC STEM CELL TRANSPLANTS

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Background. Minimally toxic conditioning regimens using low dose (200cGy) total-body irradiation (TBI) along with mycophenolate mofetil (MMF) and ciclosporin (CSP) have been used in non myeloablative haematopoietic stem cell transplants. We present for the first time haematopoietic stem cell allografts using non chemotherapy based conditioning using alemtuzumab and MMF/CSP. Aims: We present 4 cases of haematopoietic stem cell allografts using non chemotherapy based conditioning with alemtuzumab and MMF/CSP. *Design and Methods.* Patient 1 was a 58y old male who had heavily pre-treated T-cell prolymphocytic leukaemia (T-PLL) with chlorambucil, alemtuzumab, MCOP (mitoxantrone, cyclophosphamide, vincristine, and prednisolone) and cladrabine. He received peripheral blood stem cells from matched unrelated donor. Patient 2 was a 59y old male with acute myeloid leukaemia (AML) who was treated with 2 courses of FLAG-Ida and had prolonged cytopenias. He was rescued with bone marrow stem cells from matched sibling. Patient 3 was a 54y old male with acute myeloid leukaemia who had prolonged cytopenias after 2 courses of FLAG-Ida and had transplant using bone marrow stem cells from matched unrelated donor. Patient 4 was a 42y old male with chronic myeloid leukaemia which failed to respond to imatinib and transformed to myeloid blast crisis. His acute myeloid leukaemia was treated with FLAG-Ida and dasatinib resulting in prolonged cytopenias. He was rescued with peripheral blood stem cells from his sibling. Conditioning regimen used in all 4 patients was campath-1H 30 mg/d i.v from day-4 to day-2 i.e. 90mg in total. Ciclosporin 5 mg/kg/d and mycophenolate mofetil 1gram/d was commenced on day-1 as GVHD prophylaxis. *Results*. Follow up ranged from 2 - 60 months. All patients and donors were CMV negative. Donor stem cell CD34 counts ranged from 3.83x10^6/kg to 6.6x10^6/kg. G-CSF was initiated in all 4 patients. Patient 1 was heavily pre-treated with chemotherapy while the remaining 3 patients had prolonged severe cytopenias following chemotherapy and had fungal pneumonia at the time of transplant. Patient 1 had normal full blood count prior to conditioning and had no pancytopenic period and at 3 months donor chimerism was 22%. He received one dose of DLI (1x10^6/kg CD3+ve cells) and at 6 months this achieved 100% donor chimerism. Engrafted was prompt in patient 2 and 4 within 21-32 days. Patient 3 started to engraft but died from progressive pneumonia 15 days post transplant. All patients alive were transfusion independent within 50 days post transplant. Patient 1 developed grade 2 chronic GVHD while other patients did not develop GVHD. Patient 1(T-PLL) remained in complete remission for 30 months before relapsing. Patient 2 (AML) continues to remain in complete remission at 60 months post transplant. Patient 4 (AML) is 60 days post transplant in complete remission. *Conclusions*. In patients who are unable to tolerate non myeloablative cytotoxic based conditioning, haematopoietic stem cell engraftment can be achieved using non chemotherapy based conditioning with alemtuzumab, mycophenolate mofetil and ciclosporin. This can induce durable remissions and needs further evaluation in future clinical trials.

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EFFECTIVE PERIPHERAL BLOOD PROGENITOR CELL MOBILIZATION WITH G-CSF AND PLERIXAFOR: A PROMISING MOBILIZATION TREATMENT?

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Background. In different circumstances, basically depending on age, type and number of prior treatments, patients with hematological diseases have problems in mobilizing sufficient peripheral blood hematopoietic stem cells (PBSCs) for autologous hematopoietic stem cell transplantation (auto-SCT). Plerixafor is a novel molecule used as mobilizing agent in combination with G-CSF. Plerixafor is a reversible inhibitor for the binding of stromal derived factor-1 α to its receptor CXCR4. This complex plays a role in the retention of HSC within the bone marrow and its inhibition can lead to an increased number of PBSCs. *Aim.* To describe the results of the Plerixafor use in patients with at least one previous failure in PBSCs mobilization. *Design and Methods.* Five patients from a single institution were retrospectively analyzed, 1 female and 4 males, with a median age of 52 (range 30-62) and diagnosis of Non-Hodgkin's lymphoma (4) and Hodgkin's disease (1). The patients were exposed to different mobilization regimens in order to achieve a minimal number of PBSCs (2x10^6 CD34$^+$ cells/kg) sufficient to proceed to auto-SCT. The cells obtained in each collection attempt were added until the minimum required amount was reached. Four patients had received one previous conventional mobilization (G-CSF, 10 µgr/kg/daily, or G-CSF in combination with cyclophosphamide, 1,5 gr/m^2) and one patient two previous treatments (Firstly G-CSF, secondly G-CSF plus cyclophosphamide), without collecting enough CD34$^+$ cells. In the last mobilization, Plerixafor (240 gr/kg/daily SC) was associated to G-CSF in the fourth day. PBSCs were collected on a COBE Spectra apheresis system (Gambro BCT, Lakewood, CO, USA). *Results.* In Plerixafor mobilization, G-CSF was administered a mean of 5,8 (range 5-7) days, whilst with the other treatments the mean was of 6,6 (range 5-8). The minimum number of CD34$^+$ cells could be completed with only one Plerixafor dose in three patients. After the first Plerixafor exposure, the patients had peripheral blood CD34$^+$ count and collected CD34$^+$ cells/kg means of 18,64/µL (range 4.4-31.5) and of 1,35x10^6/kg (range 0.4-2.94), respectively. With the other schedules, the counts were: mean of peripheral blood CD34$^+$ cells 9,1/µL (range 4,96-17,92) and mean of collected CD34$^+$ cells 0,47x10^6/kg (range 0.41-0.62). No adverse events were reported. Two patients underwent auto-SCT, with median time to neutrophil engraftment (>0.5x10^9/L) on day 12 and platelet engraftment (>20x10^9/L for 7 days without transfusion) on day 15. *Conclusions.* Plerixafor administered in conjunction with G-CSF could be an effective way to salvage patients who have failed other mobilization attempts and need to proceed to auto-SCT. With this drug, our patients have improved

the peripheral blood basal CD34+ cells and CD34+ cells in the apheresis products, without observing side effects. However, it is necessary to include more patients to confirm this hypothesis.

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AMD3100 IN POOR MOBILIZER PATIENTS WITH HEMATOLOGIC MALIGNANCIES

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Background. About 20% of patients (pts) affect by haematological malignancies is considered 'poor mobilizer' with a very low CD34 yield at collection without the possibility to perform hi-dose chemotherapy and autologous stem cell transplantation (ASCT). CXCR4, a stromal derived growth factor, interacts with the receptor of the stem cell stopping the bound with the stromal chemokine factor-1 (SDF-1α) expressing a regulator action on stem cells flow in the microvessel from and toward the bone marrow.

Table 1, 2, 3, 4.

Table – 1 Patient clinical characteristics	
Diagnosis	
NHL	3
MM	1
Gender	
Male	2
Female	2
Age	58,5 (52 – 67)
Patient 1 – NHL	ARAc HiD+Peg
	ARAc HiD+G-CSF + growth hormone
Patiens 2 and 3 – NHL	DHAP protocol + GCSF
Patient 4 – MM	EDX +Peg
	EDX+ G-CSF + growth hormone

Table – 2 Plerixafor mobilization	
Patient 1 – NHL	EDX / Lenograstim d.5 – d. 17 / AMD3100 d.10 – d.17
Patiens 2 and 3 – NHL	DHAP / Lenograstim d.1 – d.8 / AMD3100 d.10 – d.13
Patient 4 – MM	EDX / Lenograstim d.1 – d. 13 / AMD3100 d.10 – d.13

Table – 3 Apheresis data	
Patients blood volume	4,7 Lt (3,9 – 5,2)
WBC pre-apheresi	$32x10^3$ (4 – 62)
GN pre-apheresi	24,9 $x10^3$
Plts pre-apheresi	$45x10^3$ (11 – 134)
CD34 positive cells	12/μL (2 - 27)
Apheresi number	2,5 (2 – 4)
Blood volume proc	12,4 Lt (9,89 – 17,7)
CD34 positive harvest	39/μL (9 – 117)
CD34/Kg/apheresi	1 (0,7 - 2)
%Efficiency apheresi	37 (10 – 60)

Table – 4		
	CD34/Kg harvest	*Day of apheresis*
Patient 1 – NHL	0,37/ μL	+16 +17
Patient 2 – NHL	0,35/ μL	+12 +13
Patient 3 – NHL	3,71/ μL	+12 +13
Patient 4 – MM	1,96/ μL	+11 +12 +13

The antagonist molecule of CXCR4 (plerixafor - AMD3100) allows the release of the HPC determining an increase of the CD34 positive cells in the peripheral blood. AIMS In the attempt to reduce the risk of an unsussesfull mobilization of HPC in high risk pts, poor mobilizer with previous regimens, we utilized the new mobilizing factor plerixafor, in combination with G-CSF and chemotherapy. *Design and Methods.* We treated 4 pts, 3 high risk NHL (2 in CR; 1 PR) 1 MM in PR. All of 4 patients had failed previous mobilization with a number of CD34 after mobilization minor than 10/μL. in table 2. Pt-1 was treated with Hi-Dose ARAC+Peg-filgastrim; ARAC HD+G-CSF + growth hormone. Pt-2 and 3 with DHAP protocol + GCSF, while Pt-4 with MM by EDX +Peg-filgastrim, EDX+ G-CSF + growth hormone. Clinical pts characteristics and previous regimens of mobilization are listed in Table 1. After chemotherapy, G-CSF was started from day 5 at a dose of 10 g/Kg/die and having associated to Plerixaflor from day 10, with evening administration at the dose of 240 mg/kg/die. HPC collection data are summarized in table 4 and 5. Two pts (nb 3 and 4) collected a sufficient number of CD 34^+ HPC able to perform ASCT. *Conclusions*. This study confirm the efficacy of Plerixafor to mobilize CD34 positive cell in a subset of patient unresponsive to previous mobilizing strategy. Harvest was negatively influenced by chemotherapy load, use of some chemotherapic agents (so fludarabina, lenalidomide), and by chemorefractory disease status. Our data in a small series of pts confirm the possibility to reduce the risk of poor mobilizer phenomena in about 50% of haematological pts.

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PLERIXAFOR IS ABLE TO MOBILIZE A HIGH PROPORTION OF LYMPHOMA AND MYELOMA PATIENTS THAT PREVIOUSLY FAILED A PBSC MOBILIZATION: A SINGLE CENTER STUDY

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We have employed PLERIXAFOR (AMD3100) in patients who failed a previous cycle of PBSC mobilization. Each patients was used as an internal control and thus results of plerixafor -mobilization were compared to data obtained during the previous PBSC mobilization in the same patient. We studied six patients affected with NHL (3), MM (2) and HL (1), patients were aged 23-63 years. All patients had failed a first cycle of mobilization chemotherapy using disease-oriented chemotherapy (VP 16 containing cycle in 3 patients, CTX containing cycle in 3 patients) plus G-CSF at dose of 10 mcg/Kg. Failure was defined as a CD34+ cells in P.B. less than 20/mmc (5 pts) or by an CD34+ harvest < $1.5x10^6$/Kg (1 pts). 4/6 of all patients had at start of second mobilization using plerixafor a Platelet count <$140x10^9$/L and 4/6 patients had WBC count <$4.0x10^9$/L. G-CSF therapy was administered daily at 10 mcg/Kg/d and starting from the evening of, the forth day, Plerixafor was administered at dosage of 240 mcg/Kg as s.c. injection in outpatient clinic. Median value of maximum $CD34^+$ cells obtained in P.B. during the first unsuccessful PBSC mobilization cycle was $12,8x10^6$/L while $CD34^+$ peak after G-CSF+Plerixafor was $26.7x10^6$/L (t-test for paired data: p=0.07). Percentage of patients reaching criteria for a successful mobilization (threshold of $CD34^+$ cell in P.B. >$20x10^6$/L) after G-CSF+Plerixafor was 83% (5/6 pts), significantly higher than results achieved during first cycle (16%) (χ^2 test: p=0.02). Mean apheresis number was 2 for each patients and median total $CD34^+$ harvested were $4,6x10^6$/Kg (range 0.8-8.2). Median collection efficiency during plerixafor+G-CSF therapy was 35%. Plerixafor was well tolerated and the only adverse event registered in 2 patients was rash at injection site. 2/6 of patients mobilized using Plerixafor had already received autologous PBSC transplant at our institution after BEAM and HD-PAM schedules and neutrophils engraftment was obtained at day+15 and + 18.

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COTRANSPLANTATION OF THIRD PARTY MSC TO FACILITATE ENGRAFTMENT OF HAPLOIDENTICAL STEM CELLS IN A PATIENT HAVING EXPERIENCED GRAFT-FAILURE

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Background. Graft failure is a life-threatening complication after allogeneic hematopoetic stem cell transplantation (HSCT) and efforts to achieve secondary engraftment are challenging. *Design and Methods.* Here, we describe the clinical course of a young AML patient suffering from graft-failure after haploidentical peripheral hematopoietic stem cell

transplantation. In the current case, third party mesenchymal stem cells (MSC) were cotransplanted to facilitate engraftment of a second T depleted graft from the same HLA-mismatched donor. In order to increase the progenitor content of the second graft, the CXCR-4 antagonist AMD3100 was added to the second cycle of G-CSF mobilisation. *Results*. The higher number of CD34+ cells transplanted and the cotransplantation of MSC resulted in a rapid and sustained trilineage hematologic engraftment. Complete donor chimerism in myeloid and lymphatic cells was achieved. Graft-versus-Host disease occurring after the second transplantation required combined immunosuppressive treatment. *Conclusions*. Alternative progenitor cell mobilisation and MSC cotransplantation might be useful in the management of graft-failure after HLA-partially matched HSCT, but further studies have to be performed to confirm the safety of this approach with respect to the incidence of GvHD and relapse.

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PATIENTS UNDERGOING ALLOGENEIC BONE MARROW TRANSPLANTATION IN NON-ONCOLOGICAL DISEASES: EXPERIENCEOF A SINGLE CENTER

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Background. From 1995 to today, in our center were performed 69 transplants of allogeneic bone marrow donor from sibling. *Design and Methods*. In 17 children, 19 transplants have been performed for non-oncological diseases (2 patients underwent a second transplant). The diseases for which have been used for transplantation are as follows: 3 patients with Fanconi Anemia, 2 patients with Thalassemia intermedia, 1 patient with HBS, 2 patients with Cooley's disease, 2 patients with Aplastic Anemia, 1 patient with bthal/HbS, 1 patient with Blackfan-Diamond, 1 patient with Osteopetrosi, 3 patients with SCID (Severe Combined Immunodeficiency Disease), 1 patient with deficiency of FAS. The distribution regard the sex is the following: 11 patients were males, 6 females. The average age was 8.8 years (range: 4 months - 15 years). Donors have been nine cases in males and females in eight cases, the source of stem cells was bone marrow, except one case where we used a cord. *Results*. The TRM at 100 days was 0%. No patient died within the first hundred days from bone marrow reinfusion. In 6 patients there was a graft versus host disease (GVHD) skin grade I-II, 3 patients in a limited chronic GVHD of the skin to load in two cases of hepatic parenchyma in a case. Two patients died in the following complications: the first, after four months from transplantation to engraftment failure, remaining in aplasia associated with infectious episodes and exitus for acute renal failure, the other after six months from transplantation, for complications related to acute bacterial infection and a liver disease post-anesthetic. The event free survival was 2.8 years. The overall survival was 88%. *Conclusions*. Our experience, although on a limited number of patients, allows us to believe that the transplantation of allogeneic bone marrow in patients with non-oncological diseases, some very severe and disabling (such as osteopetrosi undermining the development of the brain parenchyma, vision and hearing, as well as abnormal skeletal development and psycho-motor-somatic and SCID, severe immunodeficiencies and potentially lethal) is a valide therapy, with a fatality and an acceptable toxicity.

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IS FEASIBLE CD34 CELLS ENUMERATION BY HEMATOLOGY AUTOANALYSER?

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Background. CD34+ detection methods based on cytofluorometric (CF) assays is, nowadays, without any doubt the best method to monitorise the moment the haematopoietic precursors (HP) apheresis should be initialised. It is also an outstanding method for CD34+ quantification in the afore mentioned product. Some autoanalysers are capable to semi-automatically process blood samples using monoclonal antibodies (MoAb) marked with florochromes. An excellent correlation has been demonstrated between lymphocyte population analysis by Cell-Dyn Sapphire /4000 autoanalyser and the reference method (flow cytometry). Aim The aim of this study was to compare CD34+/CD45dim progenitor count using both methods mentioned above (Cell-Dyn Sapphire and flow cytometry), in peripheral blood and apheresis product samples from patients and donors enrolled in HP transplantation programs. *Material and Methods*. 28 PH apheresis products and 11 peripheral blood samples in EDTA were processed after mobilization with growth factors. MoAbs used were antiCD45FITC/CD34PE (Becton Dickinson) and prepared using a standard method for FC. At the same time, these samples were also marked with the same MoAbs following the open tube technique, 5 minutes incubation, lysis and washing being automatically performed by Cell-Dyn Sapphire. Files were downloaded into a memory device USB and were then analysed through the FCS Express v3.0 program. *Results*. Time consumed per sample using the autoanalyser was 7 minutes, compared to 20 minutes per sample using FC analysis. Bland-Altman and Passing-Bablock tests were applied, where the identity line in both measurements of the apheresis product was (y= 0.885x +0.0144) with a slope of 0.086 for a CI of 95%. In peripheral blood a correlation was obtained at (y= 0,75x + 0.0175) with a slope of 0.750 for the same CI. *Conclusions*. In our series of results, CD34 count using Cell-Dyn Sapphire showed a good correlation with the reference method (FC). However, flow cytometry comparison was better when dealing with apheresis samples. These differences could be explained by the different quantity of positive events on both sample types. HP quantification by the autoanalyser could be used as a deciding tool as to when to start the apheresis and to estimate their concentration in the obtained product. A bigger amount of samples should be analysed in order to confirm our data. The technique should be enhanced so that in the near future CD34 quantification could be performed by an autoanalyser with total confidence.

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TRANSFORMING GROWTH FACTOR β-1 LEVELS IN INFLAMMATORY BOWEL DISEASE

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Background and aim. Transforming growth factor β 1(TGF-β1) levels was intended to increase during the inflammation of human colon mucosa. In this study we aimed to show the correlation between TGF-ß1 levels that increase in the activation period of inflammatuar bowel disease (IBD)and in healty group. *Material and Method*. For the study, 70 patients were taken. 40 of the patients were Ulcerative colitis(UC) while 30 of them were Crohn's disease(CD). In the UC group 18 of them were women and 22 of them were men. The average of the UC group was 40.7+ 1.6(16-69) years. In the CD group, 13 patients were women and 17 were men. The average age of the group was 36.9+1.9(18-63) years. In the healty control group, 10 women and 10 men were taken and the average age of the group was 35.65+8.21(22-49) years. All the patients had stopped to use the drugs for one month before the sample were taken. Crohn disease activation index and Rachmilewitz Endoscopic Index were used for the estimation of clinical activation of IBD . The activation period was accepted when the Crohn disease activation index was over 150 and the Rachmilewitz Endoscopic Index was over 4. At the same time CRP, sedimentation rate, fibrinogen level, thrombocyte and white blood cell count were measured fort he estimation of activation period. TGF-β1 levels were studied with ELISA (Bender-Med, Austria). *Results*. The average level of TGF-β1 in CD was 1230.0+572.71 pg/ml, in UC group it was 1362.5+880.6 pg/mL and in control group it was 1133.3+766.5 pg/ml. No statistical difference was found between these groups. In the activation group of CD,TGF-β1 level was 1310.0+719.9 pg/mL and in the remission group it was 956.6+795.0 pg/ml. No statistical difference was found between these groups and the control group. Also between activation and remission groups of CD, the levels of TGF-ß1 were showed no statistical difference. In UC during the activation period, TGF-β1 was found 1952.5+5543.7 pg/mL and during the remission period TGF-β1 was found 772.5+167.8pg/ml. We found statistically difference between the levels of activated group of UC and the healty group ($p<0.05$).Between the activated and remission groups of UC we also found statistically difference for the TGF-β1 ($p<0.05$). *Conclusions*. We found that in the UC patients group, if there was activation, TGF-β1 levels were statistically higher than the patients in remission and the control group. This result supported that during the activation, as cytokines were increased due to the Inflammation, TGB-β1 was also increase. This increase had also affected the inflammation. Because TGF-β1 was produced and secreted from the small intestine and colon mucosa. Although TGF-ß1 controled the proliferation and differentation of healty epithelial cells, it also had a role during the mucosa healing and the increase of fibrosis. In our study we couldn't find any correlation between the patients in activation and the remission periods of CD. In some studies this correlation was estimated, through some studies were

supported our results. In the study of Del Zoto, he said that, in CD and UK the stimulation of cytokine activation was occurred with different mecanisms and these cytokines had affected the production of TGF-β1 with different ways. As the results of the studies, the increase of TGF-β1 levels was accepted as one of the activation criteria in ulcerative colitis.

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THE ROLE OF TGF-β-1 PROTEIN AND TGF-β-R-1 RECEPTOR IN IMMUNE ESCAPE MECHANISM IN BLADDER CANCER

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Background. Tumor cells have numerous immune surveillance escape mechanisms as well as means of resistance to apoptosis. This study tried to clarify one of these mechanisms in bladder cancer with the hope of being able to develop targeted therapy that will sensitize the tumor cells to immune mediated apoptosis. *Design and Methods*. In this study, light and electron microscopic examination and expression of TGF-β-1protein and TGF-β-R-1 receptor using immunocytochemical and immunoelectronmicroscopic techniques in urine and peripheral blood mononuclear cells (PBMNCs). Samples were obtained from 5 healthy controls (Group 1) and 60 patients are studied and classified according to the cytopathologic examination of their urine into 2 main subgroups: chronic cystitis (bilharzial and nonbilharzial, (Group 2, n = 15) and bladder cancer (transitional cell carcinoma and squamous cell carcinoma, (Group 3, n=45), whether associated with schistosomal infection or not associated. Results: Urine examination by both immunocytochemical and immunoelectronmicroscopic techniques revealed a statistically significant decrease in the percentage of positive cases expressing TGF-β-R1 receptor in bladder cancer in comparison with either chronic cystitis cases or controls ($p<0.01$), while TGF-β-1 protein was significantly increased ($p<0.01$). By light and electron microscopic examination, exfoliated necrotic malignant epithelial (urothelial) cells and many inflammatory cells were detected in malignant

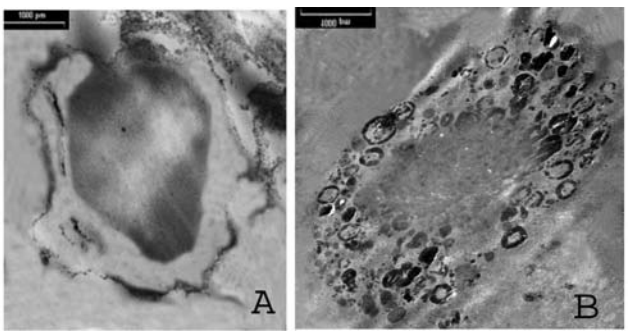

Figure 1. TEM photomicrograph of immunoperoxidase-labeled PB.

Examination of PBMNCs by immunocytochemical and immunoelectronmicroscopic techniques showed a significant increase in the percentage of positive cases expressing both TGF-β-1 protein and TGF-β-R-1 receptors in bladder cancer in comparison to the control group ($p<0.01$ and $p<0.05$, respectively) and to chronic cystitis cases ($p<0.05$). By light and electron microscopic examination, 42 out of 45 bladder cancer cases (93.3%) revealed remarkable apoptotic changes represented by cell shrinkage, surface blebs, nuclear chromatin condensation, and vacuolated cytoplasm. *Conclusions*. This work helps researchers and clinicians to better understand one of the escape mechanisms in bladder cancer that may facilitate the reverse of tumor escape from the immune system. It also draws attention to TGF-β-1 protein and TGF-β-R1 receptor; TGF-β-1 protein can be used as attractive target for anticancer therapy, and the absence of TGF-β-R1 can be considered a marker for malignant transformation of urothelial cells in bladder cancer.

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INACTIVATION OF ICSBP BY PROMOTER METHYLATION IS LIKELY INVOLVED IN PROGRESSIVE LEUKEMOGENESIS

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Background. Screening of PubMed GEO database showed a down-regulation of ICSBP (interferon-consensus site binding protein), a transcription factor of the IRF family involved in myeloid cell differentiation, preferentially in AML with t(8;21), t(15;17) and del(7q)/7, and in therapy-induced MDS/AML presenting del(5q). *Aim*. The aim of this study was to investigate whether ICSBP promoter methylation may be responsive for down-regulation in these cytogenetically defined subtypes of AML *Design and Methods*. DNA extracted from blood or bone marrow from patients diagnosed with primary AML (15: 5 t(15;17), 8 t(8;21), 2 with 5q-), secondary AML (14: 6 complex aberrant, 4 with 7-/7q-, 2 with trisomy 8, 4 with normal karyotype), childhood MDS (18: 1 with complex aberrant, 8 with 7-/7q-, 1 with trisomy 8, 1 with der(1) t(1;6), 7 with normal karyotype), and adult MDS (63: 8 complex aberrant, 16 with 7-/7q-, 8 with trisomy 8, 28 with 5q-, 8 with 5q- and additional aberrations) were screened for *de novo* methylation in the proximal promoter of ICSBP by methylation specific PCR. Additionally, *de novo* methylation of the ICSBP promoter was analyzed in four cancer cell lines and the restoration of the ICSBP function was investigated in one cell line after 5-azacytidine treatment. *Results*. Taken together, we observed ICSBP promoter methylation in 27 out of 116 patients (23%). Hypermethylation occurred most frequently secondary AML (8/20; 40%), compared to AML (4/15; 27%), adult MDS (13/63; 21%), and childhood MDS (2/18; 11%). Within the cytogenetically defined subtypes, ICSBP promoter methylation was found most frequently in patients with trisomy 8 (4/11, 36%), 7-/7q- (6/23, 26%), 5q- (7/30, 23%), complex karyotypes (2/15, 17%), t(8;21) (3/8, 37,5%), and t(15;17) (1/5, 25%). The ICSBP promoter was methylated in the Kasumi-1 and HL-60 cell lines, and unmethylated in the U937 and F-36P cell lines. Accordingly, ICSBP expression was absent in the cell lines with methylated promoter, as detected by Western blot analysis. 5-azacytidine treatment of the Kasumi-1 cell line induced a demethylation of the promoter, and after addition of interferon-g, a reexpression of ICSBP protein. *Conclusion*. Loss of expression ICSBP due to promoter methylation is a frequent event in myeloid neoplasias, particularly in secondary AML and in distinct cytogenetic subgroups like complex karyotype, 5q-, 7-/7q- and trisomy 8. ICSBP may be a target for epigenetic therapy, since reexpression was induced by 5-azacytidin treatment.

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DEATH-ASSOCIATED PROTEIN KINASE 1 IS EPIGENETICALLY SILENCED IN NK AND NK/T CELL LINES, BUT NOT IN T-ALL CELL LINES

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Background. T-cells and natural killer (NK)-cells are derived from pluripotent stem cells and can be distinguished by the expression of various marker proteins. The most important markers are the T-cell receptor and specific NK-cell receptors. For quite some time now there is the focus on epigenetic markers for the discrimination of different cell types and neoplasias. Epigenetic inactivation of tumor suppressor genes (TSG) by promoter CpG island hypermethylation is a common hallmark of malignant diseases. Specific profiles of hypermethylated CpG islands have been described to be unique for different neoplasias. *Aims*. Here, we screened for TSG promoter hypermethylation to find out whether the methylation status of a defined set of TSG allows for the discrimination of T-acute lymphoblastic leukemia (T-ALL) from NK-cell-derived cell lines. *Design and Methods*. We analyzed the methylation status of 24 different TSG in 20 cell lines representing T-ALL, NK, NK/T and T-anaplastic large cell lymphoma malignancies. For this approach we used a methylation-specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay. Characteristic methylation patterns were verified by methylation-specific PCR (MSP). Furthermore, data were validated at the mRNA expression level by quantitative real time PCR and at the protein level by western blot analysis. *Results*. The screening for TSG methylation showed that one of the analyzed genes - Death-associated protein kinase 1 (DAPK1) - was typically methylated in NK and NK/T cell lines, but not in T-ALL cell lines. The observed DAPK1 methylation

pattern could be confirmed by MSP. Furthermore, hypermethylation of DAPK1 promoter inversely correlated with DAPK1 mRNA and protein expression. Incubation of DAPK1 hypermethylated cell lines with the demethylating agent 5-Aza-2'deoxycytidine reactivated DAPK1 gene expression confirming that promoter hypermethylation was indeed responsible for transcriptional silencing of DAPK1 in NK and NK/T cell lines. *Conclusions.* Hypermethylation of the TSG DAPK1 differentiates NK and NK/T cell lines from T-ALL derived cell lines. Future investigations with patient samples will show whether the DAPK1 methylation status can be used to differentiate between T-ALL and malignant NK-cells.

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MODULATION OF ALTERNATIVE RNA SPLICING BY AMILORIDE IN K562 CELL LINE

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Background. Alternative splicing plays an essential role in the control of many biological processes, such as embryonic development, cell growth, and apoptosis. Since many apoptotic genes have alternatively splice variants with antagonistic function, aberrant splicing of these genes is associated with malignant growth and is a characteristic of different kinds of cancer including leukemia. Modulation of apoptotic genes by alternative splicing, therefore, has been proposed to be potential for cancer

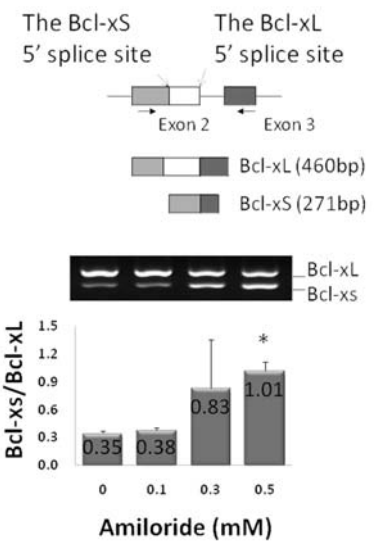

Figure 1.Modulation the Bcl-x alternative splicin

Aim. Although several small molecules have been proposed to modulate alternative splicing of RNA recently, additional molecules are still needed to be surveyed. *Design and Methods*. First, we detected the Bcl-x mRNA alternative splicing in k562 cell line under the treatment of a clinical proved drug, amiloride, by RT-PCR. To evaluate the mechanism of alternative splicing, we analyzed the expression of serine-arginine-rich proteins (SR proteins) by Western blot. To validate whether amiloride regulates Bcl-x alternative splicing has related to the cell apoptosis, we examined cell cycle and viability by flow cytometry. The signal pathway of apoptosis including Bcl-2 family proteins and multiple protein kinases were evaluated by Western blot. Finally, exon array was performed to genome-wide analyze the alternative splicing by Human Exon 1.0 ST Arrays (Affymetrix). *Results*. Treatment of K562 cell line with amiloride downregulated the expression of Bcl-xL mRNA and upregulated the expression of Bcl-xS in a dose- and time-dependent manner (Figure1).5-(N-ethyl-N-isopropyl)- amiloride, the amiloride analogue, however, did not modulate alternative splicing of Bcl-x mRNA. The expression of SF2/ASF was reduced prominently but hnRNPA1 was highly expressed after amiloride treatment. Moreover, most of the SR proteins, including SRp 55, SRp40, SRp30, and SRp20, were dephosphorylated after amiloride treatment. In addition to the modulation effects of alternating splicing, cell cycle analysis demonstrated S phase arrest and apoptosis after amiloride treatment.We also found that the proapoptotic protein Bax and Bcl-xS were upregulated and the antiapoptotic protein Bcl-2 and Bcl-xL were downregulated during amiloride-induced apoptosis. Besides, ERK1/2 dephosphorylation, a dose-dependent increase of phospho-JNK, and phospho-p38 MAPK were also found in amiloride-treatment cells. Exon array analysis showed that there are 59 genes have alternative splicing after amiloride treatment. *Conclusions*. In this study, we found that amiloride can modulate Bcl-x alternative splicing. Consistent with its RNA expression, the antiapoptotic protein Bcl-xL was down- regulated and the proapoptotic protein Bcl-xS was up-regulated and cause the cell apoptosis. The modulation effect of amiloride to alternative splicing is mainly through SR proteins, and amiloride may play a role in the cancer treatment.

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SOCS-1 EXON 2: DEMETHYLATION BY AZACYTIDINE INCREASED THE RELATIVE EXPRESSION OF SOCS-1 AND ASSOCIATED WITH TRANSCRIPTIONAL SILENCING IN LEUKAEMIC CELL LINES

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Background. Mutations in genes such as FLT3, KIT and RAS have been identified to provide a proliferative signal in acute myeloid leukaemia (AML) and serve as targets for drug treatments. In addition, epigenetic may play a major role in the pathogenesis of AML where aberrant methylation is a major mechanism for silencing of tumour suppressor genes. Hypermethylation of the suppressor of cytokine signalling-1 (SOCS-1) gene has been reported to cause inactivation in leukaemogenesis. However, there is an issue in the use of different locations of SOCS-1 gene being amplified (exon 2 and 5'UTR) that gave discrepant methylation results in AML. *Aims*. The aim of the study was to determine the role of SOCS-1 exon 2 methylation in haematopoietic cell lines and the relative expression of the gene after the addition of either azacytidine or deoxyazacytidine as the conversion agent of methylcytosine. *Design and Methods*. The relative expression level of SOCS-1 gene in four leukaemic cell lines was compared to determine the gene expression in SOCS-1 exon 2 methylated and unmethylated cell lines. Demethylation technique was established by treating the cell lines using either azacytidine or deoxyazacytidine. Methylation-specific PCR (MS-PCR) was employed to determine if demethylation using the conversion agent would convert methylcytosine into cytosine in the SOCS-1 exon 2 methylated cell lines. Quantitative reverse transcription PCR (RQ-PCR) were used to analyse the relative expression of SOCS-1 in haematopoietic cell lines and calculated using the 2-ΔCT equation. *Results*. There was a decrease in the relative expression level of SOCS-1 gene in SOCS-1 exon 2 methylated cell lines (ME-1 and Jurkat) compared to unmethylated cell lines. The demethylation technique was established using 1 μM of azacytidine and harvested after three days. The use of azacytidine is favoured instead of deoxyazacytidine because of the greater toxicity of the latter to the cells. The demethylation experiments showed that there was a marked increase in relative expression of SOCS-1 in ME-1 and Jurkat cell lines after the addition of azacytidine with MS-PCR results showed evidence of cytosine conversion in both. The mean expression level of SOCS-1 gene in ME-1 and Jurkat cell lines were 17 folds and 7 folds higher respectively compared to untreated controls. RQ-PCR results showed that SOCS-1 exon 2 methylation is associated with the transcriptional silencing of SOCS-1 gene in haematopoietic cell lines. *Conclusions*. The treatment of SOCS-1 exon 2 methylated cell lines with demethylating agent azacytidine resulted in the re-expression of SOCS-1 gene and it was the methylation of SOCS-1 exon 2 but not 5'UTR may lead to epigenetic silencing of SOCS-1 in leukaemic cell lines.

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RELEASE OF CYTOCHROME C FROM MITOCHONDRIA PRECEDES BAX TREANSLOCATION/ACTIVATION IN TRITON X-100-INDUCED APOPTOSIS

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Background. The mechanisms of apoptosis have been profoundly investigated in the last decade. In the mitochondrial pathway,

cytochrome c is released from mitochondria into the cytosol and forms the apoptosome, leading to activation of executioner caspases. Among various stimuli, sublytic concentrations of nonionic detergents, such as Triton X-100 (TX) have been shown to induce apoptosis. However, the mechanisms by which detergents induce apoptosis remain unclear. 2. *Aims*. Recently, it was reported that conformational change of Bax was observed when Bax translocated from the cytosol to mitochondria in apoptosis induced by various stimuli including anti-Fas antibody and TNF-a. Therefore, we investigated whether activation of Bax might play a crucial role in Triton X-100- induced apoptosis (TXIA) in cells. 3. *Design and Methods*. 1) Human monoblastic leukemia cell line U-937 was used in our studies. 2) Apoptosis was assessed by typical apoptotic morphological changes (under light microscope), nuclear condensation and fragmentation (under fluorescent microscope), cellular DNA content (propidium iodide staining and FACS analysis) and exposure of phosphatidylserine on the surface of apoptotic cells (stained with FITC-conjugated Annexin V and PI, and analyzed by FACS). 3) Caspase activity in TX-induced apoptotic cells was detected by fluorescence of DEVD-MCA (by using ARVO 1420 multilabel counter). 4) Effects of caspase inhibitors (z-VAD-fmk and z-DEVD-fmk) were assayed by cellular DNA content of those cells. 5) Western blot analysis for cytochrome c and Bax was performed in the cytosolic and mitochondrial fractions of those cells. The images were obtained using VersaDoc imaging system. 6) To confirm the results obtained by Western blot analysis, those cells were immunostained with anti-activated Bax antibody or anti-cytochrome c antibody. Images were obtained by FV300 laser scanning confocal microscope. 4. *Results*. 4.1) Most of U-937 cells showed typical apoptotic membrane blebbing and nuclear changes at treatment of 0.01% TX. 4.2) Rapid activation (at 5min after TX treatment) of caspases-3 and -9 was observed, whereas activation of caspase-8 seemed slower than other two caspases. 4.3) Pretreatment with caspase inhibitors completely inhibited TXIA. Although Bax translocation/activation was inhibited by caspase inhibitors, they did not affect cytochrome c release from mitochondria. 4.4) TX induced rapid release of cytochrome c from mitochondria to the cytosol in TXIA. 4.5) Translocation and activation of Bax from the cytosol to mitochondria occurred in TXIA. 4.6) The Immunostaining experiments of conformational changes of Bax and cytochrome c confirmed the results obtained by Western blot analysis. *Conclusions*. We suppose that in TXIA, mitochondria seems to be the primary target and cytochrome c release from mitochondria induces caspase activation, which further induces Bax translocation/activation. Furthermore, our results suggest that activation of caspase-3 may occur in the mitochondrial pathway of apoptosis through formation of the apoptosome including caspases-9. Since Fas-induced apoptosis depends on caspase-8, the mechanism of TXIA is quite distinct from that of Fas-induced apoptosis. At the very least, the extremely rapid kinetics of apoptosis induction by sublytic concentrations of TX represents a novel and powerful tool to probe specific mechanisms of apoptosis.

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CYTOTOXIC EFFECT OF TETEACYCLINE ANALOGUES (DOXYCYCLINE, MINOCYCLINE AND COL-3) ON ACUTE MYELOID LEUKEMIA HL60 CELLS

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Background. Despite progress in the treatment of acute myeloid leukemia, so far the treatment outcome is not satisfactory. Side effects of chemotherapy are major limiting factors due to unspecific cytotoxicity. New effective treatment strategies with fewer side effects are required for further improvement. New mechanisms-based targeted drugs have been developed. Tetracycline analogues (TCNAs) have been shown to possess besides antibiotic properties also non-antibiotic activities. They have been shown to induce apoptosis in several cancer 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 is chemical modified tetracycline-3. Chemically modified tetracyclines do not possess antibiotic properties. Aim In this study, we investigated the cytotoxic effect of TCNAs DOXY, MINO and COL-3 in human myeloid leukemia cell line HL60. *Design and Methods* HL60 cells were cultured in complete RPMI 1640 medium (supplemented with 10% fetal bovine serum). Stock solution of DOXY and MINO were prepared in sterile water, while COL-3 was dissolved in DMSO. Cells were treated with DOXY, MINO and COL-3 in final concentrations ranging from 0.5 to 100 µg/mL until 24 h. The control cells were incubated with complete drug-free medium. Cells treated with DMSO in a final concentration of 0.2% were used in the experiments with COL-3 as a control for solvent toxicity. Cell viability was studied using resazurin assay. Apoptosis was assessed using morphological criteria using May-Grünwald-Giemsa staining. Loss of mitochondrila membrane potential was investigated using tetramethylrhodamine methyl ester and flow cytometry. Protein cleavage pattern was studied using SDS-PAGE and immunoblotting. Pretreatment with pancaspase inhibitor Z-VAD-FMK in concentration 100 µmol/L for 1 h followed by incubation with TCNAs was used to confirm the caspase-dependent apoptosis. *Results*. All three drugs induced concentration-dependent decrease in viability of HL60 cells at 24 h of incubation. The greatest effect was observed after incubation with COL-3 (IC50 was 0.9 µg/mL for COL-3 and 9.6 µg/mL for DOXY and MINO, respectively). Apoptotic morphology was detected already 6 h after the incubation start and it became more pronounced in a concentration and time-dependent manner. Apoptosis was caspase-dependent as detected by caspase activation and confirmed by Z-VAD-related decrease in percentage of apoptotic cells. Loss of mitochondrial membrane potential and cleavage of Poly-ADP-ribose polymerase (PARP) and BCL-2 were detected. The order of apoptotic events differed among the TCNAs. *Conclusions*. Cytotoxic effect of TCNAs on leukemic HL60 cells was exposure dependent. All three TCNAs induced apoptosis in the HL60 myeloid cell line in a mitochondrial and caspase-dependent way. COL-3 had the most potent anti-proliferative and pro-apoptotic effect and thus, TCNAs may have treatment potential in acute myeloid leukemia.

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EVALUATION OF PHOSPHOLIPIDS CHANGES WITH APPLICATION OF IN VITRO 31P MRS DURING APOPTOSIS INDUCED BY ARA-C ON PATIENTS WITH ACUTE LEUKAEMIA

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Background. Phospholipids play an important role in the biological events of the progression of cancer. 31P magnetic resonance spectroscopy (MRS) can be a powerful method for non-invasive investigations aiming at an explanation of phospholipid mechanisms in the tumor tissue. The 13P MRS spectra of the phospholipid extracts from bone morrow mononuclear cells (BMMC) consisted of 7 resonant peaks due to PC - phosphatidylcholine, CPLAS - phosphatidylcholine plasmalogen, SM - sphingomyelin, PE - phosphatidylethanolamine, PI - phosphatidylinositol, PS - phosphatidylserine, CL - cardiolipin. *Aims.* To continue our previous studies on the application of 31P MRS to the analysis *in vitro* of phospholipids changes in bone marrow mononuclear cells from patients with AL, we decided to evaluate this method to see if it gives satisfactory results during apoptosis of blast cells induced with Ara-c *in vitro*. *Design and Methods.* The investigation was carried out on BMMC from 20 patients with acute leukemia (AL). Mononuclear cells were cultured without and with 20 g/mL Ara-C for a 24-hour period. Subsequently, flowcytometric analyses were performed with Anexin-V and Propidium Iodide staining, measured by apoptosis. Phospholipids were extracted with a modified Folch method from the same BMMC. The spectra 31P were received on an AMX 300 Brukner (7.05T) spectrometer. Calculation of phospholipid concentration was based on peak integral intensities in the 31P NMR spectra for the individual compounds and methylenediphosphonic acid (MDPA). *Results.* On the basis of our investigations we divided the patients into two groups. The first one (A), from which the percentage of apoptotic cells after 24 hours of incubation with Ara-C was less than 25% (the average being 17%) we noted as non-responsive to Ara-C. The second group (B), from which the percentage of apoptotic cells was over 25% (the average being 46.8%) was classified as responding to Ara-C. In group A, a significant difference between the concentrations of phospholipids in cells cultured with and without Ara-C only for CPLAS could be verified. The average increase of the concentration is 7.0 mol/l ($p<0.15$). In group B, a significant difference between the concentration of phospholipids in cells cultured with and without Ara-C for the following phospholipids PI and PE was observed. An average decrease PI 17.9 µmol/l ($p<0.20$) and PE 20.1 mol/l ($p<0.15$) with the given level of significance could be verified. *Conclusions.* The study of the above application of *in vitro* 31 MRS for early detection resistance of Ara-C in blast cells in patients with AL, may show a difference in the concentration of phospholipids observed during apoptosis induced by Ara-C *in vitro*. Moreover, investigated changes of phospholipids concentrations with 31P MRS may suggest their participation in Ara-C induced cell apoptosis.

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ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF AN ORAL IRON-CHELATING AGENT DEFERASIROX ON HUMAN LEUKEMIC CELL LINES

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Background. Iron chelation therapy has been proved to have antiproliferative and apoptotic effects in rat and human cell lines, including hepatoma, neuroblastoma and leukemia. However, most studies were regarding the effect of deferoxamine (DFO), and it is still unknown whether a new oral iron chelating agent deferasirox has the antiproliferative effect on human leukemic cell lines or not. *Aims.* The purpose of this study was to investigate the antitumor effect of deferasirox on human leukemic cell lines. *Design and Methods.* Two human leukemic cell cultures (HL-60 and KG-1) were maintained in the RPMI1640 with L-glutamine (300 mg/L), 25 mM HEPES, 25 mM NaHCO3 and fetal bovine serum. Deferasirox was added at the concentrations of 50 or 100 uM. DFO was used as an iron chelator reference. Cell proliferation was evaluated daily by MTT assay for 72 hours. Cell cycle was analyzed by flow cytometry. Apoptosis assay was done by flow cytometry with use of Annexin V and propidium iodide (PI). We also studied the change of proteins involved in the apoptosis pathway by western blot. All experiments were repeated at least three times. *Results*. MTT assay showed that deferasirox had antiproliferative effect on both cell lines. As early as day 1, cell proliferation was started to be suppressed in the deferasirox-treated group ($p<0.001$), and this suppression effect continued until culture day 3. This antiproliferative effect was dose-dependent. Cell cycle analysis revealed that increased subG1 fraction compared to control (no treatment group). On day 1, subG1 fractions were 6.6% (control), 88.9% (50 uM), and 96.4% (100 uM), respectively. On day 2 and 3, this fraction increased up to 95-99% in the deferasirox-treated group. Increased apoptosis was also confirmed by Annexin V and PI staining. Western blotting showed that decreased expression of bcl-2 in deferasirox-treated KG-1 cell line. *Conclusions*. We demonstrated that a new oral iron-chelating agent deferasirox had antiproliferative and apoptotic effects on human leukemic cell lines. *in vivo* study and investigation of synergism with current antileukemic drugs are warranted.

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THE INFLUENCE OF FORODESINE USED ALONE OR IN COMBINATION WITH OTHER CYTOTOXIC DRUGS ON THE VIABILITY AND APOPTOSIS OF CLL CELLS IN VITRO- PRELIMINARY RESULTS

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Background. Recent observations suggest that forodesine (F), a novel inhibitor of purine nucleoside phosphorylase (PNP), may have clinical activity in chronic lymphocytic leukemia (CLL). The main mechanism of action of F is induction of apoptosis by intracellular accumulation of deoxyguanosine triphosphate (dGTP) unlike other purine analogs, cladribine (2-CdA) and fludarabine (FA) which are incorporated into DNA. Bendamustine (BEN), an alkylating drug and another promising agent in CLL, may also exert an antineoplastic effect by triggering tumor cell apoptosis. *Aims.* The aim of our study was to evaluate the effect of F alone or in combination with 2-CdA, FA or BEN on CLL cell viability and apoptosis. The type of interaction was also tested. *Design and Methods*. Peripheral blood mononuclear cells (PBMCs) were obtained from 30 untreated CLL patients who gave informed consent. In preliminary experiments, the following concentrations of drugs were used: 1, 2, 3 or 4uM of F alone; deoxyguanosine (dGuo): 10 or 20uM alone or in combination with F; 2-CdA: 25, 50 and 100nM, FA: 0.8, 1.6 and 3.2uM, BEN: 20, 40 and 80 ug/mL. Concentrations of 1 or 2 uM F + 20uM dGuo were chosen for further experiments and the effects of these drugs alone and in combination with 2-CdA, FA or BEN were tested. Cultures with initial PBMCs' concentration of $0.5x10^6$ cells/mL, were maintained for 24 and 48h. Cells maintained in cultures without drugs were used as controls. Cell viability was measured by flow cytometry after staining with propidium iodide, whereas apoptosis was detected by Annexin V assay. Compensating apoptotic index (CAI) was calculated by subtraction of spontaneous apoptosis in parallel control cultures from apoptosis measured in cultures with drug additions. Statistical differences between obtained values were evaluated by Wilcoxon signed-rank test. The combination index (CI) was used to estimate synergistic, additive or subadditive interaction. A CI value<0.8 was defined as synergism, 0.81-1.2 as addition and >1.21 as subaddition. *Results*. F or dGuo as single agents did not significantly influence CLL cell viability, whereas the combination F+dGuo resulted in significant apoptosis compared to either control or those agents alone ($p<0.01$). CAIs for all combinations of F used jointly with different dGuo concentrations, after 48h incubation were higher than 10%. The highest proapoptotic effects were obtained for 2uM F+20 uM dGuo and 3uM F+20uM dGuo (CAIs 22.5% and 23.8%, respectively). Moreover, median CAIs in samples treated with 2 uM of F +20 uM dGuo in combinations with the highest concentrations of BEN (40 ug/mL), 2-CdA (50nM) and FA (1.6uM) were significantly higher than in controls ($p=0.001$, $p=0.002$, and $p=0.0009$, respectively). F in combination with BEN acted synergistically (CI=0.8), whereas F used either with 2-CdA or FA showed subadditive interaction. *Conlcusions*. Our results demonstrated, that F combined with dGuo induce apoptosis of CLL cells. Additionally, the combination of F with BEN exerts synergistic effects, suggesting that clinical studies on the efficacy of F alone or in combination with BEN in CLL patients are warranted.

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THE STUDY OF APOPTOTIC MECHANISMS INDUCED BY ARSENIC TRIOXIDE AND AMIFOSTINE IN HUMAN ACUTE PROMYELOCYTIC LEUKEMIA CELL LINES HL-60 IN VITRO

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To explore the mechanisms of apoptosis induced by arsenic trioxide and amifostine in human acute promyelocytic leukemia cell lines HL-60. *Design and Methods*. HL-60 cells were given different concentrations of arsenic trioxide alone and combined with amifostine.The inhibitory ratio of the cells were measured by MTT assay, cell cycle was examined by flow cytometry(FCM), and the expression of survivin was detected by semi-quantitate RT-PCR.Result Proliferation of HL-60 cells exposed to arsenic trioxide dropped down with increasing dose of the drug and this effect was significantly higher when arsenic trioxide was used in combination with amifostine. HL-60 cells was arrrested at G1 phase and decreased remarkably in S phasein in time and dose-dependently manner after exposure to arsenic trioxide combinated with amifostine. Furthermore,there was a more significant decrease in survivin expression in HL-60 cells trated with arsenic trioxide in combination with amifostine as compared to the cells trated only with arsenic trioxide. *Conclusions.* Arsenic trioxide induced HL-60 cells form apoptosis by arrresting at G2/M phase and probably downregulating the expression of survivin.Amifostine enhanced the sensitivity of HL-60 cells to arsenic trioxide by arrresting at G1 phase (perhaps this is the non-expression phase of survivin) thus educed its promoting apoptosis effect.

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THE ROLE OF SIMVASTATIN IN INDUCING APOPTOSIS AT PATIENTS WITH MALIGNANT HAEMOPATHIES

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Background . The inhibition of protein geranylgeranylation by statins led to reduction of cell viability of malignant cells in a time and dose-dependent way, process mediated by both induction of apoptosis and inhibition of proliferation. *Aims.* Our aim was to study the effect of Simvastatin on the expression of annexine V at patients with chronic lymphoproliferations. *Design and Methods*. We have studied a group formed by 23 consecutive patients with malignant lymphoproliferations which were hospitalised in the Haematology Department of the County Clinical Hospital from Sibiu during April-June 28 and had no contraindications for statins. They all received Simvastatin 12 mg/day, three days. Before, and after, we have performed the next immunological, haematological and biochemical tests: annexine V, haemoleucogram, glycaemia, creatinine level, ALAT, ASAT, bilirubin, cholesterol, triglycerides and total lipids. The dosing of anexine V was performed with a CYTOMICS FC5 flowcytometer, which can simultaneous recognize 4

different fluorocroms. The protocol for annexine V for patients with chronic lymphoproliferations (except patients with multiple myeloma) was Annexin V-FITC, PIPE, CD2-ECD, CD5-PC5, and for those with multiple myeloma was Annexin V-FITC, PIPE, CD45-ECD, CD38-PC5. The results were statistically analysed using the SPSS program. *Results*. The average age of the patients from the studied group was 65.78±1.91 years. The medium survival period from the moment of diagnosis was 28.6±3.39 months. At them, there was performed an average number of 1.17±1.11 therapeutic lines and they had the next diagnoses: chronic lymphocytic leukemia - 8 patients, non-Hodgkin malignant lymphoma - 8 patients and multiple myeloma - 7 patients. The cholesterol and total lipids level decreased significantly after the treatment with Simvastatin (from 192.17±41.95 mg/dL to 166.83±45.91, and from 691.76±193.96 to 597.74±189.17 mg/dL, respectively) (p<0.001, respectively p=0.016). The increasing level of ASAT was at the limit of statistically significance and did not overgrow the normal range, while ALAT registered a decrease, but without statistically significance. The early and late apoptosis, estimated by the flowcytommetric dosage of the annexine V, increased significantly after the treatment with simvastatin (p=0.007, respectively p=0.003). *Summary.* The Simvastatin administration could have an adjuvant effect in the treatment of patients with malignant lymphoproliferations, by inducing apoptosis.

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PROTEOMIC ANALYSIS OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Acute lymphoblastic leukemia (ALL) is a malignant disease of the blood and the bone marrow and is considered the most common type of childhood cancer. Although the rates of success in treatment of ALL are steadily increasing, further attempts may lead to a more favorable therapeutic outcome. The use of proteomic technology could prove beneficial on knowledge regarding early cancer detection, monitoring response to treatment, or identification of the most informative diagnostic markers. *Aims*. In a clinical practice, plasma represents an opportunity for proteomic research to benefit prognosis and response to therapy. The purpose of this study was to verify and validate a list of protein candidates relevant to the prediction of the clinical behavior and personalized treatment of childhood ALL when performed on plasma from bone marrow (BM) and peripheral blood (PB). *Design and Methods*. Twenty eight plasma samples from BM and PB collected from patients with confirmed childhood ALL at diagnosis were analysed. Diagnosis was based on morphology, cytochemical staining, cytogenetic analysis, molecular and immunophenotypic study. As controls, four plasma samples from BM and PB from non-leukemic pediatric patients were studied in parallel. Differential proteomic analysis was performed by two two-dimensional gel electrophoresis (2-DE) followed by protein identification by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry. In the first dimension, in order to increase sensitivity, all samples were run on several gels each with different pH range starting from 3-10NL and following with the smaller 4-7L pH range. The second-dimension electrophoresis was carried out in 12% SDS-polyacrylamide gels in a PROTEAN apparatus. The gels were stained with colloidal Coomassie blue, scanned in a GS-800 Calibrated Densitometer and analyzed using the PDQuest image processing software. The Swiss-Prot database (*http://ca.expasy.org/sprot/*) was used to search for protein identification. *Results*. Fifty proteins were found differentially expressed from ALL BM plasma samples and 60 proteins were found to be expressed from PB plasma as compared to control. By these, 35 proteins were detected in both the BM and the PB plasma of the ALL pediatric patients. The majority of the identified proteins included suppressor genes, metabolic enzymes, structural proteins and signal transduction mediators. *Conclusions*. Our study demonstrates the potential of proteomic technology for the identification and confirmation of protein composition in plasma. However, a comprehensive knowledge of the value of these proteins is required in order to elucidate its relevance on childhood ALL screening, diagnosis and treatment outcome.

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PROTEOMIC ANALYSIS OF CHILDHOOD ACUTE MYELOID LEUKEMIA

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Background. Childhood acute myeloid leukemia (AML) is a frequent hematological malignancy that shows a high degree of heterogeneity in response to therapy. Given the diverse nature of AML and the inherent variability in individual protein levels, it seems likely that the best approach to screen for AML will be to determine the protein profile using proteomic technology. *Aims*. We studied two cases of childhood AML, in order to identify proteins, whose function could regulate the clinical characteristics and the treatment outcome of the disease. By these, one was diagnosed with myelodysplastic syndrome (MDS) before AML diagnosis, whilst the other with confirmed AML. *Design and Methods*. Six bone marrow plasma (BM) and six peripheral blood plasma (PB) samples were obtained before diagnosis, at diagnosis of AML and three months following initiation of treatment. As controls, two plasma samples from BM and two from PB from non-leukemic pediatric patients were studied in parallel. Differential proteomic analysis was performed by two-dimensional gel electrophoresis (2-DE) of overlapping pH ranges from 3 to 10, followed by protein identification by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. For the detection of the differential expressed proteins, gels were scanned in a GS-800 Calibrated Densitometer and analyzed using the PDQuest image processing software. *Results*. Comparison of plasma samples from BM and PB of the AML pediatric patients with the controls revealed differences in various molecular mass ranges with different isoelectric points. Forty one proteins were found differentially expressed between the BM samples as compared to the control. Among these, 10 were found expressed from the BM sample derived from the MDS case before AML diagnosis as compared to the control, whilst the remaining 31 proteins were found differentially expressed from the BM samples at diagnosis. In addition, 8 proteins (Vitamin D-binding protein precursor, Serotransferrin precursor, Complement C3 precursor, Clusterin precursor, β-2-glycoprotein 1 precursor, Apolipoprotein E precursor, Apolipoprotein C-II precursor and Antithrombin-III precursor) were found differentially expressed from the follow-up BM samples. Regarding the proteins extracted from PB plasma samples, 26 were found differentially expressed between the PB samples as compared to the control. Among these, 4 proteins were found expressed from the PB sample derived from the MDS case before AML diagnosis as compared to the control, whilst the remaining 22 proteins were found differentially expressed from the PB samples at diagnosis. Eight proteins (α-2-macroglobulin precursor, Apolipoprotein A-I precursor, Clusterin precursor, Gelsolin precursor, Keratin type I cytoskeletal 10, Leucine-rich α-2-glycoprotein precursor, Prothrombin precursor and Transthyretin precursor) were found differentially expressed from the follow-up PB samples. In general, functional analysis showed that the majority of the detected proteins were metabolic enzymes, structural proteins, signal transduction mediators and immunoglobulins. *Conclusions.* Proteome analysis of plasma seems to offer a useful approach for profiling pediatric patients with AML. Additionally, the current technology was shown to provide significant insight into the presence and/or absence of several proteins that might serve as useful biomarkers for childhood AML. However, further analyses are warranted to validate and uncover the role of these findings.

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A COMPARISON OF IN SILICO AND IN VITRO SEARCH FOR A GENE EXPRESSION PATERN SPECIFIC FOR STEROID RESISTANCE OF LEUKEMIC CELLS COLLECTED FROM PEDIATRIC PATIENTS WITH ALL

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Background. Among recent research concerning THE treatment of ALL, neoplasmatic cell genetic profile analysis is one of the most far-reaching. The expression of genetic material is surely one of the main areas of interest for molecular analysis. The involvement of bioinformatics into molecular research may be a way to avoid inconsistancy of the results, and establishing new genetic prognostic factors. Aims: Using a bioinformatic analysis for objective selection of candidate, prognosticaly important for ALL, genes, which have not been yet associated with this disease's clinical outcome. Analysis of expression of selected genes in

leukemic cells, and correlation between them. *Design and Methods*. The bioinformatic analysis was performed with genomic data fusion method. The ENDEAVOUR software was used. As a training set we used 3 genes which have been already recognized as important for response to chemotherapy in ALL (NR3C1, FKBP5, NFkBIA). In a group of 36 children with ALL, the molecular analysis was performed on leukemic cells collected at the day of diagnosis. To evaluate the increase of product of amplification reaction in real-time the RT-PCR technique was used. *Results*. The algorithm of bioinformatic analysis results with selection of for genes to further analysis. Among those genes there were: JunB - encoding transcription factor, CCL3 - encoding chemokine, NCC27 - encoding chloride channel in nuclear membrane and RBM14 - encoding the co-activator protein for transcription factors.

Table 1. Correlation matrix between gene expression levels.

Values given as Spearman rank correlation

Variable	Delta NFKBIA	Delta FKBP5	Delta JUNB	Delta CCL3	Delta RBM14	Delta NCC27	Delta NR3C1
Delta NFKBIA		0,24	0,12	0,21	0,51 p<0,01	0,42 p<0,05	0,31
Delta FKBP5	0,24		-0,12	-0,28	0,11	0,24	0,51 p<0,01
Delta JUNB	0,12	-0,12		0,14	0,54 p<0,001	0,34 p<0,05	-0,56 p<0,001
Delta CCL3	0,21	-0,28	0,14		-0,01	-0,05	-0,24
Delta RBM14	0,51 p<0,01	0,11	0,54 p<0,001	-0,01		0,87 p<0,001	0,02
Delta NCC27	0,42 p<0,05	0,24	0,34 p<0,05	-0,05	0,87 p<0,001		0,21
Delta NR3C1	0,31	0,51 p<0,01	-0,56 p<0,001	-0,24	0,02	0,21	

Those genes where selected as never before associated in research reports with ALL biology and outcome. The expression level of reported genes was given as delta value in relation to GAPDH expression (housekeeping gene) and mean values was; ΔNR3C1 = 4.40±1.55, ΔNFkBIA = 1.59±1.59, ΔFKBP5 = 4.15±1.35, deltaJUNB = -0.65±5.2, ΔCCL3 = 5.48±3.6, ΔRBM14 = 3.15±2.13, ΔNCC27 = -0.11±1.67. Non-parametric statistical analysis revealed strong correlations between the expression level of RBM14 and NCC27, JUNB plus between NR3C1 and JUNB (p<0.001). The correlation between NFkBIA and RBM14 expression, and also in apply to NR3C1 and FKBP5 expression was also statistically significant (p<0.01). For the relation of expression of NCC27 gene with JUNB and NFkBIA (p<0.05). Correlation matrix of mRNA expression of selected genes is shown in Table 1. *Summary*. Our results shows that the usage of bioinformatics leads to objective selection of a small group of genes connected with glucocorticoid resistance of ALL cells, what have been proved with correlation matrix of mRNA expression in leukemic cells *in vitro*.

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DESIGN AND DEVELOPMENT OF HIGHLY SPECIFIC CLONAL ANTIBODIES FOR THE CHARACTERIZATION HUMAN, BOVINE AND OVINE PRION PROTEIN

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Quantification of differences in structural conformation of two physiologically different but by aminoacid composition identical prion proteins, PrPC and PrPSC, is one of the main target in basic studies concerning the spongiform encephalopathy (SE). The characterisation of those two forms of the disease-related main infectious agent, including the related proteome, is crucial for the characterisation of signalling pathways involved in potential transmission of SE from bovine spongiform encephalopathy (BSE) and sheep (scrapie) to human, developing Creuzfeld-Jacob Disease (CJD). The new approach in design and production of clonal antibodies based on detailed proteomic characterization of antigen molecule was applied for the prions. The basic idea was to develop the antibody which will recognize both forms of protein, and the antibodies which recognize only PrPC. Difference in obtained signals may quantify the potential conformational change and formation of pathological PrPSC. Outside of antibody production, we introduced the mammalian cell lines for stable and transient expression of PrPC, which may serve for potential studies of prion transmission, as well as studies for its pathological conversion. Four clonal antibodies were produced and their quality characterized by western blot. First clonal antibody recognizes human, bovine and ovine prions, and was designed to the epitope at the N-terminal sequence of prion protein (within the sequence T33 - R48). The second antibody was produced to the epitope within aminoacid sequence R136 - E152 corresponding to the part of α-helical domain 1 of PrPC. This region may be changed to β-sheet structure in PrPSC, therefore may be used for structural changes in prions for human, ovine and bovine proteins. C-terminal sequence of α-helical domain 3 (Q223 - F235) is characterized by the third clonal antibody and may be applied for the studies of conformational changes as well. This clone is human prion specific. The fourth clone, which covers the bovine and ovine prions (E232 - F246) recognizes PrPC of human and ovine. The COS-7 and HEK293 cells were PrP depleted by knock-out and dually co-transfected with combination of vectors containing the gene of human, bovine and ovine PrP. The transfected cells were used for investigation of PrP interspecific interactions simulating disease transmission among humans, beef-cattle and sheep. The present work demonstrate the potential of clonal antibodies for application in prion conformational changes studies. The quick, efficient and easy to use test may help in testing the potential of PrPSC in human, bovine and ovine tissues/biological fluid samples. This is of intense need in today's diagnostics/prevention studies.

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HIGH PREVALENCE OF MTHFR GENE A1298C POLYMORPHISM IN IRAN

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Background. Mutations in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene could reduce the enzyme activity and via different mechanisms can lead to several diseases such as vascular disease in particular, coronary artery disease and deep vein thrombosis and also cancers. *Aims*. The aim of this study was to assess the prevalence of the two most common polymorphisms, C677T and A1298C, which have not been studied in the Iranian population so far. *Design and Methods.* We randomly selected 160 healthy individuals. Using PCR and RFLP analysis, we studied the prevalence of the C677T and A1298C MTHFR genotypes in these cases. *Results*. We found that for C677T, the prevalence of C/C, C/T, and T/T genotypes was 63.1%, 29.4%, and 7.5%, respectively with an allelic frequency of 0.22. However, the A1298C genotypic prevalence of A/C, A/A, and C/C was 56.3%, 30.6%, and 13.1%, respectively, with an allelic frequency of 0.41. *Conclusions*. Our results about the prevalence of C677T variant between Iranian populations were similar to other reported studies in Asia. Otherwise, Compared to most other populations reported so far, the Iranian population showed a high prevalence of the MTHFR A1298C polymorphism. This is an important finding to be followed in terms of clinical significance in this community. Atashrazm F, Hematology department, IUMS, Tehran, Iran

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INCIDENCE OF MUTATIONS OF CLASS III RECEPTOR TYROSINE KINASE (KIT AND FLT3) IN PEDIATRIC AND ADULT ACUTE LEUKAEMIC PATIENTS

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Background. C-kit and FLT3 are member of the class III receptor tyrosine kinase family. The mutations in these genes lead to uncontrolled proliferation of leukemic cells and unfavorable prognosis. *Aims*. The data concerning the incidence and associations with patient's characteristics vary in different studies. The purpose of this study was molecular charcacteristic of leukemia, diagnosis and frequency determination of these mutations with different subtypes in AML and ALL patients. *Design and Methods*: Blood or Bone Marrow samples from newly diagnosed acute leukemic 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 diagnosis and the assignment of FAB classification were based on morphology and

immunophenotype. DNA extraction was performed on all the blood samples by proteinase K and phenol method. 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 were analyzed by PCR followed by CSGE and RFLP respectively. Subsequently, PCR products of positive ITD and exon 8 of c-kit have been confirmed by sequencing techniques. *Results*. The median age of adult AML was 47 +/- 12 (range from18-75) years and in pediatric acute leukemia was 5.5 +/- 1.6 (range from 1-17) 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). In 91 pediatric acute leukemia (18 AML and 73 ALL), Flt3 ITD and D835 mutations occurred in 7.7% and 2.1% respectively. The frequency of ITD mutations in AML found 23% with highest occurrence in M3 subclasses (11.2%) characterized by the t (15;17). The frequency of ITD mutations in ALL found 4.1%, which occurred only in early pre B subclasses. Interestingly, ITD not found in pre B, B and T ALL. The frequency of D835 mutations in AML found 5.2% and it was found only in M3 subclasses characterized by the t (15;17). The frequency of D835 mutations in ALL found 1.3%, which occurred only in pre B subclasses. Interestingly, D835 not found in early pre B, B and T ALL. The sequencing of PCR products of ITD showed different insertions of nucleotides in JM region of ITD such as 27, 47 and 63 bp insertions which were in similar with those literature reported previously. *Conclusion*. 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 frequency of ITD in ALL was significantly lower than AML and it was only found in early pre B. Mutations of c-kit resulted in 5.6% of adult AML (one patient had both mutation together), which was significantly associated with M2 and M4 with T8-21 and INV16.

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MEGAKARYOCYTIC BLAST CRISIS IN PATIENT WITH RARE VARIANT OF PHILADELPHIA REARRANGEMENT T(9;22;22) AND ADDITIONAL TRANSLOCATION T(3;7)

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Megakaryoblastic crisis is extremely rare and accounts for less than 3% of chronic myelogenous leukemia (CML) in blastic transformation. The variant translocation in CML is reported for 2-10% of CML. We present an unusual case of megakaryocytic blast crisis with variant Philadelphia rearrangement t(9;22;22) and additional translocation t(3;7). Patient, a 73-year old woman diagnosed for chronic myeloid leukemia with suggested an accelerated phase of CML was admitted to the Department of Hematology. Past history was significant typical attributable for CML in a chronic phase (2003). The patient started therapy with alfa-interferon. Treatment with alfa-interferon was immediately discontinued because of intolerance and the patient was treated further with imatinib mesylate (2004). In November 2006 she was admitted to the Departament because of deep venous thrombosis of left inferior extremity. Laboratory studies showed platelets 3144 G/l. Afterwards the secondary therapy with imatinib mesylate combined with hydroxyurea was started. The patient showed a positive haematologic response. In Jun 2007 she was diagnosed as having CML in megakaryoblastic transformation. CBC showed anemia, WBC 30 G/l, platelet count 5972G/l. The bone marrow aspirate was hypercellular with megakaryocytic hyperplasia and increased in the myeloblast count (12%) was detected. Molecular analysis by RT-PCR showed an occurrence of D276G mutation of BCR/ABL oncogen. The presence of this mutation is correlated with resistance to the treatment with imatinib mesylate. The patient was treated with dasatinib. All 20 metaphases analysed showed the abnormal chromosomes 3, 7, 22 and second homologue chromosome 22. The suggested karyotype was described as follows 46,XX,t(3;7)(p25;q34),t(9;22;22)(q34;q11;q13). The FISH analyses have confirmed an occurrence of chromosome translocations as showing classical cytogenetics. The karyotype analysed after 8 months of continous treatment with dasatinib was showing 6 cells with the translocations of both types and 14 cells only with translocation between short arm of chromosome 3 and long arm of chromosome 7. It means 70% metaphases did not contain Philadelphia chromosome. In clinical terms in relation to the beginning of treatment the patient was found responsive to dasatinib treatment. The translocation t(3;7) could be constitutional translocation because it is relatively stable during treatment. The alternative explanation is that translocation t(9;22) is of secondary origin and arises after translocation t(3;7) which is persistent in the treatment. The breakpoint in 22q13 region could be causative for an increase number of megakaryocytes in our patient. This region includes the gene MKL1, which is involved in a specific translocation that creates a fusion with RBM15 gene (1p13), this translocation has been associated with acute megakaryoblastic leukemia. Some genes, which play a role for platelets growth and their function {PDGFB (22q13.1), TCF20 (22q13.3), and FBLN1 (22q13.31)} are also located in 22q13 region.

1201

BONE MARROW VERSUS PERIPHERAL BLOOD: COMPARISON OF THE CHIMERISM ANALYSIS

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Background. Bone marrow (BM) examination is routinely performed for minimal residual disease monitoring in leukemia patients. By contrast, chimerism is usually tested from peripheral blood (PB), and, surprisingly, little is known about the clinical impact of the chimerism status in BM. *Patients and Methods.* 226 pairs of PB and BM samples obtained from 102 adult patients were investigated. Frequencies of diagnoses within samples were as follows: AML 42%, CML 24%, ALL 11%, CLL 7%, MDS 7%, other hematological malignancies 9%. Assessment of chimerism was performed using either capillary electrophoresis with fluorescence detection, or quantitative real-time PCR with one order of magnitude higher sensitivity of mixed chimerism detection. *Results.* Mixed chimerism in BM was detected even more than 9 years after transplantation, however, on the other hand, complete donor chimerism was also observed as early as day +35. Proportion of autologous cells detected in BM comparing to PB was: higher in 140/226 cases (62%), equal in 67 (30%), and smaller in 19 instances (8%). Within the group with higher autologous fraction in BM, 75/140 (54%) pairs were those with complete donor chimerism in PB and mixed chimerism in BM at the same time; the majority (61/75; 81%) of such BM samples contained only very small amount of autologous cells (bellow 0.1%). Nevertheless 18/140 cases (13%) possessed clear clinical relevance and all but one were from AML patients: chimerism detected in both compartments varied from 0.1% to 60% and corresponded to either impending relapse (n=16) or a graft failure (n=2). *Conclusions.* Our data indicate that chimerism analysis in BM can provide new information in contrast to PB analysis in AML patients. Highly discrepant results with high autologous recovery in BM can be considered as a sign of imminent relapse or a graft failure. Two important questions are remaining to be further explored: what are the residual autologous cells causing the persisting mixed chimerism in the BM, and what is the best timing for BM chimerism analysis.

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1202

PROGNOSTIC SIGNIFICANCE OF FLT3-ITD, FLT3-D835 MUTATIONS AND NPM1 MUTATIONS IN ACUTE MYELOGENOUS LEUKEMIA

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Background. Cytogenetic aberrations are one of the most important independent prognostic factors in AML. However 40-50% of the patients have a normal karyotype conferring an intermediate risk, but their survival is highly variable. FLT3 mutations or internal tandem duplication (ITD) and NPM1

mutations play an important role in these patients. The presence of FLT3 aberrations is associated with an unfavourable prognosis whereas NPM1 mutations, when isolate, confer better prognosis. Some studies have described that increased values of the ITD/wild-type (WT) allele ratio confer a worse outcome. The aim of the study is to characterise the prognostic significance of the ratio of ITD/WT alleles, FLT3-D835 mutations and NPM1 mutations. *Patients and Methods*. One hundred fifty-eight patients with AML (93 patients with non promyelocytic AML, 15 with acute promyelocytic leukaemia, 36 AML with multilineage dysplasia and 14 with secondary AML) were examined for the presence of FTL3 and NPM1 mutations. Most of them were treated according the AML-03 multicenter protocol of the Spanish Group (CETLAM). Risk groups were defined according to results of standard cytogenetic analysis as high risk, intermediate risk and low risk. Patients with promyelocytic AML were included in the PETHEMA APL-05 trial and had been excluded for the prognosis analysis. The presence of FLT3-ITD was performed on genomic DNA. Polymerase chain reaction (PCR) products were analysed on standard 3% agarose gels. Ratio from patients with ITD and NPM1 mutations were established using a fluorescently labelled primer (Nakao M et al. Leukemia 1996 and Gale R et al. Blood 2008, respectively) and analysed by Genescan. FLT3-D835 mutations were determined using an ECORV restriction enzyme digestion after PCR amplification. *Results*. From the 158 patients, 84 had an intermediate-risk karyotype (53.1%) and 31 harbour an ITD (19.6%). Twenty-two of these 31 patients (71%) had an intermediate-risk karyotype. FLT3-ITD/WT ratio ranged from 0.041 to 5.431 (median 0.46). In patients with intermediate-risk karyotype the presence of FLT3-ITD was associated with more percentage of blasts in bone marrow (p=0.014), high WBC count (p<0.001), inferior OS (p=0.001) and CR (p=0.002). Neither the FLT3-ITD/WT ratio nor the FLT3-D835 mutations had impact on DFS or OS in the present series. Patients with FLT3-D835 mutations had a trend for higher relapse (p=0.060). A total of 21% of patients had NPM1 mutations. These mutations were seen more frequently in intermediate-risk patients (p<0.001) and the patients had more percentage of blasts in bone marrow (p<0.001) and high WBC count (p=0.012). The good prognosis of NPM1 mutations disappeared with the concomitant presence of FLT3-ITD (p=0.055). *Conclusions*. The presence of FLT3-ITD and NPM1 mutations is associated with higher percentage of blasts in bone marrow and high WBC count. Although FLT3-ITD has poor prognostic significance in patients with AML and intermediate-risk karyotype, the FLT3-ITD/WT ratio, FLT3-D835 and NPM1 mutations (and FLT3-ITD) did not have impact on prognosis in these patients from the present series.

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1203

POLYCYTHEMIA VERA WITH JAK2V617F AND JAK2C618R MUTATIONS: A MOLECULAR AND FUNCTIONAL STUDY

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Polycythemia vera (PV) is a chronic myeloproliferative disorder (MPD) characterized by somatic activating mutations in the JAK2 gene. JAK2V617F is the predominant mutation occurring in over 90% of patients. We describe a molecular and functional study of two combined JAK2 mutations which were identified in an 83 year-old caucasian man with PV. Allele-specific polymerase chain reaction (AS-PCR) assay for JAK2V617F confirmed the presence of this mutation in peripheral blood cells of the patient. Direct sequencing of exon 14 showed heterozigosity for JAK2V617F but also revealed a heterozygous thymine for cytosine substitution at cDNA position 1852 (T1852C). This alteration leads to incorporation of an arginine residue instead of a cysteine at codon 618 (JAK2C618R). To exclude that T1852C is a rare polymorphism, we confirmed its absence in 200 chromosomes of normal caucasian individuals by PCR and restriction enzyme digestion using a BtgI restriction site created by the T1852C substitution. In addition, sequencing analysis of JAK2 exon 14 in DNA obtained from peripheral blood mononuclear cells of the patient showed that both nucleotide alterations were present only in a minor proportion comparatively to whole blood cells, indicating that T1852C was also an acquired alteration. Cloning of exon 14 PCR product followed by screening of individual bacterial colonies showed that JAK2V617F and JAK2C618R mutations were present in cis. We then designed a reverse primer for detection of JAK2C618R and included it in the AS-PCR assay instead of the JAK2V617F-specific primer. Using this approach we analysed peripheral blood DNA samples of 48 JAK2V617F-positive PV patients, 43 JAK2V617F-negative patients with erythrocytosis, and of 74 patients with essential thrombocythemia (39 JAK2V617F-positive and 35 JAK2V617F-negative). The JAK2C618R mutation was not detected in any of the 165 patients. For functional analysis, the full-length JAK2 cDNA was amplified from a normal individual sample and cloned into a N-terminal GFP expression vector. The JAK2V617F and JAK2C618R mutations were introduced alone or in conjunction using site-directed mutagenesis. Proliferation assays are now being performed to compare proliferative potential of differentially-mutated JAK2 clones. In conclusion, the results of DNA analysis show that JAK2C618R is a rare mutation in MPD patients and also suggest that selection of a mutant clone carrying JAK2C618R may be dependent on the acquisition of the JAK2V617F mutation.

1204

TRISOMY 8 IN PDGFRB-NEGATIVE CELLS IN A PATIENT WITH IMATINIB SENSITIVE CHRONIC MYELOMONOCYTIC LEUKAEMIA AND THE T(5;16)(Q33;P13)/PDGFRB/NDE1 FUSION

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Background. The development of clonal chromosome abnormalities in Philadelphia-chromosome negative cells was well documented in chronic myeloid leukaemia (CML) responding to interferon-α (IFN) and imatinib (IM). Fusion genes involving the platelet-derived growth factor receptor β (PDGFRB) were identified in Ph-negative chronic myeloproliferative disorders (CMPD) generating a constitutive tyrosine kinase activity usually sensitive to IM. We identified a novel PDGFRB translocation partner (i.e. NDE1) in a patient affected by chronic myelomonocytic leukemia (CMMoL) with t(5;16)(q33;p13). *Aims*. Here we describe the clinical evolution of this patient who responded to IM while developing a clone with trisomy 8 in PDGFRB negative cells. *Design and Methods* and *Results*. The patient, affected by Noonan syndrome with PTNP mutation and normal constitutional karyotype, was diagnosed with CMMoL in august 2004, age 30. Sequential hematological and cytogenetic features are shown in Table 1. Following the demonstration of PDGFRB involvement by fluorescence *in situ* hybridization (FISH), the patient was commenced on IM 400 mg daily on October 2004. After one month, complete hematological response with normal white blood cell count and differential and disappearance of splenomegaly was recorded, along with a major cytogenetic response (Table 1).

Table 1. Hematologic and molecular cytogenetic findings in a patient with CMMoL and t(5;16)(q33;p13) - PDGFRB/NED1 fusion.

date / phase	WBC (a) (monocytes)	BM findings	Hb (a)	Plts (a)	Karyotype (b)	FISH analysis (c)	
						Trisomy 8	*PDGFRB* translocation
May 2004 / diagnosis, hydroxyurea 20 mg/Kg/day	38,6 (13,0)	M/E 6/1; increased monocytes, DysE; DysMeg ALIP+	13,5	336	46,XX, t(5;16)(q32;p13) [13] / 46,XX [1]	<1%	83%
October 2004 / Imatinib 400 mg/day	8,5 (0,90)	M/E 5/1 Increased monocytes	9,5	70	46,XX, t(5;16)(q32;p13) [7] / 46,XX [12]	<1%	ND
November 2004 Imatinib 400 mg/day	7,0 (0,35)	ND	10,1	319	46,XX, t(5;16)(q32;p13) [3] / 46,XX [33]	<1%	11%
October 2005 Imatinib 400 mg/day	4,3 (0,27)	normal	11,8	161	46,XX [20]	<1%	<1%
April 2007 Imatinib 400 mg/day	4,1 (0,21)	normal	11,8	208	46,XX [20]	<1%	<1%
December 2007 Imatinib 400 mg/day	4,1 (0,90)	normal	10,2 (d)	165	47,XX, +8 [3] / 46,XX [20]	16,5%	<1%(e)
April 2008 Imatinib 400 mg/day	4,5 (0,82)	normal	12,1	178	46,XX [20]	3%	<1%

Abbreviations: DysE: dyserythropoiesis; DysMeg: dysmegakaryocytopoiesis; ALIP+: presence of abnormal localization of immature precursors
WBC: white blood cells; Hb: Hemoglobin (g/dL); Plts: platelets; ND: not done
(a) WBC, monocytes and Plts are expressed as absolute counts X 10^9/L; Hb is expressed as g/dL
(b): Constitutional karyotype on PB lymphocytes: 46, XX [20]
(c): % cells with the anomaly; the cut-off point for positivity was 3% for trismy 8 and 3% for *PDGFRB* translocation
(d): iron-deficiency anemia responsive to treatment
(e): RT-PCR for *PDGFRB/NDE1* fusion negative

A complete cytogenetic remission was achieved in October 2005. In December 2007 BM aspirate showed normal morphology, whereas cytogenetic and FISH documented the appearance of a clone with trisomy 8. No evidence of t(5;16) was found by cytogenetics. FISH and molecular (RT-PCR) studies excluded the presence of PDGFRB/NDE1 fusion. Hybridization of chromosome-8-centromeric probe was performed on BM smear and granulocyte precursors showed trisomy 8 in 12/50 cells, whereas only a minority of erythroid precursors (2/30 cells) displayed three signals. Four months later the patient was still on IM, her hematological conditions stable. Trisomy 8 was no more detectable by cytogenetics and FISH. *Conclusions.* Our finding of a complete response by RT-PCR in a patient with a new variant PDGFRB translocation is noteworthy. The appearance of an unrelated trisomy 8 in IM-sensitive Ph-negative CMPD was not reported before. In our patient +8 was noted 38 months after the start of IM and was no more detectable after 4 months. Fluctuations of the size of the abnormal clone were both described in cases with or without disease transformation (acute myelogenous leukemia, myelodysplastic syndrome). Fluctuating trisomy 8

populations were previously described in MDS. We documented the involvement of the granulocytic lineage by trisomy 8, whereas erythroid cell involvement was of borderline significance. The development of an unrelated clonal abnormality in a patient with IM-sensitive CMPD with PDGFB rearrangement shows that this phenomenon is not restricted to IM-sensitive CML and suggests that it may represent a general phenomenon in CMPD in which the founder clone is inhibited in its growth by an effective drug. Though the development of aneuploidy unrelated with the dominant clone appears to be part of the natural history of , in part reflecting genetic instability, a possible effect TK inhibitors on the mechanisms underlying chromosome segregation at anaphase cannot be ruled out, since TK may be involved in the assembly of the mitotic spindle.

1205

BONE AFECTATION IN TYPE 1 GAUCHER DISEASE: ROLE OF PROIMMFLAMMATORY CYTOKINES

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Background. Skeletal alterations are observed in 80% of patients with Gaucher Disease (GD). Bone disease may be the most debilitating and disabling aspects of GD, leading to bone marrow infiltration by Gaucher cells, failure of remodelling, osteopenia, osteonecrosis, osteosclerosis, bone crisis and chronic bone pain including pathological fractures. The mechanism of skeletal manifestations is unclear, but they appear to result from infiltration of the medullar space by Gaucher cells. They do not cause bone resorption directly, but could induce secretion of cytokines increasing osteoclast activation with secondary bone resorption. Therefore, the pathogenesis of the bone changes may be due to the increased bone resorption caused by Gaucher cells. Aims of study: To indentify a cytokine profile in type 1 GD patients with or without bone disease. *Design and Methods*. Two panels of different cytokines including: a) IL2, IL4,IL13, IL17, EGF, Fractalkine, INFg, MCP1, MIP1b, TGFa; b) IL4, IL6, IL7, IL10, IL13, MIP1a, MIP1b and TNFa were analysed in plasma samples by Luminex®100 platform and Millipore cytokine kit. A total of 16 type 1 GD patients (females 50%, mean age: 42 year; range:14-83) and 18 controls (females 63%; mean age: 45 years; range 23-63) were studied at diagnosis. Descriptive analysis and mean comparative non parametric χ^2 test was performed. Results: We had observed in type 1 GD a significant high plasma IL-2 (p=0.01), IL-17(p=0.001), TGFa(p=0.001)and lower IL4 (p=0.001), IL13 (p=0.001)concentrations vs controls. In addition we have detected a significant increase of IL13 and IL17 (p<0.01) in patients with vs without bone disease. No significant differences the cytokine profiles related age and gender had been observed. *Comments*. In our experience Luminex®100 technology is a sensible and accurate technique useful to determine cytokine profile in plasma. A different significant cytokine profile was observed between GD patients and controls as well as between GD patients with and without bone affectation.

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1206

THE VALUE OF BONE MARROW ASPIRATE SMEARS COMPARED WITH BONE MARROW TREPHINE BIOPSIES IN ROUTINE DIAGNOSTICS - A COMPARATIVE STUDY OF 77 CASES

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Background. Bone marrow examination is often requested for the diagnosis and staging of the haematological diseases. It is also useful in the assessment of patients with fever of undetermined origin as well as in the diagnosis of various storage and infiltrative disorders. Current knowledge indicates that both procedures (bone marrow aspirate smears, BMAS, and bone marrow trephine biopsies, BMTB) have advantages and disadvantages in establishing the final diagnosis.*Aim.* The present study compares the value of the two methods in terms of the clinical outcome, as they are routinely processed and analysed by haematologists, pathologists and internists. *Design and Methods.* 77 samples were collected. All samples were subjected for diagnostic purposes in both methods. Smears were prepared according to standard procedures and routinely stained with May-Grunwald-Giemsa and iron technique. At least 300 cells were valued microscopically. Marrow cores involved samples ≥1 cm and valued using also mAbs. For the comparisons of proportions Fisher's exact test was used.10 patients (13%) had lymphoma, 5 patients (6.5%) had monoclonal gammopathies, 11 patients (14.3%) had myeloproliferative disorders (MPD), 23 patients (29.9%) had myelodysplastic syndromes(MDS), 26 patients (33.8%) had non-malignant alterations (bone marrow reactive disease) and 2 patients (2.6%) had metastases of solid tumors. *Results.* The sensitivity for the detection of bone marrow diseases was 68,8% and 96,1% for BMAS and BMTB respectively. The proportion of agreement, independently of the disease category was 66,2 % (95% CI:55,4%-77,0%). The higher level of agreement between the 2 methods 88% (95% CI:75,3%-100%) was found for non malignant alterations. The afore mentioned level of agreement was significantly lower in the cases concerning MPD 27,3% (95% CI: 0%-58,7% p=0.01). The proportion of agreement concerning MDS was 69,5% (95% CI:49.2-89,9% p=0.10). As far as lymphoma was 60% (95% CI:23.1-96.9% p=0.076), while agreement concerning the five cases of monoclonal gammopathies and two cases of metastases was 60% and 50% respectively. *Conclusions*. Our data show that bone marrow trephine biopsy is more accurate in the final diagnosis than bone marrow aspirates. Regarding MPD BMAS is believed to be of lower diagnostic accuracy (p<0.005) due to the presence of fibrosis, the absence of specific diagnostic findings as well as the overlap (cover) with other accompanying lesions. Aspiration alone may be adequate for non malignant alterations.

1207

NEW STRUCTURAL CHROMOSOME ALTERATIONS IN PLASMA CELL DISORDERS

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Background. Multiple myeloma (MM) and related disorders are neoplasms of terminally differentiated B-cells. Cytogenetic information is often limited by the low proliferative activity of plasma cells. Finding of clonal chromosome aberrations is considered one of the most important and independent factor of adverse prognostic in plasma cell disorders. Aim: To report new chromosome structural aberrations observed in patients with MM and plasma cell leukemia (PCL). *Design and Methods*. A total of 40 patients with plasma cell disorders cytogenetically studied in our laboratory showed chromosome alterations. Among them, 10 (25%) cases (7 males: mean age 65 yr; range 45-80 years) were selected for inclusion in the present report based on the presence of new structural rearrangements. Seven patients were at diagnosis and three at relapse. Nine patients had diagnosis of MM and one case showed PCL. All patients gave their informed consent and the study was approved by the Ethics Committee of our Institution. Chromosome analysis was performed on BM cells processed by direct method and unstimulated 24-72 hs cultures, in F-12 medium supplemented with 20% fetal calf serum. G-banded technique was used. Karyotypic abnormalities were described according to the International System of Human Cytogenetic Nomenclature. FISH (fluorescence *in situ* hybridization) analysis using centromeric, locus specific, painting and Spectra Vision DNA probes (Vysis-Abbot) were performed according to manufacturer´s protocols. *Results.* In MM, a total of 24 new chromosome structural alterations were found: 19 translocations (16 unbalanced) and 5 complex rearrangements. Chromosome 1 was the most frequently involved (10 alterations), followed by chromosomes 13 (4), X, 2, 11 and 16 (3 each) and, 3, 5 and 7 (2 each). The most common aberrations of chromosome 1 were unbalanced translocations involving chromosomes: X, 2, 5, 7, 20 and 21, most of them determining 1q gains (60%). Four patients showed more than one abnormality of this chromosome. Complex rearrangements of chromosome 1 were: psu dic(X;1)(q24;p11), der(1)dup(1)(q25q32)t(1;4)(p12;p12) and psu dic(1;11)ins inv(1;11)(q10;p11q25)dup(1p?). New alterations of chromosome 13 were: t(6 ;13)(p15;q14), der(11)t(11;13)(p15;q14), der(13)t(2;13)(q11;p11) and der(14)t(13;14)(q14;q32). The patient with PCL had five new abnormalities: psu dic(15;1;22)(p12;q11;q10), del(4)(p15.2), del(6)(q25), del(7)(q11q32) and del(10)(p12.2). Almost alterations were confirmed by FISH using painting and/or Spectra Vysion DNA probes. FISH analysis also detected del13q14 (2 cases), IGH rearrangements (2), and del17p13 (1). *Conclusions.* We found new chromosome alterations and regions of cytogenetic deletion in patients with plasma cell disorders. More studies must be necessary to determine the recurrence of these abnormalities and their potential importance in lymphomagenesis.

1208

PROGNOSTIC RELEVANCE OF FLT3/ITD, NPM1 AND CEBPA MUTATIONS IN ACUTE MYELOID LEUKAEMIA PATIENTS WITH NORMAL KARYOTYPE- THE SINGAPORE GENERAL HOSPITAL EXPERIENCE

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Background. In the management of patients with Acute Myeloid Leukaemia (AML), the identification of prognostic markers is an important part of the evaluation as it helps to predict response to therapy and long term outcome.

Conventionally patients with normal karyotype- AML are classified into the intermediate risk category, but in reality, this is a highly heterogeneous group as the clinical outcomes of these patients vary greatly. Recently, it has been reported that a comprehensive assessment of known molecular prognostic markers would help identify clinically relevant subgroups among normal karyotype-AML patients. *Aims*.: To assess the incidences and prognostic relevance of mutations in the FLT3 (FMS-like tyrosine kinase 3), NPM1 (Nucleophosmin) and CEBPA (CCAAT/enhancer-binding protein α) genes in a series of adult normal karyotype- AML patients seen at our institution. *Design and Methods.* Consecutive newly diagnosed AML patients (n=61) with normal karyotype were analyzed for the presence of FLT3/ITD, NPM1 and CEBPA gene mutations. We compared the mutational status of these genes with clinical outcome in patients who were below 65 years of age and had undergone standard induction/consolidation chemotherapy with a minimum 1-year follow-up. *Results*. FLT3/ITD, NPM1 and CEBPA gene mutations were detected in 43% (26/61), 51% (31/61) and 23% (14/61), respectively, of the patients tested. While FLT3/ITD mutations occurred at similar incidences in M1, M2 , M4 and M5 subtypes, the incidence of NPM1 mutations was higher in M5 subtype (8/10, 80%). CEBPA mutations, however, appeared to be greatly associated with M1 and M2 subtypes. FLT3/ITD mutations varied in size from 18 bp to 210 bp. NPM1 mutations always resulted in a net insertion of 4 bp with Type A mutation being the most frequently occurring. About 52 % (16/31) of NPM1 mutated patients were also FLT3/ITD-positive. Of the 14 patients who had CEBPA N-terminal nonsense mutations, 13 patients had at least an additional in-frame C-terminal mutation. In this study, seven novel CEBPA N-terminal mutations which would result in a predicted loss of C/EBPα function were identified. Relapse-free survival (RFS) and overall survival (OS) analyses were calculated by Kaplan-Meier method and statistics were compared with the log-rank chi-square test. Patients with FLT3/ITD mutations, regardless of their NPM1 and CEBPA mutation status, had a significantly shorter median RFS (16.63 months, p=0.03) and shorter median OS (20.17 months, p=0.07) compared with patients without FLT3/ITD mutations (last event was at 66.67 months). Patients with NPM1 or CEBPA mutations without ITD mutations had longer median RFS and OS compared with patients with other genotypes (ITD-positive, triple negative). Median RFS and OS of the former cohort were not reached whereas they were 13.73 months and 20.17 months respectively for the cohort with other genotypes (p=0.002, p=0.06). *Conclusions.* Our study that mutational status of CEBPA, NPM1 and FLT3-ITD in normal karyotype-AML patients has significant prognostic implications. Although this is a small scale study, the results do suggest statistically significant impact in some aspects, thus corroborating the importance of systematic analysis of these three markers in newly diagnosed AML patients, particularly those without cytogenetic abnormality.

Table.

Effects of FLT3-ITD, NPM1 and CEBPA mutation status on clinical outcomes				
	Median RFS, months		Median OS, months	
All with normal karyotype (n=37)	20.17		*	
ITD+ (n=20)	16.63		20.17	
Vs		*p=0.03*		*p=0.07*
ITD- (n=17)	*		*	
NPM1+/ITD- (n=5), CEBPA+/ITD- (n=7)	*		*	
Vs		*p=0.002*		*p=0.06*
ITD+, others (n=25)	13.73		20.17	

*Value has not been reached at the end of study

P values of less than 0.05 were considered to indicate statistical significance

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IDENTIFICATION AND CHARACTERIZATION OF NEW MOLECULAR PATTERNS IN SOME DIFFICULT THALASSEMIA CASES

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Background. Thalassaemia is one of the most serious hereditary disorders in the Mediterranean and worldwide. It results from an inherited abnormality of globin gene production: α-globin genes in α-thalassemia and β-globin genes in β-thalassemia. In our project we are focused on characterizing the genetic determinants that lead to a better understanding of genotype-phenotype interaction in thalassemia, namely α-thalassemia mutations, interaction of β-thalassemia, determinants of HbF level, etc. *Aims*. The strategy of the project is based on multiple feedback from phenotype-to-genotype and genotype-to-phenotype, submitting the samples through different filters performing haematological tests, haemoglobin electrophoresis, HPLC, biochemical tests and molecular analysis. *Design and Methods.* As we demonstrated in our previous works the majority of β-thalassemia mutations in Romanian population are of Mediterranean origin and the patterns of allele distribution are according with the specific profile of population from South-East Europe. In this work, some of dificult cases samples were analyzed. Usually, we performe the mutational analysis in two steps: gene scanning by Denaturing Gradient Gel Electrophoresis (DGGE), followed by direct detection PCR methods based on specific identification (ARMS-PCR, PCR-RFLP, Real-Time Genotyping). After the application of this methodology, there were selected a number of 26 samples suspected for β-thalassaemia and a number of 8 samples presenting a genotype that was not entirely consistent with the phenotype. *Results*. Scanning for mutations of the α-globin gene. For the Romanian population there is not any known data on the mutation screening of the α-globin gene causing β-thalassaemia. Although it is expected that the β-thalassaemia mutations will be more frequent, in generally their study is a difficult task because the severe forms have a much lower occurrence. The majority of a-thalassaemias (80%) are given by gene deletions according with the mutation analysis follows a Gap-PCR approach. According to the haematological data there were selected a group of 26 patients suspected of β-thalassaemia due to the low MCV / MCH and low HbA2 values and ruling out other causes of hypochromia and mycrocytosis. They were submitted to the Gap-PCR molecular analysis for the most common mutations in the Mediterranean region: α3.7, αααα, MED I, α20.5, MED II, αPolyA. The results are shown in the Table 1. Sequencing of β-globin gene was performed for those samples that revealed a new pattern by the previous DGGE analysis or for those samples without any abnormal DGGE pattern but the patients presented some features that indicated the presence of a mutation in the β-globin gene. By direct DNA sequencing method it was possible to identify the presence or the absence of any mutation or polymorphism. The results are shown in the Table 2.

Table 1 and 2.

Table 1.

Nr	Genotype β	Genotype α	Hb A	F	A2	RBC	Hb	MCV	MCH
1	N/N	A MED/N	97,7		2,3	5,36	10,8	63,1	20
2	N/N	A MED/N				4,28	9,4	75.5	22
3	N/N	A MED/N	97.6		2.4	5.3	12	71.5	22.5
4	cd8/N	AAA/N	94,5		5,5	4,45	8,4	55,5	18,9
5	N/N	A 3.7/N	97.1		2.9	5.24	12.5	72.9	23.9

Table 2.

No. of patients	β-globin mutation	Silent polymorphisms	Polymorphism status
1	IVS I-1 (G→A)	IVS II-74 (G→T)	homozygote
2	cd 51 (-C)	IVS II-74 (G→T)	heterozygote
2	IVS II-1 (G→A)	IVS II-74 (G→T)	heterozygote
1	+1570 (12 nts 5' to the polyA site)		
1	cd17 (A→T)	IVS II-16 (C→G) IVS II-74 (G→T)	heterozygote homozygote
1	+3 (A→T)	IVS II-74 (G→T)	heterozygote

Also, direct DNA sequencing gave us possibility to identify the presence of one novel mutation and some silent polymorphisms, previously unknown in the Romanian population. The new pattern belongs to a young thalassaemic patient with an intermedia clinical phenotype transfusion dependent. The new CAP +3 (A-T) mutation is inherited from the father and is linked to IVS I-1 (G-A) mutation inherited from the mother. This genotype was found resulting in a β-thalassaemia intermediate status and was confirmed by allele specific amplification (ARMS-PCR). *Conclusions*. These data have a major implications on the genotype-phenotype correlation of intermedia status of β-thalassaemia disease. Molecular diagnosis of homozygotes and identification of carriers of β-thalassemia may improve clinical management of patients with this disorder and can help reduce the birth rate of new homozygotes cases in our country. Also a better prediction of the phenotype starting from the genotype will substantially increase the genetic counselling services.

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QUANTIFICATION OF MUTATED NPM1 IN COMPARISON TO WT1 IN THE FOLLOW UP OF ADULT AML PATIENTS

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Background. The expression of Wilms' tumor gene (WT1) is a reliable marker for monitoring minimal residual disease (MRD) in peripheral blood (pB) and bone marrow (BM) of AML patients without specific molecular markers. However, due to low WT1 expression in normal hematopoietic cells, an artificial cut-off limit defining WT1 overexpression has to be introduced into real-time RT-PCR assays. Therefore, WT1 expression near cut-off limits are difficult to translate into clinical decisions. Mutations in the nucleophosmin-gene (NPM1) are present in about 50% of AML patients with normal karyotype and are a feasible MRD marker for these patients, too. *Aims*. Comparison of both markers in the follow up of AML patients. *Design and Methods*. For quantification of WT1 transcripts, a TaqMan RT-PCR assay was used (*Ogawa H et al: Blood 101 [2003], 1698ff*). NPM1 mutations were screened via a melting curve based LightCycler assay (*Schnittger S et al: Blood 106 [2005], 3733ff*) and quantified with TaqMan RT-PCR (*Dvorakova D et al: Leukemia Oct 2 2008 epub ahead of print*). *Results*. In 20 AML patients (13 female, 7 male, median age 59 years [35-84]) NPM1 mutations were detected and in 19 of them transcripts were quantifiable with TaqMan RT-PCR. During follow up, 212 pB and BM samples of these patients were tested for WT1 and mutated NPM1 in parallel. 204 samples yielded concordant results: NPM1 was positive when WT1 was overexpressed and negative in samples with normal WT1 expression. However, three NPM1 negative samples (1 pB, 2 BM) expressed WT1 at high levels at that time, conversely five NPM1 positive samples (4 pB, 1 BM) had normal WT1 expression. *Conclusions*. Quantification of WT1 and NPM1 mutations yields highly concordant results in the follow up of AML patients. NPM1 facilitates the interpretation of WT1 results, especially in samples with WT1 expression near cut-off limits and is a valuable supplement to routine MRD monitoring.

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CEBPA GENE MUTATIONAL STATUS: A COMPLETE SCREENING USING HIGH-RESOLUTION MELT CURVE ANALYSIS

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In recent years, several independent prognostic factors in cytogenetically normal acute myeloid leukemia (CN-AML) have been reported. Mutations or the expression levels of certain genes have been often used as molecular markers for patient's outcome prediction or for treatment outcome evaluation. One of them, gene encoding CCAAT/enhanced binding protein α (CEBPA), plays an important role in myeloid differentiation and when mutated, confers favorable prognosis for patients with CN-AML. Recent studies have shown that these mutations act as independent prognostic markers for overall survival and remission duration. Moreover, they can be used as markers for real-time monitoring of minimal residual disease, considering the fact that the same CEBPA gene mutations occur (if presented) in AML diagnosis and relapse, but simultaneously disappear during remission. Regarding this, the intimate knowledge about CEBPA gene mutational status is clinically important and it is necessary to identify CEBPA mutant leukemias as quickly as possible after diagnosis. Complete mutation screening of the CEBPA gene is therefore necessary and requires fast, precise and sensitive diagnostic tool. Using direct sequence analysis (i.e. gold-standard screening approach) we identified among a cohort of 90 AML patients a subset of 4 (4%) CEBPA mutations, 25 (28%) CEBPA gene polymorphisms and 61 (68%) CEBPA wild type genes, what indicates that more than 50% of all samples are CEBPA mutation/polymorphism (alteration) negative. Due to this, we found HRM analysis useful as feasible and reliable criterion to distinguish between alteration positive/negative samples. Thus, for routine diagnostics, we developed screening method using high resolution melt curve analysis prior to direct sequencing where only alteration positive samples (according to reference) are further sequenced. It leads to significant time and cost effectiveness comparing to "gold-standard" considering the fact that more than half of all samples may not be further analyzed and are considered wild types. Reported methodology was tested and evaluated on cohort of 28 AML patients, where 11 were analyzed retrospectively and 17 were investigated *de novo*. With this approach, all alteration positive/negative patients were successfully distinguished and the results obtained were in absolute concordance with the direct sequence analysis. The estimated robustness is 10-4 (i.e. 0.005 ng of gDNA in initial sample) and detection limit reaches 4% of mutated allele. Additionally, detailed comparison with "gold-standard" approach is given and discussed.

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LATINAMERICAN STANDARDIZATION IN BCR-ABL Q-PCR, FIRST STEPS TO INTENT A REGIONAL HARMONIZATION

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Background. With the introduction of new drugs for the treatment of CML such as TKIs, quantification of the bcr/abl mRNA by Q-PCR has shown a good correlation in clinical trials. However diversity of molecular approaches and different techniques lead to incomparable results between laboratories. This situation makes standardization an important step towards the optimization of CML management in the region and the plan represents a first attempt to attune the different methodologies in the region and make them comparable among them. *Design and Methods*. Thirteen laboratories in six Latin-American countries (Argentina-2, Brazil-5, Colombia-2, Costa Rica-1, Mexico-2 and Venezuela-1) planned to start this project in October 2007. At the first meeting, the group discussed the process that involves four essential steps: Total RNA extraction: 10-30x10^6 WBC do not select cellular populations. 60% use Trizol® reagent and 40% a commercial kit. cDNA synthesis: All labs use MMLV-RT and random hexamers. PCR amplification: three different PCR platforms (7 AB7500, 5 LightCycler 2.0, 1 RotorGen) with different control genes (BCR 2, ABL 7, GUS 1, G6PDH 3). All labs use a commercial plasmid dilution to generate a control curve. *Data evaluation*. Labs will report the results in absolute transcripts counts (bcr-abl/control gen) expressed in percent.

Table 1.

Samples	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
cDNA1	45 (69)	Nd	58 (77)	190	61 (120)	51 (57)	85	9 (170)
cDNA2	28 (44)	Nd	110 (147)	122	35 (70)	43 (48)	101	4.4 (83)
cDNA3	21 (32)	Nd	27 (36)	59	20 (39)	31 (34)	84	2.4 (45)
ARN1	4.1 (6.3)	458 (199)	Nd	453	15 (30)	Nd	108	0.9 (17)
ARN2	0.21 (0.32)	91 (40)	Nd	68	0.014 (0.028)	Nd	22	0.032 (0.6)
ARN3	0.016 (0.025)	42 (18)	Nd	4.3	0.005 (0.01)	Nd	3.1	0.0018 (0.03)
Lab Baseline	65%	230%	75%	Nd	51%	89%	Nd	5.3%
PCR platform	AB7500	LC 2.0	AB7500	AB7500	Rotor Gen	AB7500	LC 2.0	LC 2.0
Control Gene	BCR	ABL	ABL	ABL	BCR	ABL	GUS	G6PDH

Each laboratory will send the baseline count that will be established calculating the median BCR-ABL levels of 30 CML patients in chronic phase without treatment whose samples was received in the laboratory. In order to define a common starting point, each laboratory's baseline percentage will become 100% (International Scale). In November 2007, 8 labs (Argentina-1, Brazil-5, Colombia-1, Venezuela-1) received the first samples, 3 different cDNA from 3 new CML-CP patients. RNA was extracted and reversed transcription performed with the techniques agreed previously. Each laboratory processed the three samples with their current Q-PCR methodology. Samples were random labeled, so the BCR/ABL levels were not known by the participants and every lab received a lab-code to send results to be centrally decoded. In August 2008 Labs received a second group of samples, 3 different Trizol® pre-

served PB cell lysates from patients after sign an informed consent. Labs perform RNA extraction and cDNA synthesis proceeding to Q-PCR. Results: 7/8 Labs reported results of cDNA samples and 6/8 RNA samples, 6/8 Labs sent the baseline count then only was possible express results using International scale in 5 Laboratories. Table 1 (image) summarizes results of samples expressed in %bcr-abl/control gene, (%International Scale), Nd: not done. *Conclusions*. Results of pre-treatment CML patients in chronic phase cDNA samples expressed in (IS) are quite similar. 75% of the Labs show good correlation in results of RNA samples but 50% of them show more than 0.5 log differences. In December 2009 we started a new phase, Laboratories proceed to test a β-version Kit (MDmolecular) that contain plasmids, reference sample, positive, negative and MMR controls, primers and enzymes.

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THE CORRELLATION BETWEEN METHYLATION STATUS OF THE MDR-1 GENE AND YB-1-GENE EXPRESSION IN PATIENTS WITH HEMATOLOGY MALIGNANCIES

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Recently the success of treatment of hematological malignancies is very dependent on the understanding molecular mechanisms of disease creation and development. The MDR1/ P-gp expression closely correlates with clinical prognosis and outcome of leukemia patients. Y-box binding protein (YB-1) is well-known as member of the cold-chock domain protein family. At the same time YB-1 regulates gene transcription and translation, including of MDR1. The correlation between MDR1 methylation status and YB-1-gene expression was investigated. The investigation was performed on the DNA of lymphocyte cultures of healthy volunteers and on the DNA of leukemic cells of patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia, chronic myeloid leukemia (CML) and chronic lymphoid leukemia (CLL) patients was investigated. Totally 44 nucleic acid samples (from 6 ALL, 15 AML, 3 CML, 10 CLL and 10 normal donors) obtained from mononuclear cells of peripheral blood or bone marrow were involved in the study. Both whole blood and fractionated samples of blood were used. The methyl sensitive (HpaII) and methyl insensitive (MspI) restriction endonucleases were used for the digestion of the DNA-samples, as well as Real-Time PCR-method was applied. The results were also compared with the expression of cell surface P-glycoprotein (Pgp). Overexpression (and demethylation) of the MDR1 gene has been known in association with poor clinical outcome in various hematological malignancies, including acute leukemias. We have revealed a reverse correlation between the methylation status of the MDR-1 gene and YB-1-gene expression in patients with with progressive status (resistant forms, relapse or late stage of chronic leukemias). The intensity of the MDR-1 gene methylation progressively decreased, whereas YB-1-expression was going up. Our results suggests, that these two markers (MDR-1 methylation and YB-1-gene expression) might be a good prognostic tandem and a promising tool in clinical disease evaluation.

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CYTOGENETIC ABERRATIONS IN MONOCLONAL B-CELL LYMPHOCYTOSIS

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Background. Monoclonal B-cell Lymphocytosis (MBL) is currently under intensive investigation as a preclinical stage of chronic lymphoproliferations. However, certain important aspects of the disorder, including cytogenetic features, remain unclear. We report here on our attempt to detect cytogenetic aberrations in 10 individuals with documented MBL. *Design and Methods*. The study included 4 men and 6 women with MBL, according to the currently established diagnostic criteria. Flow cytometry showed a CLL-like phenotype in 9 cases and a "non-indicative"profile (CD5-, CD23-) in 1 case. An interphase FISH study was performed on peripheral blood cells for the detection of chromosomal abnormalities frequently observed in chronic lymphoproliferative diseases, namely 13q-, +12, -11/11q-, -17/17p-, -6/6q-, t(11;14) and rearrangement of the BCL2 and IGH genes. In cases with a low B-cell count (<15% of nucleated peripheral blood cells) the FISH study was performed on immunomagnetically purified B-cells. *Results*. The only aberration detected was 13q14 deletion, found in 6 cases, including that with the atypical (CD5-, CD23-) phenotype. Deletion was hemizygous in 4, homozygous in 1 and hemizygous coexisting with homozygous in 1 case, respectively. No deletion of 13q34 region was seen. In all cases, 13q14 deletion involved the whole population of the target-cells. All individuals were followed-up for at least 18 and up to 36 months. There was no evolution into clinical disease during the follow-up period. *Conclusions*. 13q14 deletion seems to be a frequent feature of MBL, at a rate comparable to that of overt CLL. Though this correlation implies that MBL may evolve into low-risk CLL, evidence for clonal evolution (namely homozygous and concurrent hemizygous/homozygous 13q14 deletion) indicate genetic instability already at the preclinical stage. Thus, further investigation on more individuals and with longer follow-up is warranted to elucidate whether transformation into clinical disease results form the acquisition of additional genetic lesions or whether MBL is a prodromal stage of low-risk lymphoproliferations.

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ABSENCE OF MUTATIONS IN THE TYROSINE KINASE AND JUXTAMEMBRANE DOMAINS OF VEGFR-1 (FLT-1) AND VEGFR-2 (KDR) GENES IN CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML)

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Background. Chronic myelomonocytic leukemia (CMML) is a heterogeneous disease sharing features of myelodysplastic syndromes (MDS) and chronic myeloproliferative disorders (cMPD). Recent analyses driven to characterize cMPD have shown the important role that tyrosine-kinase cell signalling pathways plays in their pathogenesis. Several activating mutations in tyrosine-kinase genes like RAS, FLT3 and JAK2 have been described. Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with biologic effects that include regulation of hematopoietic stem cell development. Autocrine production of VEGF may contribute to leukemia progenitor self-renewal and inflammatory cytokine elaboration in CMML and MDS. The activities of VEGF are thought to be mediated primarily by its interaction with two high-affinity tyrosine kinase receptors, VEGFR-1 (FLT-1) and VEGFR-2 (KDR). Mutations in the activation loops and/or juxtamembrane domain on FLT1 or KDR could lead to constitutive tyrosine kinase activity. *Aim*. To screen the exon coding sequences of the activation loop and juxtamembrane domain of FLT1 and KDR genes by direct sequencing for the presence of mutations in patients with CMML. *Patients and methods*. We studied bone marrow samples from CMML patients at the time of diagnosis (47M/10F; median age, 71 yr; median WBC, $15.2x10^9$/L; median hemoglobin level, 11.3 g/dL; and median platelet count: $163x10^9$/L) for the presence of FLT1 (TK domain n=38; JM domain n=40) and KDR (TK domain n=50; JM domain n=20) mutations. Genomic DNA was amplified using specific primers for the activation loops and/or juxtamembrane domain (*Loriaux et al, 2008. Blood.111:4788*). PCR products were cleaned with standard procedures and direct sequencing was performed on an ABIPRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was checked with GeneBank Accession NC_000013.9 to FLT-1 and NC_000004.1. Bone marrow DNA from healthy donors was used as negative control. *Results*. No mutations in the amplified sequences of the activation loop and/or juxtamembrane domain of the FLT1 and KDR genes were evident in any of the samples analyzed. *Conclusions*, These results demonstrate that the incidence of FLT1 and KDR tyrosine kinase and juxtamembrane domain mutations in patients with CMML is, if present, extremely rare.

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VARIANT T(4;12;13)(Q21;P13;Q12) TRANSLOCATION INVOLVING ETV6 AND FLT3 GENES IN A CASE OF ACUTE MYELOID LEUKEMIA WITH EOSINOPHILIA

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Background. Several different genes partners have been described to be rearranged with the ETV6 gene, which is located at band 12p13, resulting in the formation of a chimeric gene. Aims. We characterized a complex translocation t(4;12;13)(q21;p13;q12) by using fluorescence *in situ* hybridization (FISH) and reverse transcriptase polymerase chain reaction (RT-PCR) analysis. *Design and Methods*. Conventional cytogenetic analysis (CCA) was performed on 24-hour unstimulated bone marrow cells. Chromosomes were G-banded with Wright stain. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (2005). FISH technique was performed with probes for 13q12/FLT3 (RP11-179F17, RP11-153M24), 12p13/ETV6 (cos148B6, cos179A6), 4q12/CHIC2 (BAC 3H20, BAC 120K16, BAC 24O10). RT-PCR for ETV6-FLT3 rearrangement was performed using previously reported primers and conditions. *Results*. A 65-year-old man was diagnosed with an acute myelomonocytic leukemia with eosinophilia. CCA revealed a 46,XY,t(4;12;13)(q21;p13;q12)[12]/50,XY,+X,t(4;12;13)(q21;p13;q12),+10,+11,+12[4] karyotype. Metaphase-FISH showed a breakpoint on chromosome 13, inside the clone RP11-153M24 containing the FLT3 gene and on chromosome 12, cos148B6 remained at 12p13 and cos179A6 was translated on 13q12 confirming that ETV6 gene was disrupted by the translocation. RT-PCR analysis revealed the presence of ETV6/FLT3 fusion transcript. Amplified band (1241 bp) was sequenced with forward and reverse primers. Additional FISH and RT-PCR studies showed that FIP1L1, PDGFRA and CHIC2 genes were not rearranged. *Conclusions*. Translocation t(12;13)(p13;q12) involving ETV6 and FLT3 genes has been reported in a case of myeloproliferative disorder with hypereosinophilia. We suggest that the chromosome rearrangement described here may be considered a variant of the t(12;13) translocation associated with myeloid hematological malignancies with eosinophilia.

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DETECTION OF NPM1 MUTATIONS IN AML PATIENTS

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Background. NPM1/Nucleophismin/B23 is a phosphoprotein with high expression in the proliferating cells. C-terminal mutations of NPM1 gene in exon 12 are very frequently reported in AML cases with normal karyotype. Regarding the discriminating role of NPM1 mutations in prognosis of AML, assessing the frequency of these mutations in AML patients seems necessary. *Aims*. The purposes of this study are: the assessment of the frequency of NPM1 mutations among Iranian AML patients, the relationship between these mutations and FAB classification, and also the assessment of sequence of these mutations. We decided to use CSGE technique in detecting of NPM1 mutations. *Design and Methods*. Bone marrow and peripheral blood samples of 131 AML patients were randomly collected, and their DNA was extracted through standard methods. Then, PCR was applied to the fragment of NPM1 gene with specific primers. PCR products were electrophoresed using CSGE method. In the end, the positive samples were sequenced to confirm the presence of NPM1 mutations. *Results*. Out of 131 patients, 23 (17.55%) were known to have NPM1 gene mutation. The highest frequency of occurrence of such mutations was found to be among the subtypes of M4 (30.4%), M3 (21.7%), and M5 (13%). 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%). *Conclusions*. The results of this research was in line with other reports in terms of the frequency of NPM1 gene mutations in monocytic subtypes of FAB classification (M4, M5), although the total frequency of NPM1 mutations among Iranian AML patients seems to be lower, compared to other reports. High frequency of these mutations in M3 subtypes as well as allele D in all subtypes can be considered as interesting points of the results.

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OCCURRENCE OF THE ACTIVATING JAK2 TYROSINE KINASE MUTATION V617F IN INDIAN PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Chronic myeloproliferative disorders (CMPD's) are clonal hematopoietic stem cell disorders and include polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF). Sometimes it is not possible to differentiate some cases from reactive disorders however, recently described point mutation JAK2V671F a potential candidate aids in this differentiation. Since, its frequency varies in different populations we looked for the presence of JAK2V617F mutation in Indian patients with CMPD's. *Design and Methods*. A total of 150 patients; PV (n=80), ET (n=19), IMF (n=51), 35 non MPD patients and 25 healthy controls were studied. Suspected MPD cases were subjected to haemogram, red cell mass, erythropoietin. All cases and controls were screened for JAK2 V617F mutation detection by two sensitive PCR-based methods; allele specific (ASO) polymerase chain reaction (PCR) followed by restriction enzyme analysis and DNA tetra-primer amplification refractory mutation system. *Results*. The JAK2 V617F mutation was positive 85% of PV, 70% of ET and 52 % of IMF. The mean age of JAK2 positive patients was 53years (range 28-73years) and JAK2 negative was 44years (range15-83 years) (p=0.01). The overall presence of JAK2 mutation was associated with a higher hemoglobin level (p=0.041), a higher white blood cell count (p=0.007), higher age (p=0.01). Homozygosity was observed in 75% of PV, 100% of ET and 56% of IMF. The mutation was not detected in any of the non MPD patients or the healthy controls. *Conclusions*. The JAK2 V617F mutation can be frequently detected in Indian patients with MPD disorders hence should be incorporated into the initial evaluation of patients suspected of MPD. Thus, mutation screening for V617F serves as a tool to diagnose MPD's and may determine a subgroup which would require special therapy.

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PREDICTORS OF SKELETAL CHANGES IN PREPUBERTAL CHILDREN WITH B- THALASSAEMIA MAJOR

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Background. The pathogenesis of thalassaemic osteopathy is a multifactorial, limited information exists about bone accrual and bone mineral density (BMD) in prepuburtal thalassaemic children. Aim: To investigate some potential genetic and biochemical bone markers as possile early predictors of osteopathy and bone mineral density variations in children with β- thalassaemia major before puberty. *Method*. The study included thirty one prepubertal children with β- Thalassaemia major, and forty three children as control group.Patients were recruited from Pediatric Haematology Unit, Children´s Hospital, Ain Shams University and the Pediatric Haematology Clinic of the National Research Center in Egypt. Patients and controls were subjected to the followings: medical history and clinical examination, BMD assessment by DEXA, VDR gene polymorphisms (BSM1, FOK1) and biochemical bone markers (serum osteocalcin , propeptide I procollagen (PIP) and urinary Deoxypyridinoline (DPD) excretion assay). *Results*. BMD was reduced in 25% of thalassaemics at the spine and 15.4% at hip . Significantly higher levels of urinary deoxypyridinoline and lower serum propeptide I procollagen levels were found in thalassaemic children compared to controls (p<0.001). Significant correlation was present between reduced BMD in either spine or hip and the patients' age (r - 0.64, p 0.0002 and r - 0.62, p 0.001 respectively). There was significant gender difference of BMD. Reduced BMD were found more frequently in male patients with genotype bb and Ff but not in females. Significantly higher serum osteocalcin levels were detected in thalasseamic males with bb genotype than in either BB or Bb alleles. No significant differences of the biochemical indices were detected between patients with reduced and those with normal BMD. Reduced BMD did not correlate with the mean pretransfusion hemoglobin or serum ferritin. *Conclusions*. Routine BMD screening with DEXA is proposed to be a sensitive predictor for early bone changes, in particularly at lumbar spine. Egyptian male thalasseamic children with genotype bb and Ff had higher rate of bone turnover; support-

ing the involvement of Bsm1and FOK1 gene as determinants of BMD before puberty.

Table 1. Comparison of the distribution of Bsm1 genotypes.

Bsm1 Genotypes	Total cases: Z score for Spine(28) <-1 (7) N	%	>-1 (21) N	%	p value	Z score for Hip(29) <-1 (4) N	%	>-1 (25) N	%	p value
BB	3	25.0%	9	75.0%	0.84	1	8.3%	11	91.7%	0.30
Bb	2	20.0%	8	80.0%		1	9.1%	10	90.9%	
bb	2	33.3%	4	66.7%		2	33.3%	4	66.7%	
	Male cases: Z score for Spine(11) <-1 (2) N	%	>-1 (9) N	%	p value	Z score for Hip(12) <-1 (2) N	%	>-1 (10) N	%	p value
BB			4	100.0%	0.004			4	100.0%	0.002
Bb			5	100.0%				6	100.0%	
bb	2	100.0%				2	100.0%			
	Female cases: Z score for Spine(17) <-1 (5) N	%	>-1 (12) N	%	p value	Z score for Hip(17) <-1 (2) N	%	>-1 (15) N	%	p value
BB	3	37.5%	5	62.5%	0.33	1	12.5%	7	87.5%	0.65
Bb	2	40.0%	3	60.0%		1	20.0%	4	80.0%	
bb	0	.0%	4	100.0%		0	.0%	4	100.0%	

1220

COMPARISON OF DIFFERENT REFERENCE GENES TO DETERMINE JAK2 ALLELE BURDEN BY TAQMAN PCR TECHNIQUE

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Background. JAK2V617F is a somatic point mutation of the pseudokinase autoinhibitory domain of Janus kinase 2 (JAK2) that occurs in a significant number of patients with myeloprofilerative disorders. There is now growing evidence that the allele burden of JAK2V617F mutation plays a role in clinical phenotype, vascular complications and disease evolution to myelofibrosis. Several groups reported different estimation techniques of JAK2V617F mutation. The most sensitive technique relies on polymerase chain reaction which allows theoretically a reliable quantification of allele burden. We developed a highly sensitive Taqman qPCR method for detection of JAK2V617F mutation in samples of peripheral blood. We have determined the % JAK2V617F by comparison to a control gene (Hemopoietic cell kinase, HCK).With a high sensitivity (0.01%) this technique proved to be reliable in monitoring minimal residual disease (MRD) after allogeneic stem cell transplantation (ASCT) for myelofibrosis. *Aims and Methods*. To determine allele burden of JAK2V617 mutation in patient samples we compared this sensitive Taqman PCR method with the same method but using the wild type of JAK2 as a reference gene. *Results*. We have tested this method using known dilutions of the UKE-1 cell line in Buffy coat DNA from healthy donors reliably quantified using pyrosequencing technique resulting in a good accuracy for the levels of interest when estimating the allele burden (mutational load > 10%). We controlled our results for these known dilutions by measuring the samples again using a Taqman PCR based commercial kit (JAK2 V617F MutaQuant' Kit, Ipsogen, *http://www.ipsogen.com/*). Using wild type of JAK2 mutation as reference gene resulted in a better correlation (Correlation factor of 0,99) with the known concentrations in the high mutational load samples in comparison to the HCK gene, and it was also able to detect and quantify the mutation in dilution up to 0,01%. *Conclusions*. We conclude that measuring the allele burden of JAK2V617F mutation using the wild type JAK2 gene as a reference gene is feasible and yield a good accuracy for the higher mutational loads and is as sensitive as our original method using HCK gene.

This work was supported with an EBMT-AMGEN fellow-ship grant.

1221

HIGH RESOLUTION MELTING ANALYSIS FOR RAPID DETECTION OF BCR-ABL KINASE DOMAIN MUTATIONS IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS WITH IMATINIB RESISTANCE

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Background. Imatinib, a targeted BCR-ABL tyrosine kinase inhibitor (TKI) has become the standard drug for the treatment of chronic myelogenous leukemia (CML). Mutations in the kinase domain (KD) of BCR-ABL contribute to imatinib-resistance which occurs in 10-15% of patients with CML. High resolution melting (HRM) analysis is an emerging technique for detection of nucleic acid sequence variations. Aims of our study was to develop HRM assay for the rapid screening of BCR-ABL KD (aa. 230-490) mutations. *Design and Methods*. We designed oligonucleotide primers to selectively amplify the BCR-ABL KD in 3 overlapping fragments. For the HRM-analyses, a LightCycler 480 instrument (Roche) was used to scan amplicons of the BCR-ABL KD after two step nested PCR. 38 patients (36 CML and 2 ALL) with imatinib-resistance were tested for 3 fragments (total fragments tested: 114). KD mutation(s) (aa. change M244V, G250E, Y253H, E255V, D276G, E279K, T315I, M351T, F359I/V, L384M, L387M) were confirmed in 21 patients by direct sequencing. Results: Following HRM analysis with an instrument sensitivity setting of 0.43, 11 mutations of 12 (91%) were detected. A single mutation, F359I (caused by an A>T substitution) could not be detected by HRM probably as a consequence of the reduced efficiency of melting curve discrimination. HRM results became fully concordant to sequencing if, 100% mutant F359I samples were mixed with wild type cDNA to create heteroduplexes. Alternatively, if the instrument sensitivity setting was increased to 0.65, 12/12 (100%) mutations were detected by HRM-analysis without the need for creating heteroduplexes. However at this instrument setting, the number of false positive fragments increased from 14/95 to 30/95. Thus, increasing instruments sensitivity did not prove to be a favorable alternative. Summary: HRM is a rapid and efficient method of screening for BCR-ABL point mutations prior to sequencing.

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AUTOMATED EXTRACTION OF HUMAN RNA AND DNA FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF HAEMATOLOGICAL PARAMETERS: COMPARISON OF THE NUCLISENS EASYMAG, THE QIASYMPHONY AND MANUAL COLUMN METHODS

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Background. The accurate analysis of gene expression and genetic aberrations in haematological cells derived from whole blood, bone marrow and lymphoid tissues is of great interest in the diagnosis of haematological malignancies. Since clinical laboratories are facing an increasing number of samples each year, the application of automated extraction of high quality nucleic acids is emerging. Different possibilities for automated extraction for microbiological samples have been developed the last decade. However, the automated extraction of human RNA and DNA for applications in haemato-oncology is still challenging. Aims In a comparative study we evaluated the automated Nuclisens easyMAG (EM, Biomerieux, France, Marcy l'Etoile) and the QIAsymphony (QS, Qiagen, Germany, Hilden) versus the manual Qiagen column technique. The presence of clonal IGH gene rearrangements out of blood, bone marrow, fresh lymphoid and paraffin tissues (91 samples) for the diagnosis or follow-up of B-cell malignancies and the quantitative analysis of BCR-ABL aberrant fusion transcripts in whole blood and bone marrow (60 samples) in Chronic Myeloid Leukemia (CML) was tested. *Methods/Results*. The isolation of human DNA was similar in ratio for the manual method and the QS; the yield was slightly higher for the manual method. For the isolation with the EM, the starting volume had to be reduced to avoid bead contamination in the eluate and inhibition of subsequent PCR and fragment analysis, impairing sensitivity. Evaluation of amplified IGH genes by fragment analysis showed the best performance with the QS in peak intensity and minimal inhibition. Human RNA isolated by the specific B protocol on EM demonstrated inhibition of up to 3 to 10 times less ABL copies, depending on the amount of starting cells, in comparison to the manual method and the QS. The isolation of human RNA with the manual and QS method was equally efficient in yield and ratio. Evaluation of RNA quality with the Agilent

Bioanalyser showed severe genomic DNA contamination in EM eluates, while the quality of the QS eluates were similar or even better than for the manual method. Correlations of the ratios of the two methods were highly significant ($p<0.001$). Quantitative RT-PCR analysis showed similar BCR-ABL/ABL ratio. Sensitivity evaluation was similar for both the manual method and the QIAsymphony: ratios of BCR-ABL/ABL of 0,002% could be demonstrated. In view of work load relieve, QS met expectations, in contrast to EM for which many off-board handlings were still required. *Summary*. In conclusion, the automated extraction of DNA with both instruments was similar to the manual method, but EM DNA analysis was hampered by bead contamination. For automated RNA extraction, only the QS could reach similar results as the manual method. The implementation of the QS in a haemato-oncology diagnostic laboratory would greatly facilitate work load, both for RNA and DNA tests, and standardise the extraction, allowing reduction of variation which is of great clinical importance e.g. in CML follow-up.

1223

MOSAIC ISOCHROMOSOME XP IN A GIRL WITH SHORT STATURE AND BONE MARROW FAILURE

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A 18-month girl, second daughter of unrelated parents, was diagnosed with bone marrow (BM) failure and treated with cyclosporine ad steroids, with partial response. At 6 years of age, she was referred to us for anemia, low white bood cell and platelet counts. BM aspirate showed no blasts or myelodysplasia; bone biopsy showed reduced cellularity and megakaryocyte number. Her height was <3rd centile due to partial GH deficiency. MR showed Chiari I malformation. Slight somatic dysmorfisms were observed. DEB tests and cell cycle analysis on peripheral blood (PB) lymphocytes and fibroblasts were negative. NBS1 protein was present in western blot and the lymphoblastoid cell line was not sensitive to ionizing radiation. Nijmegen Breakage Syndrome was excluded. c-Mpl gene associated with amegakaryocytic congenital thrombocytopenia, is not mutated.The Q-banded karyotype of PHA-stimulated PB lymphocytes was 46,X,i(X)(p10)[6]/46,XX[43]. Dual color FISH analysis using Xp- and Xq-arm partial paints confirmed the staining of the entire two isochromosome p-arms and revealed a subtle q-arm signal in the centromeric region. Compared to the normal X, the size of centromere signal (DXZ1 coupled with STS Xp22.3) was larger, suggesting an isodicentric chromosome. FISH with subtelomeric Xpter (RP11-215A12) probe and RP13-216E22 BAC probe, which specifically hybridizes to the XIST locus on Xq13.2, showed the absence of the inactivation center on i(X)(p10). Subtelomeric Xpter FISH analysis on BM cells demonstrated three signals in 11/300 nuclei. Fibroblast karyotype was 46,XX. Uncommon acquired i(X)(p10) associated with hematologic disorders as well as one case of constitutional mosaic i(X)(p10) in a girl with failure to thrive and thrombocytopenia have been reported in the literature. Our case seems to confirm the i(X)(p10) involvement in bone marrow failure.

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UNCOMMON PML-RARA TRANSCRIPT VARIANTS IN PATIENT WITH ACUTE PROMYELOCYTIC LEUKEMIA TREATED WITH ATRA

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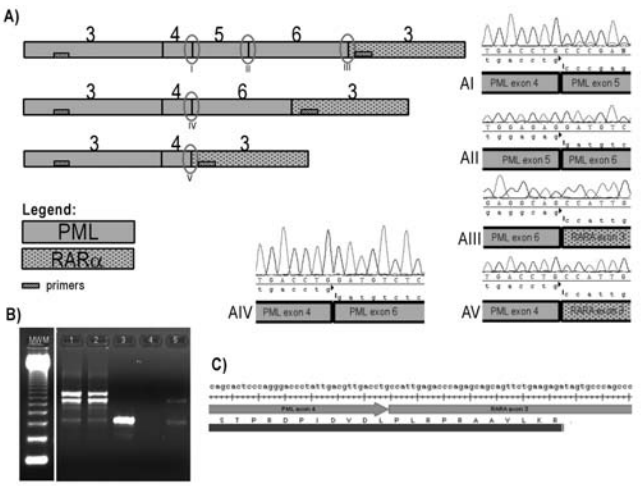
Background. Acute promyelocytic leukaemia (APL, AML M3), a subtype of acute myelogenous leukaemia, is a haematological disorder characterized by a differentiation block of myeloid cells at the promyelocyte stadium, usually caused by translocation t(15;17)(q22;q12) resulting in PML-RARA fusion gene. Recently some publications suggested that the presence of uncommon PML-RARA transcript variants could be ralated to all-trans retinoic acid (ATRA) insensitivity. *Aims*. In this study we attemted to identify the character and origin of uncommon PCR products observed at diagnosis in APL patient and determine if there are any significant changes in response to ATRA-based treatment. *Design and Methods*. Blood samples were collected from the patient in regular periods during and after the treatment. DNA and RNA was extracted from leukocytes acquired from peripheral blood. Reverse transcription of total RNA was performed with Qiagen Omniscript reverse transcriptase. PML-RARA fusion transcript was detected using BIOMED-1 protocol. After electrophoresis, bands representing various transcript variants were excised from gel, purified and directly sequenced. Acquired chromatograms were analyzed with DNA Star Lasregene 8.0 in comparison to sequences obtained from GeneBank. *Results*. Three isoforms of PML-RARA transcript were identified: a normal bcr-1, a variant lacking exon 5 and an out-of-frame variant without exons 5 and 6. No changes in the intron areas surrounding exon 5 (in the splicing signalling sequences) were foundin comparison to reference sequence. During the treatment transcripts disappeared sequentially, which suggests different sensitivity of cells expressing certain isoforms to ATRA. In molecular relapse only two atypical variants were detected, however further treatment eradicated them as well. For now, the patient is in molecular remission for 8 months. *Conclusions*. Our observations suggest that PML-RARA variant lacking exon 5 has slightly lesser sensitivity to ATRA than more common ones. The character of out-of-frame variant lacking exons 5 and 6 still remains unlcear, however it's highly probable that it doesn't play any role in leukemic transformation, and cells expressing this transcript have normal phenotype, therefore it's lack of response to ATRA should not be any surprise. However, coexistence of out-of-frame transcript and variant lacking exon 5 may lead to assumption, that the non-malignant clone carrying t(15;17) can switch to produce transcript leading to APL, and therefore could be indirectly responsible for a relapse.

Figure 1. Observed PML-RARA transcript variants.



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CYTOGENETIC ABNORMALITIES, CLINICAL FEATURES AND OUTCOME IN A SERIES OF 106 PEDIATRIC ACUTE MYELOID LEUKEMIA (AML) PATIENTS IN GREECE

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Background. Acute myeloid leukemia (AML) accounts for approximately 17% of all childhood acute leukemias. Cytogenetics is considered one of the most valuable prognostic determinants in AML while current risk-group classification in the limited cases of pediatric AML, is mainly based on cytogenetics and early treatment response. *Aims*. We reviewed the clinical and cytogenetic characteristics and the outcomes of 106 cases of childhood AML in order to investigate the incidence of the main FAB subtypes, the incidence of chromosome abnormalities, and the correlation between specific chromosome abnormalities and outcome in greek pediatric AML patients. *Design and Methods*. Chromosome studies were performed on unstimulated bone marrow cells, derived from 105 pediatric AML patients, who were ≤21 years of age at the time of diagnosis. Results: Sixty one patients were male and 45 were female. The median age of patients at diagnosis was 11.52 years. Eighty seven patients were classified according to FAB classification while in 19 patients FAB classification was not available. Five patients were classified as M0 (5.7%), 8 as M1 (9.2%), 29 as M2 (33.3%), 14 as M3 (16%), 11 as M4 (12.6%), 10 as M5 (11.5%), 5 as M6 (5.7%) and 5 patients as M7 (5.7%). Cytogenet-

ic analysis was performed at diagnosis in 105 patients and results were obtained in 99 (94.2%). Normal karyotype was found in 29 patients (29.3%) and abnormal in 70 patients (70.7%). Chromosome analyses showed t(8;21)(q22;q22) in 12 cases, t(15;17)(q22;q21) in 10, +8 in 11, -7/del(7q) in 7, del(9q) in 5, abn(7p) in 5, inv(16) in 4, del(6q) in 4, t(9;11)(p22;q23) in 3, other abnormalities of 11q in 5, -X in 5, -Y in 4, acquired +21 in 4, t(16;21) in 2, abn3q in 2, del(5q) in 1, del(16q) in 1, other abnormalities in 14 and complex karyotype (≥3 abnormalities) in 17 cases. The majority of children with t(15;17), t(8;21) and inv(16) had a good outcome. Patients with -7, inv(3q), del(5q) and 11q rearrangements had a bad outcome. Surprisingly, patients with del(7q), and dup(3q) had a good outcome. In children younger than 2 years, the most common FAB subtypes were M5 and M7, the most common abnormality was 11q rearrangement and the 5-year overall survival (OS) was 46%. In children older than 2 years and younger than 15 years the most common FAB subtype was M2, the most frequent abnormality was t(8;21) and 5-year OS was 53.6%. In children older than 15 years and younger than 21 years, the most common subtypes were M2 and M4, the most frequent abnormalities were +8, and t(8;21), and 5 year OS was 47%. *Conclusions*. The main FAB subtypes showed a distribution similar to that reported in the literature with the exception of M4 and M5 which were significantly lower, and M3 which was higher in our study. The 5-year OS ranged from 46% to 53.6% among the different age groups. The incidence of t(8;21), t(15;17) and -7/del(7q) was higher compared to that reported in other countries.

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A CYTOGENETIC STUDY OF 310 CHRONIC LYMPHOCYTIC LEUKEMIA CASES IN GREECE

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Background. Cytogenetics is an important diagnostic and prognostic factor in Chronic Lymphocytic Leukemia (CLL), although its prognostic value is limited by the cytogenetic diversity of the disease. AIM: The aim of this study was the detection of chromosome abnormalities and their incidence in B-CLL greek patients by conventional cytogenetics. *Patients and Methods.* Chromosome studies were performed on unstimulated and stimulated with tetradecanoyl phorbol acetate (TPA) bone marrow cells, derived from 310 CLL patients, aged 16-88 years. Patients were karyotyped at diagnosis or during the course of the disease between 1998 and 2008. *Results*. Two hundred and twelve patients were male and 98 were female. The median age was 64.61 years. Cytogenetic analysis was successful in 288 patients (93%). Normal karyotype was found in 174 patients (~60%), and abnormal in 114 patients (~40%). Chromosome analyses showed a complex karyotype in 32 cases (28%), +12 in 33 cases (29%), -Y in 20 (17.5%), abnormal (abn) 14q in 18 (15.8%), abn 17 in 17 (14.9%), del(6q) in 11 (9.6%), abn 3 in 10 (8.7 %), abn 18 in 9 (7.9%), del(13q) in 7 (6.1%), add(12p) in 3 (2.6%), del(7q) in 6 (5.3%), del(11q) in 6 (5.3%), t(11;14) in 3 (2.6%), t(11;18) in 1 (0.9%), -X in 6 (5.3%), abn 19 in 5 (4.4%), +8 in 5 (4.4%), del(5q) in 3 (2.6%), chromosome markers in 13 (11.4%), and other abnormalities in 51 cases (44.8%). *Conclusions.* The sex ratio was 2.2M/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. The low percentage of abnormal karyotypes, due to the low mitotic *in vitro* activity of B-CLL cells, indicates the need for new B-CLL cell stimulators. The cytogenetic complexity and heterogeneity of CLL emphasizes the value of conventional cytogenetics which permits an overview of all microscopically visible chromosome abnormalities.

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NESTED DUPLEX PCR BCR/ABL (B2A2, B3A2) IN COMPLETE AUTOMATIZED SYSTEM

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Introduction. Gene expert is a new complete system in real time pcr for Rna extraction, Cdna and nested real time duplex PCR. Once hematology diagnosis by quantification of P210 (b2a2, b3A2 isoforms) of BCR/ABL gene in chronic myeloid leukaemia was utilized. Objective i)To characterize BCR/ABL rearrangements for chronic myeloid leukemia diagnosis. ii)To utilize at diagnosis, follow-up and in minimal residual disease evaluation. Peripheral blood, bone marrow and crio persevered with standardize protocol.3) The understanding of pathology process in chronic myeloid leukemia diagnosis and in all myeloproliferative disease for development of other new target therapy in specific target therapy resistance. *Materials and Methods.* Were analyzed 209 cases; 6 CML at diagnosis, 192 CML in follow up, 6 myeloid acute leukemia at diagnosis, 9 post BMT follow up and 29 control with negative BCR/ABL gene transcripts. Of this case/control, 188 (78,9%) were peripheral blood and 50 (21%) were bone marrow samples. Ten samples were cryopreserved cells from peripheral blood obtained by ficoll separation, 200 of peripheral blood, or 200 microliter of bone marrow with 1;20 dilution or 200 microliter of cryopreservated cell in PBS dilution within 48 hours were used. In cartridges were performed lisys , sds- tween and guanidium salt, bind to column, wash for elute pcr inhibitor and water DEPC elution. were performed RT-pcr- nested and duplex pcr. As endogenous control Abelson gene was used. Results were validates with ABL end point fluorescence value (≥200) and BCR ABL endpoint fluorescence value (≤40). *Results and conclusions*. Excellent result was obtained with 200 microliter of peripheral blood, but if we use bone marrow samples of 50 of these 10(20%) were aborted for pump problems. If we use 1: 40, PBS 1x dilution all samples were evaluated. Oresults were obtained with cry preserved cell from wbc (by dry pellet diluted in 1 mL PB 1x at 1:40 dilution before 1 week of cry preservation. Gene expert is easy and highly sensitive, Patients in imatinib mesylate therapy with CML require in clinical laboratory new rapid equipment for level transcript monitoring. Gene expert is easy, speed, with 4 way for fluorescence revelation, utilize micro fluidic cartridge minimize all contamination risks in nested duplex PCR real time reaction.

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MOLECULAR CYTOGENETICS OF ETV6/RUNX1 GENE FUSION AND OTHER ABNORMALITIES INVOLVING ETV6 AND RUNX1 GENES IN PEDIATRIC B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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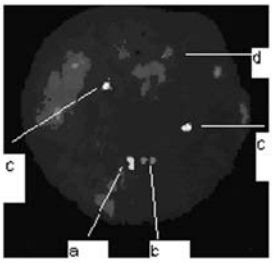
Background. The translocation (12;21)(p13;q22)/ ETV6-RUNX1 gene fusion is the most common genetic abnormality found in childhood acute lymphoblastic leukemias (ALL). This cryptic translocation is not detected by conventional cytogenetic techniques but it can be readily detected using fluorescence *in situ* hybridization (FISH). Aim: We carried out cytogenetics and FISH studies on 50 children with B-lineage ALL in order to measure the frequency of this translocation in the Egyptian population in comparison to other genetic subgroups, the frequency of ETV6 and/or RUNX1 gene alterations, and their correlation with clinical evolution and prognosis. *Design and Methods*. Bone marrow samples were obtained from 50 pediatric B-lineage ALL patients who presented to the National Cancer Institute in Cairo. An informed consent was obtained from parents of all participants and from participants above the age of 12. Karyotyping and FISH using probes for ETV6-RUNX1 extra signal, BCR-ABL dual fusion, and MLL break apart (Vysis) were performed. The signal pattern of RUNX1 and ETV6 genes were analyzed using FISH. Patients were treated according to the National Cancer Institute protocols and followed up for a period of 18 to 42 months (median 19 months) *Results.* In the current study, successful karyotyping was obtained in 43/50 (86%); when adding FISH 46/50 (92%) cases showed informative results. Using FISH, ETV6-RUNX1 gene fusion was detected in 7/50 cases (14%) while cytogenetics could not reveal the translocation (12;21). Normal karyotype was found in 13/50 when using conventional cytogenetics (CC) only, while when combining both FISH and CC two cases revealed ETV6-RUNX1 gene fusion. Other chromosomal abnormalities that were frequently encountered in ETV6-RUNX1 positive cases were either, deletion of the nontranslocated allele of ETV6 in 2 cases, gain of der(21)t(12;21) in one case and gain of normal chromosome 21 in one case. These abnormalities correspond to: lack of ETV6 signal, extra ETV6-RUNX1 fusion signal and extra RUNX1 signals, respectively as detected by FISH. Whereas CC failed in 7 cases, when FISH was performed MLL gene translocation was detected in one case and ETV6-RUNX1 gene fusion in 2 cases. Hyperdiploidy was found in 22%, t(1;19)(q23;p13) in 8%, BCR-ABL in 6%, MLL gene translocation in 6%, t(8;14)(q24;q32) in 6%, hypodiplody in 2 cases, and RUNX1 gene amplification in one case. Best overall survival correlated with ETV6-RUNX1 positive cases and high hyperdiploid cases with mean overall survival (OS) being 28.3 (p=0.103) and 26.3 (p=0.0757)months respectively. Worst overall survival was associated with all other chromosomal abnormalities having mean OS of 14.6 months (p=0.0134). Cases with normal karyotype had a mean OS of 22.2 months (p=0.6337), while cases with abnormal-

ities of ETV6 or RUNX1 genes other than ETV6/RUNX1 fusion had a mean OS of 21.8 months. *Conclusions*. ETV6/RUNX1 fusion is found in 14% in our series and had a favorable outcome. Other ETV6 and RUNX1 genes abnormalities are detected in 14/50 cases (28%) and associated with intermediate OS.



FISH using ETV6/RUNX1 dual color extra signal probe (Vysis) showing translocation ETV6/RUNX1 with two fusion signals from an additional derivative 21.
a: Normal ETV6 gene. b: Normal RUNX1.
c: ETV6/RUNX1 gene fusion with an extra fusion signal. d: Residual RUNX1.

Figure.

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GENETIC COUNSELLING DIFFICULTIES IN THALASSEMIA: NEWBORN PHENOTYPE UNCERTAINTY IN ASSOCIATION WITH A NOVEL β GLOBIN MUTATION

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Background. The risk determination for β thalassemia carriers of having affected children represents a significant genetic counseling question. Phenotypic predictions are difficult in the presence of rare or novel mutations. Prenatal diagnosis seeks to predict the child's clinical phenotype however this may be variable, depending on the parental mutations or the co-inheritance of other genetic determinants (i.e. α and γ genes defects). AIMS We report the case of a non-consanguineous pregnant couple who are both β globin mutation heterozygotes (novel in the mother and previously reported in the father). *Design and Methods*. Routine hematology, separation of the Hb fractions with measurement of the Hb A2 and Hb F levels and globin chain synthesis; molecular analysis by A.R.M.S.-PCR and direct sequencing (Beckman Coulter CEQTM 8000 Genetic Analysis System). *Results.* The Italian father was a heterozygote for the Mediterranean IVS I-6 (T->C) mutation, detected by A.R.M.S.-PCR. The mother, an Australian woman, with paternal Indian ancestry, presented with microcytic hypochromic parameters (Hb = 10.8; RBC = 5.18; MCV = 63; MCH = 20.8) without iron deficiency (ferritin = 38 ng/mL), a high Hb A2 level (6.0%) and Hb F (3.4%) consistent with β globin gene mutation heterozygosity carrier. The globin chain synthesis ratio was 1.55 also consistent with β thalassemia carrier status despite the absence of additional investigations (mRNA and cDNA tests) or family studies. β globin gene sequencing detected a 12 base deletion located at the start of the second intron of the β globin gene [IVS II-2 (-12 bp)]. The analysis of the most common defects in the α and in the γ genes did not detect additional abnormalities. *Conclusions*. The novel 12 bp deletion is assessed as resulting in a severe β thalassemia phenotype when in combination with known pathogenic β globin mutations due to hematological parameters, mutation position within intron 2 (splicing site) and an alpha/non-α globin ratio of 1.55. As this mutation has not been reported previously and the mRNA has not characterized, we have classified it as a *probable* mutation. The combination of this mutation and the IVS I-6 mutation is likely to result in a full β thalassaemia phenotype.

1230

MULTIPLEX RT-PCT FOR THE DETECTION OF LEUKEMIA-ASSOCIATED TRANSLOCATIONS - SINGLE CENTER EXPERIENCE

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Background. The current pathological approach to the diagnosis of acute leukemia is a complex, involving morphology, cytochemistry, and immunophenotyping, cytogenetic and molecular studies. Each of these components is important to appropriate diagnostic evaluation but, there is increasing evidence that major disease-defining, prognostic relevant, and therapy-determining data are provided by the genetic and molecular analyses. Most translocations, when evaluated at the molecular level, are detected by reverse transcription-polymerase chain reaction (RT-PCR) in the routine diagnostic setting. RT-PCR detection of the major leukemia translocations has numerous advantages over conventional cytogenetics, including shorter turn-around time, no requirement for dividing cells, detection of translocations that may be missed by conventional cytogenetics and providing a sensitive marker for subsequent minimal residual disease testing. Aim. The aim of this study was to validate the application of a commercially available multiplex RT-PCR assay (HemaVision 28 System) for 28 most common leukemia translocations for routine molecular diagnosis. *Material and methods*. We analyzed 30 samples of bone marrow and peripheral blood of 30 new diagnosed children with acute lymphoblastic leukemia (ALL). The samples were cryopreseved with RNAlater® (Ambion) and stored at -20°C. We centrifuged 200-500 mL fixed-cell suspension, and pellets were washed with ethanol. Then RNA was prepared using the RiboPure Blood Kit (Ambion) according to the manufacturer's recommendations. Samples were analyzed using a multiplex RT-PCR assay (HemaVision) according to the manufacturer's instructions. The assay discriminates between 28 different fusion transcripts. Reverse transcription was performed with a mixture of translocation-specific complementary DNA (cDNA) primers and PCR amplification in 8 parallel nested multiplex master reactions. Each of the master solutions contains several primers that are specific for particular fusion transcripts and a pair of control primers that amplifies a ubiquitously expressed gene. Thus, each of the master reactions identifies various chromosomal aberrations that, due to the heterogeneity of the breakpoints on the genomic level and/or alternative splicing, generate different messenger RNA (mRNA) variants. To verify the presence of a specific fusion transcript in a positive multiplex reaction, a nested split-out analysis with individual translocation-specific primer sets was performed. The specific translocation and splice variant was classified by comparing the respective pattern with the interpretation table provided by the manufacturer. Results. The RNA concentration from 30 samples was 10.35-1608.4 ng/μL. We obtained higher RNA concentrations from peripheral blood comparing with bone marrow samples. In 14 cases (46,66%) the test failed to identify a molecular abnormality; in 11 cases (36,66%) the low quantity of RNA isolated was insufficient for a definitive diagnosis; in 5 cases (16,66%) we identified molecular abnormalities - 2 cases (6.66%) BCR/ABL p190, 1 case (3.33%) TEL/AML1, 1 case (3.33%) E2A/PBX, 1 case (3.33%) MLL1/AF4. The results in positive cases were comparable with conventional cytogenetics. *Conclusions*. We consider that molecular based screening, via a multiplex RT-PCR system evaluated here, is still extremely valuable in clinical practice, but have to be evaluated together with other cytogenetic methods.

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SERUM BAFF (B-CELL ACTIVATING FACTOR OF THE TNF FAMILY) PREDICTS TIME TO FIRST TREATMENT IN EARLY B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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We analyzed the correlation between well-established biological parameters of prognostic relevance in B-cell chronic lymphocytic leukemia [CLL] (i.e, mutational status of the immunoglobulin heavy chain variable region [IgVH], ZAP-70- and CD38-expression) and serum levels of BAFF (B-cell activating factor of the TNF family) by evaluating

the impact of these variables on the time to first treatment [TFT] in a series of 125 previously untreated Binet stage A B-cell CLL patients. By using a commercial ELISA (R & D Systems, USA) we found that higher levels of BAFF characterized more frequently females (p=0.01), patients with Rai stage 0 (p=0.03), mutated IgVH (p=0.008) and low ZAP-70 expression (p=0.04). In contrast, age (p=0.35), peripheral blood lymphocytosis (PBL)(p=0.09), hemoglobin (Hb) (p=0.64), platelet (PLT) count (p=0.12), serum β2-m (p=0.49), LDH (p=0.85) and percentage of CD38-positive B-CLL cells (p=0.63) did not reflect circulating levels of BAFF. We used an optimal cut-point search to determine how to best split soluble BAFF data. Maximally selected log-rank statistics plots identified a BAFF serum concentration of 311 ng/mL as the best cut-off (p<0.0001). Accordingly, patients who had BAFF levels higher than 311 ng/mL experienced a longer TFT (median 108 months) in comparison to patients whose BAFF levels were lower than 311 ng/mL (median 30 months; p<0.0001). The univariate Cox proportional hazard model identified lower serum concentration of BAFF, Rai sub-stage I-II (p=0.003), lower PLT count (p=0.04), higher PBL count (p=0.01), increased LDH (p=0.01), ZAP-70 expression > 20% (p=0.02) and absence of mutation in IgVH (p<0.0001) as predictor of shorter TFT. In multivariate analysis only soluble BAFF (Hazard ratio [HR], 6.13; CI 95%, 2.31-16.25) and mutational status of IgVH , (HR= 2.99; CI 95% 1.33-6.76, p=0.008) maintained their discriminating power. The effects of BAFF on TFT were masked by mutational status of IgVH in patients with unmutated IgVH. However, serum levels of BAFF and mutational status of IgVH had a joint effect on TFT in patients with mutated IgVH which translates into a segregation of patients with mutated IgVH in two groups with different TFT according to BAFF levels (HR= 8.9; p<0.0001). Our results indicate that in early B-cell CLL biological profile including among other parameters soluble BAFF may provide a useful insight into the complex interrelationship of prognostic variables. Furthermore, BAFF along with mutational status of IgVH can be adequately used to predict clinical behaviour of patients with low biological risk.

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DELETION OF CHROMOSOME 5Q AS A RARE BUT NON RANDOM ABNORMALITY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Backround. Chronic lymphocytic leukemia (CLL) is associated with cytogenetic abnormalities at diagnosis or during the course of the disease. However, most of them have not been completely determined due to the low mitotic *in vitro* activity of B-CLL cells. Aim: We present a conventional and molecular cytogenetic study of two CLL cases with deletion 5q [del(5q)] as a sole abnormality in order to contribute to the identification of rare recurrent aberrations and their prognostic impact in CLL. *Patients and Methods*. Patient 1, a 66-year-old man, and patient 2, a 59-year-old woman were diagnosed with B-CLL in September 2008 and in October 2007 respectively. Both received no therapy and are still in excellent clinical condition 5 and 17 months later correspondingly. Cytogenetic studies were performed on unstimulated and stimulated bone marrow cells at diagnosis. Fluorescent *in situ* hybridization (FISH) studies were performed on the same specimens using commercial DNA probes for detection of deletions of 5q31 (EGR1/D5S23), 5q33-q34 (CSF1R/D5S23, DS5721), 17p13 (TP53), 11q22.3 (ATM), 13q14/13q34 (D13S319/13q34) and trisomy 12 (CEP 12). Results: Chromosome analysis revealed del(5q) as a sole abnormality and FISH demonstrated normal hybridization pattern for 13q14, 13q34, ATM, p53 and CEP12 regions in both patients. Furthermore, in patient 1, FISH showed a normal hybridization pattern for 5q31 but a single signal for 5q33-q34 region. FISH results for del(5q) were not obtained for patient 2 due to lack of cytogenetic material. *Conclusions*. This study presents the first case reports of del(5q) in CLL and demonstrates that del(5q) as a sole aberration, is a rare but non random abnormality, possibly not associated with an adverse prognosis. It also indicates that 5q33-q34 could be the potential critical region. Further studies are required to delineate the prognostic value of del(5q) in CLL, and to identify candidate genes with potential role in the pathogenesis of the disease.

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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 within 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. 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. 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. *Conclusions*. 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.

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CORRELATIVE STUDY BETWEEN IMMUNOMODULATORY ACTION OF PROPOLIS ON CULTURED PBMNCS AND ITS SCHISTOSOMICIDAL ACTIVITY

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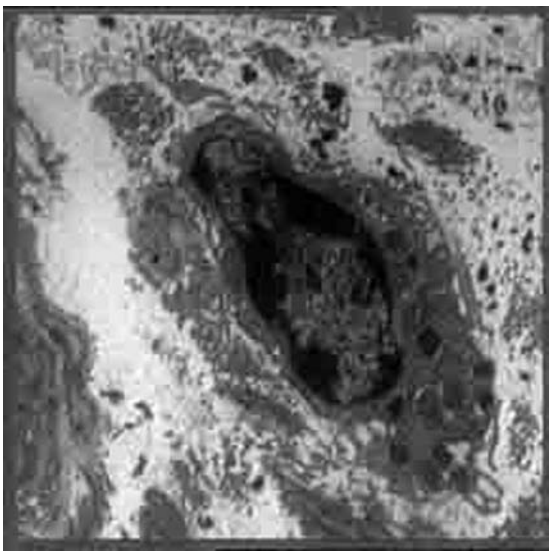
Propolis, a beehive product widely used in folk medicine as an anti-inflammatory agent, has been attracting researchers' attention to scientifically elucidate its biological properties and therapeutic activities. This study aimed to spot light on the value of propolis as an immune-stimulant and to evaluate the influence on schistosome hematobium infection cure rate. To achieve this goal we estimated the effect of propolis on cultured peripheral blood mononuclear cells activation *in vitro* by IL-2 and NO determination. We also evaluated the effect of *in vivo* treatment with propolis on schistosomL haematobium worm and bone marrow by parasitological and ultrastructural studies. Twenty S. haematobium infected golden hamsters were included in the study, subdivided into two groups each of 10 animals Group 1: Infected Control with 300+10 cercariae of S. haematobium by abdominal skin exposure. Group 2: Animals were treated with propolis three months post the infection. Our *in vitro* results revealed that propolis induces a discreet elevation in IL-2&NO release in PBMNCs cultures supernatant of S.hematobium infected hamsters. Mean level of IL-2 was 16.17±1.67 pg/mL in the presence of propolis and 3.31±0.76 in its absence with highly statistically significant difference (p<0.001). Regarding NO, Mean level of NO was 7.76±1.30 U/mL in the presence of propolis and 2.6±0.42 in its absence with highly statistically significant difference (p<0.001). Also, propolis caused observed activation and absence of apoptotic changes at the ultrastructural level of cultured PBMNCs. *in vivo* results, revealed significant reductions in mature worm loads (either male or female), tissue egg loads (either intestinal or hepatic) 21.00 and 19.79% respectively and Percentage reductions of egg developmental stages was 68.07% with statistically significant difference compared with infected control group (p<0.05). Ultrastructural study of S.hematobium worm revealed implantation and degeneration of the spines within vesiculated tegument and for the bone marrow it revealed evidence of lymphocyte and promonocyts activation in addition to remarkable increase in the num-

ber of the activated natural killer cell. Data suggest that propolis acts as immunmodulator activating immuncompittent cells. This information would provide new insights in considering propolis to have a potential therapeutic benefit if used in conjunction with anti-schistosomal drug in treatment of schistosomiasis infection. KEY WORDS: propolis, schistosome haematobium, PBMNCs, IL-2, nitric oxide ,electron microscopy

Figure 1. Electron micrograph of activated natural killer cells.



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PREVENTIVE EFFECTS OF BILE ACIDS ON BILIRUBIN-INDUCED APOPTOSIS OF HUMAN BRAIN MICROVASCULAR ENDOTHELIAL CELLS

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Background. Unconjugated bilirubin (UCB) is known for its potential neurotoxicity, particularly during the neonatal period, although remaining unclear the mechanisms underlying its access to the brain. In fact, there is only one study on the effects of UCB on bovine brain endothelial cells where it was shown that UCB induces apoptosis. So, the injury to the endothelial cell lining of the blood-brain barrier (BBB) that might explain the underlying mechanisms of UCB access to brain deserves to be studied, and novel therapeutic approaches are needed to prevent cell damage. Ursodeoxycholic acid (UDCA) is an endogenous bile acid widely used for the treatment of chronic cholestatic liver diseases. Following oral administration, it is conjugated in the liver, originating tauroursodeoxycholic acid (TUDCA) and, mostly, glycoursodeoxycholic acid (GUDCA). In previous studies, we showed several beneficial effects of UDCA or its conjugates, namely their ability to abrogate UCB-induced oxidative disruption and inflammatory responses, therefore preventing nerve cell death. *Aims*. In this study, we used a simplified model of the BBB, constituted by human brain microvascular endothelial cells (HBMEC), to investigate whether: (i) physiologically relevant concentrations of UCB induce cell death by apoptosis; (ii) UDCA and GUDCA are able to prevent apoptotic cell death from occurring and (iii) establish the temporal window of therapeutic intervention. *Design and Methods*. A HBMEC cell line, that showed to maintain morphologic and functional characteristics, was used. Cells were exposed to 50 or 100 M UCB, in the presence of 100 μM human serum albumin to solubilise UCB, for 4, 24 and 48 h at 37°C. To evaluate the protective role of UDCA and GUDCA, cells were separately treated for 1 h with 50 μM of each of the bile acids prior to the 48 h incubation with UCB. To establish the therapeutic window, another set of experiments was performed by addition of the modulators 8 hours after exposure to UCB. Assays with bile acids alone were also included. Apoptosis was assessed by the analysis of nuclear morphology after incubation with Hoechst dye 33258 (Sigma) using a fluorescence microscope. *Results.* Exposure of HBMEC to UCB led to a time- and concentration-dependent increase in apoptotic cell death (statistically significant differences were obtained for all data points vs. respective control, except for 4 h incubation with 50 mM UCB). Both bile acids were able to counteract UCB-induced cell death by apoptosis, being the greatest protection (≥50 %, $p<0.05$) achieved by addition of the therapeutic compounds prior to the beginning of the incubation with the toxic insult. Interestingly, even after 8 h exposure to UCB, the therapeutic molecules were able to abrogate cell death by nearly 30 % ($p<0.05$). *Conclusions*. The present results provide the first demonstration of the disruption of the endothelial cell lining of the human BBB by UCB. They also demonstrate the ability of the bile acids UDCA and GUDCA to abrogate UCB-induced apoptosis, therefore disclosing a novel therapeutic approach to apoptotic cell death resulting from exposure to an endogenous circulating molecule.

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NOVEL TREATMENT OF MULTICENTRIC CASTLEMAN'S DISEASE WITH LENALIDOMIDE

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Introduction. Multicentric Castleman's disease is a rare lymphoproliferative disorder that is characterized by lymph node hyperplasia, a marked systemic inflammatory response and a tendency to evolve into an aggressive lymphoma. It typically presents with systemic symptoms including night sweats, weight loss, and lethargy. Therapeutic options are limited and multiple agents including steroids, thalidomide, rituximab, and tocilizumab have been used to treat the condition. *Aims/Method*s. A 49-year-old HIV negative patient was eventually diagnosed with Castleman's disease having had an atypical presentation with progressive marrow failure due to fibrosis, marked systemic symptoms and serositis with pleural effusions and ascites. His clinical course was complicated by peritonitis and sepsis on several occasions. After initial stabilization with rituximab he had progression of disease and was retreated with rituximab in combination with cyclophosphamide, vincristine and steroids. Despite this he had on going progression of disease with worsening ascites and general deterioration. As there are anecdoctal reports indicating activity of thalidomide a trial of thalidomide 200 mg once daily was given. While there was a clinical response to thalidomide it had to be stopped due to hyperkalaemia. Based on a a similar mode of action patient was started on lenalidomide 25 mg once daily for 21 days in every 28-day cycle in combination with dexamethasone. The dose of steroids was gradually reduced and the patient has continued single agent lenalidomide since. To date he has received 18 cycles of lenalidomide and remains in clinical remission. The main toxicities that were observed during lenalidomide therapy included thrombocytopenia and an episode of desquamative rash on his hands and feet which required temporary cessation of therapy and short course of oral corticosteroids and subsequent dose reduction of lenalidomide to 15 mg. *Conclusion*. The standard therapy of Castleman' disease is not established. Published evidence consist of mainly of case reports and small case series. There is growing evidence that the use of rituximab can result in durable remissions and therefore it is emerging as the likely treatment of choice for most patients. There are anecdotal reports that thalidomide was active in symptomatic control of the disease. However there is no published evidence on the use of lenalidomide in the Castleman's disease. Our patient tolerated lenalidomide very well and achieved satisfactory disease control. This represent a novel approach and offers another treatment option in patients not responding to or intolerant of other therapeutic modalities.

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ANTITUMOR ACTIVITY OF NAMPT INHIBITION AND SYNERGY WITH CYCLOSPORIN A AND HDACIS TO PROMOTE APOPTOSIS IN PRIMARY LEUKEMIC CELLS

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Background. During the neoplastic transformation, nicotinamide phosphoribosyltransferase (NAMPTase), a key enzyme involved in NAD biosynthesis, performs an important role since becomes upregulated to compensate for increased metabolic demands. Therefore, drugs capable to inhibit this enzyme such as FK866, are highly sought for their poten-

tial as cancer therapeutics. The NAD depletion, as formerly reported by a lot of authors, is associated with mitochondrial dysfunction and autophagy triggering cell death. The cytotoxic effect of these compounds seems to be selective for malignant, as opposite to normal cells. Nevertheless NAMPT inhibitors alone induce a mild cell death on primary leukemic cells. We have made use of FK866 in combination with traditional drugs cyclosporin and valproate, to improve this results. *Design and Methods*. We collected peripheral blood samples from B-CLL (previously untreated and treated; n=22) and AML (n=8; different FAB subtypes) patients. The mononuclear cell were isolated by density-gradient centrifugation using Ficoll-Hypaque and immediately seeded in RPMI 1640-based culture medium. Consequently 2,5x105 primary leukemic cells/well were plated in 96 well plates and treated with increasing concentrations of FK866 (range 10-9-10-6 M) either alone and in combination with cyclosporin (10-6-10-4M) or valproate (10- 4-10-5 gr/mL). Viability was assessed at 96 h from the beginning of the treatment by propidium iodide exclusion using a FACS Calibur. In selected cases, Annexin-V/propidium iodide staining was done to detect early- vs. late-apoptotic leukemia cells in response to FK866 alone and in combination treatment. Intracellular NAD content was measured using a biochemical assay; the pan-caspase inhibitor (zVAD-fmk), the caspase 9 and 8 specific inhibitors were used to value apoptosis; flow cytometric assay was used to detect P-glycoprotein and to determine intracellular FK866 fluorescence. *Results*. We observed an heterogeneous response to FK866 growing doses with an average of 50% of death cell at 10nM; since primary leukemic cells were mildly susceptible to apoptosis via NAMPT inhibition, we tested the FK866 in combination with valproate and cyclosporin showing a much weaker antiproliferative effect. Also the intracellular NAD levels lowered mainly in the combination treatment. To evaluate the additive or synergistic effect, a co-operative index (CI) was calculated for each sample with following formula: CI= sum of specific apoptosis of single agent treatment/specific apoptosis upon combined treatment. CI values <1,=1 and >1 mean that the effect is synergistic, additive or infra-additive effect respectively. The co-operative index was <1 in both drugs associations showing a synergistic effect for both the two ones. This result suggests that, in addition to Nampt inhibition, the two drugs may act through others machanisms. In detail, valproate might improve FK866 antileukemic activity via concomitant stimulation of complementary apoptotic pathways; cyclosporin, through the P-gp inhibition, induces an increment of intracellular FK866 improving its owns activity. Furthermore, we investigated the role of ADP-ribosyl cyclase CD-38 and we did not observe different susceptibility to FK 866 in CD 38 +/- cells. *Conclusions*. Our data indicate a novel strategy for the treatment of hematological malignances since valproate and cyclosporin may improve the outcome of monotheray with FK866 and provide a potential alternative leukemia treatment.

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CAFFEIC ACID PHENETHYL ESTER INDUCES APOPOSIS IN ACUTE MYELOID LEUKEMIA CELLS

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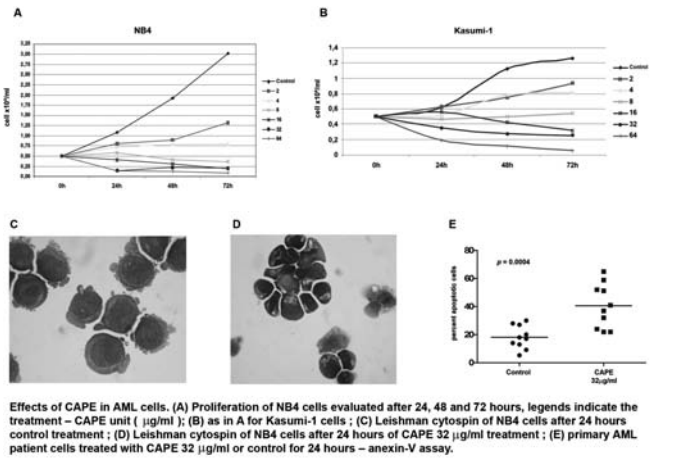
Caffeic acid phenethyl ester (CAPE) is an active phenolic compound present in propolis obtained from honeybee hives. It is reported to present a broad spectrum of biological activities including antioxidant, anti-inflammatory and antitumoral. The antitumoral activity of CAPE as evaluated by several studies *in vitro* and *in vivo* seems to be related to distinct effects like the inhibition of angiogenesis, invasion and metastasis and the induction of apoptosis or differentiation of cancer cells. However the demonstration of apoptosis induction in acute myeloid leukemia (AML) cells and the demonstration of enhancement of all-trans-retinoic acid (ATRA) induced differentiation in acute promyelocytic leukemia (APL) by CAPE is restricted to the HL-60 cell line. HL-60 is a very sensitive cell to apoptosis induction by diverse treatments and is not a model of APL differentiation by ATRA since do not posses the PML-RAR-α rearrangement. Our aim was to evaluate the effects of CAPE treatment on APL cell lines NB4 and NB4-R2 (a cell resistant to ATRA-induced differentiation) and on AML cell line Kasumi-1 (a cell representative of core binding factor leukemias with AML1-ETO rearrangement). Also to evaluate the effect of CAPE on the induction of apoptosis in primary AML samples. Proliferation and viability was evaluated by direct cell count with tripan blue exclusion dye in Neubauer chamber at fixed time intervals. Differentiation was evaluated by flow cytometer determination of CD11b expression. Cell cycle distribution and apoptosis were evaluated by flow cytometer determination of propidium iodide- DNA fluorescence and apoptotic cells were defined as sub-G0 fraction. Also apoptosis was detected by the anexin-V method. Leishman stained cytospins for light microscopy were used to confirm apoptosis or differentiation. CAPE did not induce differentiation in the cell lines NB4, NB4-R2 or Kasumi-1 and did not alter the differentiation induced by ATRA in NB4 cells. CAPE inhibited the proliferation of AML cell lines in in a time and dose dependent fashion (Figure A, B) CAPE induced apoptosis in all cell lines. In the NB4 cell line CAPE induced cell death (tripan blue assay), estimated ED50 was 32.1 mcg/ml. ED50 for induction of apoptosis in the more sensitive assay using anexin-V in NB4 cells after 24h was 7.5mcg/mL and for Kasumi-1 was 10.2mcg/ml. CAPE (32 mcg/ml) significantly induced apoptosis in cells from AML patients, mean IC50 (29.26 - 51.76) versus control treated cells 18.16 % ; IC95% (12.27 - 24.05); p=0.0004 (Figure E) One possible mechanism for CAPE cytotoxicity in leukemia cells is NFK-B inhibition. This hypothesis is currently under investigation in our laboratory. In conclusion, CAPE do not induce differentiation in APL cells but induces apoptosis and inhibition of proliferation in a dose and time dependent manner in AML cells lines and also induces apoptosis in patient samples. These properties are interesting and need further investigation.



Effects of CAPE in AML cells. (A) Proliferation of NB4 cells evaluated after 24, 48 and 72 hours, legends indicate the treatment – CAPE unit (µg/ml); (B) as in A for Kasumi-1 cells ; (C) Leishman cytospin of NB4 cells after 24 hours control treatment ; (D) Leishman cytospin of NB4 cells after 24 hours of CAPE 32 µg/ml treatment ; (E) primary AML patient cells treated with CAPE 32 µg/ml or control for 24 hours – anexin-V assay.

Figure.

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RITUXIMAB THERAPY FOR IMMUNE THROMBOCITOPENIC PURPURA

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Background. Standard therapy for autoimmune diseases (drugs with non-specific immunosoppressive capacities) is associated with infections and toxicity. As the anti-CD20 monoclonal antibody Rituximab induces complement- or cell-mediated lysis of autoreactive lymphocyte clones, we assessed its efficacy in patients who were resistant to standard therapy for immune thrombocytopenic purpura (ITP). *Design and Methods*. Rituximab was administered as a weekly infusion of 375 mg/m^2 for 4 consecutive weeks. *Aims*. The objective of this study was to evaluate the efficacy and safety of Rituximab in the treatment of patients with ITP. *Patients*. 17 patients were observed, with median age 47,5 years (range 14 - 81years), 13F, 4 M. 15 patients had idiopathic thrombocytopenic purpura (ITP), 1 had non Hodgkin Lymphoma, another had Sjogren's disease. All had platelet counts ≤10000/m^3 and had received steroids and high dose, intravenous immunoglobulin. One had received Azathioprine (Sjogren's disease), another has received Cyclophosphamide in the previous 2 months. Chemotherapy had failed in the patient with non Hodgkin Lymphoma and splenectomy in four patients with ITP. Result: Median follow-up was is 32,5 months (range 3-62). Of the 17 treated patients, complete remission was achieved in 14 patients and no response in 3 patients; 3 months after treatment platelet counts were over 120 000/mmc in 14 patients, 2 patients reached normal platelet counts after splenectomy, 1 good responder requested a second Rituximab cycle after 16 months and obtained a second response with a persistent normal platelet count after 58 months. Rituximab was well tolerated. *Conclusions*. Younger age and shorter interval from diagnosis to Rituximab appeared indicators of better outcome. Rituximab is an effective agent for the treatment of ITP resistant to standard immunosuppressive drugs with tolerable toxicity.

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RITUXIMAB TREATMENT OF REFRACTORY AUTOIMMUNE HEMOLYTIC ANEMIA AND REFRACTORY AUTOIMMUNE THROMBOCYTOPENIC PURPURA

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Background. The B lymphocyte plays a major role in the pathogenesis of autoimmune diseases. Targeted therapy against CD20 positive B lymphocytes may be an effective treatment of Autoimmune Hemolytic Anemia (AHA) and Autoimmune Thrombocytopenic Purpura (ITP). Aims: The objective of this study is to determine the role of Rituximab as a therapeutic means against the two autoimmune hematological diseases. *Design and Methods*. Patients who have been under Rituximab treatment were studied retrospectively: 3 AHA patients [2/3 with an underlying lymphoproliferative disease (LPD)] and 11 ITP patients (5/11 with no underlying disease, 3/11 with underlying LPD, 1/11 with underlying LPD and collagonosis, 1/11 with collagonosis and 1/11 with common variable immunodeficiency). Rituximab was administered IV weekly in doses of 375 mg/m^2 for 4 weeks. The monthly infusions that followed were absolutely related to each patient's response to the treatment. All the patients had been treated for their underlying conditions except one with AHA and CLL. In addition, all the ITP patients had responded poorly or not at all to any previous treatment. *Results*. Two patients with underlying LPD and AHA completely responded after 6 cycles of RItuximab treatment while another patient with refractory AHA didn't respond after 4 cycles and quit. As far as ITP patients were concerned, 4/6 with an underlying disease responded completely and 1/6 partially, maintaining his PLT levels between 30000/l-50000/μL. A female patient with common variable immunodeficiency relapsed a year after receiving 8 cycles of treatment and responded after repeating the therapy. As for the patients with no underlying conditions (5/11), one of them responded completely and three of them partially, while one did not respond at all. *Conclusions*. Despite the small number of patients, it seems that Rituximab can be a safe and effective treatment for autoimmune hematological diseases.

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INCREASING INTRACELLULAR GENERATION OR ACCUMULATION OF CERAMIDES INCREASED CYTOTOXIC EFFECTS OF RESVERATROL IN HUMAN K562 CHRONIC MYELOID LEUKEMIA CELLS

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Backround. Resveratrol, trans-3,5,4'-trihydroxystilbene, is a naturally occurring phytoalexin produced by various plants in response to stress, injury, ultraviolet (UV) irradiation, and fungal infection, and also skin and seeds of red grapes. Resveratrol affects the processes underlying tumor initiation, promotion and progression. Many studies in human cell cultures indicate that resveratrol can modulate multiple pathways involved in cell growth, apoptosis, and inflammation. Ceramides are second messenger of the sphingomyelin pathway, and they constitute a signaling mediator of various biological activities including cell cycle arrest, cell senescence, cell migration and adhesion, differentiation or apoptosis in normal and tumor cells from various tissues. While ceramide is an apoptotic molecule, conversion of ceramides to glucosyl ceramide or sphingosine-1-phosphate by glucosylceramide synthase (GCS) or sphingosine kinase-1 (SK-1) enzymes, respectively, result in cell growth and proliferation. Thus, increasing intracellular concentrations of ceramide by induction of *de novo* synthesis or inhibition of GCS by N-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decanamide, hydrochloride (PDMP) or SK-1 by sphingosine kinase-1 inhibitor may be one of possible pathways of stress-induced cell death. *Aims*. We have shown in our previous studies that resveratrol can trigger cell death in human K562 chronic myeloid leukemia (CML) cells. But there is no detailed information about the mechanisms of resveratrol-induced apoptosis. More importantly, the involvement of ceramide metabolising genes and their end products in resveratrol-induced apoptosis are not investigated in CML. In this study, we tried to show the possible roles of ceramide, glucosylceramide and sphingosine-1-phosphate in resveratrol-induced apoptosis in human K562 cells. *Design and Methods*: Cytotoxicity analyses of resveratrol, C8:ceramide, PDMP and SK-1 inhibitor and combinations of resveratrol with C8:ceramide, PDMP or SK-1 inhibitor in K562 cells were analysed by XTT cell proliferation assays and possible synergistic analyses were examined. *Results*. IC50 values of resveratrol and C8:ceramide or IC90 value SK-1 inhibitor and PDMP were found to be 80 μM and 60 μM or 7 μM and 20 μM, respectively. There were 8-, 18-, 43-, 45-, and 59% decreases in cell proliferation of K562 cells in response to 1-, 10-, 50-, 70-, and 100 μM resveratrol, respectively. Combination of the same doses of resveratrol with 20 μM PDMP decreased proliferation of K562 cells to 29-, 46-, 55-, 58-, and 68%, respectively, as compared to untreated controls. On the other hand, application of 60 μM C8:ceramide in combination with the same doses of resveratrol decreased cell proliferation to 87- to 90%, as comparing to untreated controls. 7μM of SK-1 inhibitor in combination with 1-, 10-, 50-, 70-, and 100 μM of resveratrol resulted in 23-, 42-, 54-, 72-, and 73% decreases in cell proliferation. *Conclusions*. In this study, we aimed to show cytotoxic effects of resveratrol, exogenous ceramide, C8:ceramide, GCS inhibitor, PDMP, and SK-1 inhibitor on K562 cells. The results of this study showed for the first time that there were synergistic effects of combination of resveratrol and IC50 value of C8:ceramide or IC90 values of SK-1 inhibitor and PDMP. Taking together all these results showed that ceramides may be involved in resveratrol-induced apoptosis in K562 cells.

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RITUXIMAB THERAPY FOR REFRACTORY SLE-EVANS SYNDROME

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Background. Targeted depletion of B lymphocytes is an alternate approach to treatment of autoimmune disorders. Rituximab, a chimeric, human, IgG1a monoclonal antibody, is specific for the CD20 antigen and can selectively deplete B-cells by antibody-dependent cell-mediated cytotoxicity, complement- mediated cytotoxicity, and inhibition of cell proliferation with direct induction of B cell apoptosis. Several studies have shown the efficacy and safety of Rituximab in autoimmune disorders such as chronic idiopathic thrombocytopenic purpura (ITP), idiopathic autoimmune thrombocytopenia and/or autoimmune hemolytic anemia - (Evans syndrome), rheumatoid arthritis (RA), SLE, mixed cryoglobulinemia. Case Report: We describe a 16 year-old male with SLE-Evans syndrome refractory to glucocorticosteroids, intravenous immune globulin (IVIG) therapy, Mycophenolate mofetil and plasmapheresis. The patient responded to four weekly infusions of rituximab and maintains this remission now for 36 months. Therapy was well tolerated, and no infectious complications occurred. *Result*. Rituximab appears safe, effective and feasible in a variety of autoimmune diseases. After rituximab administration the patient had a favorable response to this treatment and since 36 months, he showed no more thrombocytopenia. *Conclusions*. These datas indicate that rituximab is effective and feasible in most pediatric patients with autoimmune diseases but we still need more experience. We think that it's logical to use rituximab as therapy for Evans syndrome resistant to first-line therapy and ciclosporin/MMF as an alternative to intensive immunosuppressive therapies or splenectomy. By the recurrence of thrombocytopenia or anemia, second treatment course is feasible and may be successful in controlling the disease

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THE EFFECTS OF EMODIN ON PROLIFERATION AND APOPTOSIS IN JURKAT CELLS AND ITS MECHANISM

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Aims. To investigate the effects of Emodin on inhibiting proliferation and inducing apoptosis in leukemia cells. Acute T lymphoblastic leukemia cell line Jurkat was chosen to be investigated. *Design and Methods*: MTT assay was used to explore the cell proliferation. Cell cycle analysis, Annexin V/PI analysis, TUNEL and DNA ladder were used to investigate cell apoptosis. RT-PCR was taken to test the expression of apoptosis related gene and Western Blot was used to test the expression of related proteins. *Results*. Emodin could remarkably inhibit proliferation in Jurkat cells with the IC_{50} of 20 umol/L. Cell cycle analysis, Annexin V/PI assay and TUNEL showed that Emodin could effectively induce apoptosis in Jurkat cells with a time and does dependent manner in a range of concentration. After the treatment of Emodin, the expression

of c-myc and bcl-2 was down regulated in both gene and protein expression level. Western blot showed the expression of procaspase-3,8,9 decreased, while activated cleaved fragment caspase-3 increased in a time dependent manner. *Conclusions.* Emodin could effectively inhibit proliferation and induce apoptosis in Jurkat cells, and c-myc, bcl-2 and caspase family may have involved in the process stated above which was induced by Emodin.

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RITUXIMAB THERAPY FOR IMMUNE ANEMIA AND THROMBOCYTOPENIA: THE STUDY OF 06 CASES

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Background. Immune thrombocytopenic purpura (ITP) and autoimmune hemolytic anemia (AHA) may respond to the chimeric anti-CD20 monoclonal antibody rituximab, even when refractory to conventional therapy. Aims: to evaluate the effect of rituximab in the two autoimmune diseases cited above. *Design and Methods*. Our study is retrospective on 03 years. We have collected the files of 06 patients; 02 of whom had AHA, and 04 had ITP. Those patients have not responded to the corticotherapy, and have undergone, then, a splenectomy. After that, they have been given rituximab. The hemoglobin count was between 05 and 09 gr/dL whereas the platelet count varied between 4000 and 11000/uL. *Results.* 02 of the 04 ITP patients have shown complete response (CR), with platelet count >400000/uL. 01 partial response (PR) with platelet count 20000-50000/uL. 01 failure with platelet count <10000/uL. Both of the 02 AHA patients have shown complete response (CR), with hemoglobin count >10 gr/dL. *Conclusions*. Rituximab has given good results on this small population, it would be more interesting to include more patients, and do further multicenteric studies.

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STUDY OF THE RESISTANCE IN ANTIPLATELET DRUGS, BY DETERMINATION OF GLYCOPROTEIN ON THE PLATELET MEMBRANE

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Introduction. Several studies have been made in order to determine why some individuals that are under anti-platelet treatment have relapses of thromboembolic episodes. The platelets function in individuals under aspirin or clopidogrel was checked using aggregometer, PFA-100, thromboxane derivative 11-dehydrothromboxane B2 in the urine. In order to follow up the changes of platelet membrane receptors in patients under anti-platelet treatment, we decided to examine the glycoproteins: Gp-Ib, Gp-Ibβ, Gp-IIb, Gp-IIIa and P-selectins. The reason for this was to see if these receptors were inhibited by this treatment. Material: We studied 83 people (36M and 47F with mean age 45,2±23,9 and 52,3± 19,1 correspondingly). From these; 43 {12Ms and 31Fs} without treatment and 29 {15Ms and 14Fs) under aspirin, 7 under clopidogrel {5M and 2F} and 4Ms were under both. *Design and Methods*. We applied platelet aggregation with AA and ADP. Also PFA-100 with Colla/EPI and Colla/ADP cartridges. In the same time we determined the CD41a, 42a, 42b, 61 and 62P antibodies with flow cytometry. The determination of glycoproteins was made in PRP. In order to evaluate the accuracy of this method we determined the glycoproteins in one person 10 times. As shown by CV our method has good reproducibility. Our results are shown a significant decrease of the number of all glycoproteins receptors in the patients under antiplatelet treatment except for the P-Selectins. Comment: It is a method of confirmation of the other methods as it presents the decrease in the number of the receptors. After the ascertainment that the resistance in clopidogrel is related with mutation CYP2C19*2 (a large gene of more than 90 kb in size, including nine exons, on chromosome 10q24.1-q24.3), we tested with a strip assay 2 patients with clopidogrel resistance and confirmed the presence of the homozygote mutation CYP2C19*22. Proving in the same time the genetic base of clopidogrel resistance.

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AZACITIDINE AND PEGYLATED G-CSF: OEDEMATOUS SWELLING OF THE TONGUE AS POTENTIAL SIDE EFFECT OBSERVED IN TWO PATIENTS

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Background. Indications for treatment with azacytidine are presence of a myelodysplastic syndrome, intermediate-2 or high risk, chronic myelomonocytic leukemia with excess of blasts >10% or acute myeloid leukemia with 20-30% blasts and myelodysplasia-related changes. Reported adverse reactions concern mainly the hematological, cardiovascular, central nervous, dermatological and respiratory systems. Potential fatal side effects of pegylated G-CSF are adult respiratory distress syndrome and rupture of the spleen. We here report for the first time occurrence of a rare, but potentially life threatening risk seen after combination of these medicaments in two patients. Patient 1 (46 year, male) received azacitidine 75 mg/m^2/d for seven days, due to a relapse of an acute myeloblastic leukemia after allogeneic stem cell transplantation. Following chemotherapy pegylated G-CSF was administered. On day +18 after treatment start an asymmetrically swelling of the tongue was observed. No further complaints like dyspnoe or dyspahagia were present. An ultrasound examination was suspicious of a hematoma. The MRT-scan with contrast medium showed a diffuse enhancement indicating an inflammatory process. Antibiotics and platelets were given. The swelling disappeared within four days. Patient 2 (67 years, male) presented with a refractory anemia with excess of blasts (REAB-2, IPSS intermediate-2) and was treated with azacitidine (75 mg/ m2/d for seven days). Seventeen days after start of chemotherapy and seven days after application of pegylated G-CSF the patient complained dysphagia and hypersalivation. The CT- and MRT-scan showed similar findings interpreted with swelling of the tongue and the adjacent tissue. The antibiotic treatment was escalated and corticosteroids were given. The complaints and clinical findings resolved within five days. *Summary*. The parallel occurrence of clinical symptoms and radiological signs of tongue swelling after application of azacitidine and pegylated G-CSF is indicative for a potential association of one or both of these two drugs. For none of the other prescribed medicaments an association with tongue swelling has been reported. Furthermore, other potential causes like bacterial infection, hematoma of allergic reaction seem to be unlikely upon clinical presentation. However, the pathophysilogical background of this localized reaction remains to be clarified. Since enoral edema is a potential life threatening risk and combination of these two drugs are used routinely, we report this rare clinical complication.

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CYTOTOXICITY BY COMBINED TREATMENT OF WARFARIN AND DOXORUBICIN ON LEUKEMIC CELLS IN VITRO

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Background. Previous results from our laboratory gave evidence that warfarin treatment may enhance the toxic effect of radiation in leukemic cell. Doxorubicin (DOX) is an antitumor drug commonly used as single and in combination with other chemotherapeutic agents, for treatment of wide range of human malignancies. *Aims*. This study investigated combinations of DOX with warfarin for their cytotoxicity against leukemic cells following a 72 h exposure *in vitro*. *Design and Methods*. Cell proliferation and apoptosis assays with various concentrations of warfarin with or without DOX (1 μM) were done on two leukemic cancer lines, human chronic myelogenous leukemic K562 cells and promyelocytic leukemic HL-60 cells, and on normal human peripheral blood mononuclear cells (PBMC). Results: The warfarin at the lower concentrations (<50 μM) with DOX treatment also induced in additive manner apoptosis in K562 and HL-60 cells. Combined treatment also attenuated cell proliferation in both cell lines. Presence of 100 μM N- acetylcysteine, as antioxidant, in the cell culture did not impair the cytotoxicity of DOX and warfarin. *Conclusions*. The present results indicate that warfarin treatment may enhance the toxic effect of DOX in leukemic

cells. The mechanism by which warfarin potentiate this cytotoxicity is unclear, but it may not be directly due to toxic damage induced by warfarin-generated free radicals, since co-treatment with N- acetylcysteine was unable to prevent this toxicity.

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WARFARIN RESISTANCE INDUCED BY OXCARBAMAZEPIN

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A sixteen years old male patient who had prosthetic aortic valve replacement six years ago was referred to our clinic for low INR values despite warfarin use. The patient was taking oxcarbamazepine because of a seizure-like episode he experienced one year ago. However, INR value decreased from normal target range at the 6th months of oxcarbamazepine therapy and reached a value of 1.23 at 12th months. We discontinued oxcarbamazepine therapy, but, the patient's INR values were still at the subtherapeutic levels. We increased daily warfarin dose to 20 mg with resulting INR of 1.5. We searched for genetic defects leading to warfarin resistance and found VKORC1 1173CC genotype and CYP2C9 *2 allele. Based on these findings we decided to use clopidogrel 75 mg - aspirin 300 mg per day combination. Warfarin resistance is a rare clinical problem which can be caused by genetic or acquired causes. Although our patient had VKORC1 1173CC genotype which requires higher warfarin dose, he also had CYP2C9 *2 variant which is associated with lower warfarin dose. We suggested that oxcarbamazepine-warfarin metabolism interaction which may be via VKORC1 1173CC mutation can cause a continuous decrease in warfarin's ability to suppress vitamin K epoxide reductase enzyme in our patient.

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HIGH LEVELS OF SOLUBLE IL2R AND PDL1 EXPRESSION MAY INHIBIT ANTITUMOR IMMUNITY IN CHRONIC MYELOID LEUKEMIA

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Background. Cancer patients are known to exhibit immune escape mechanisms that complicate the development and use of immunotherapy in these patients. For example, many solid tumors are infiltrated by suppressor cells. These cells effectively inhibit tumor immunity by direct contact and the release of immune modulators such as TGFβ and IL10. Many tumors exhibit immunomodulatory function including release of suppressive cytokines. Lately, the expression of Programmed Death receptor Ligand 1 (PDL1) on tumors has been linked to escape from tumor immunity. The increased presence of suppressor cells and other immune escape mechanisms have been reported to include hematological cancers as well although little is known about their role in chronic myeloid leukemia (CML). Aims The aim of the project was to investigate the immunological profile exerted by tumor cells in CML patients. *Design and Methods*. Blood from CML patients (n=15) and healthy controls (HC, n=22) was evaluated for the presence of T regulatory cells, PDL1 expression on CD34+ cells using flow cytometry and cytokines by the use of ELISA and cytometric bead array. Results Our results demonstrate that CML patients only have a minor increase of Treg cells compared to healthy controls. However, the level of Treg cell-associated molecules such as soluble IL2R (sCD25) and IL10 in plasma was higher in CML patients than in controls. Five out of 11 evaluated patients had expression of PDL1 ranging from 1-36% of the total $CD34^+$ population demonstrating that this molecule may be involved in controlling tumor immunity in 50% of CML patients. PDL1 expression on bulk tumor cells remains to be investigated. *Conclusions.* Taken together, these results show that CML patients only have a minor increase of Treg cells but tumor immunity may be kept under control by other mechanisms such as high levels of systemic IL2R and IL10 as well as tumor expression of PDL1.

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MATURATION STIMULI-DEPENDENT DIFFERENTIAL INDUCTION FROM X-IRRADIATED HUMAN MONOCYTES TO DENDRITIC CELLS

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Background. Dendritic cells (DCs) are a type of antigen-presenting cell which play an essential role in the immune system. Recent immunotherapeutic research has been focused on the use of DCs as potential cellular vaccines against malignant tumors. However, little is known whether the DCs generation is affected after chemotherapy and radiotherapy. In particular, the influence of ionizing radiation remains to be elucidated. Therefore, the current study focused on human peripheral blood monocytes, which are the DC precursors of myeloid DCs, and found that X-irradiated monocytes can differentiate into DCs and then mature after TNF-α stimulation in terms of phenotypic characteristics, while many of their functions such as the matrix metalloproteinase-9 (MMP-9) activity and the ability to stimulate allogeneic T cells are impaired in comparison to controls (*J. Radiat. Res., 49, 2008*). [Aims] Recent reports propose various types of maturation stimuli, including proinflammatory cytokines and pathogen-derived components on processing DCs for immunotherapies. Therefore, the present study investigated whether X-irradiation of monocytes influenced the maturation of DCs in response to specific maturation stimuli. *Design and Methods*. The use of human buffy coats was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine. Monocytes were separated from the buffy coat and exposed to X-rays at 0, 2, 5 and 10 Gy. To prepare immature DCs (iDCs), irradiated monocytes were cultured in the presence of rhGM-CSF and rhIL-4. To prepare mature DCs (mDCs), iDCs were stimulated by LPS or a stimulus mixture (MIX; [rhTNF-α, IL-1α, IL-6, PGE2]) for 48 hr. The phenotype of the DCs was analyzed by flow cytometry and the MMP-9 activity was assayed by gelatin zymography. Furthermore, intracellular reactive oxygen species (ROS) were measured using 2', 7'-dichlorodihydrofluorescein diacetate. *Results.* When LPS was used as the maturation stimulus, the expression level of the co-stimulatory molecule CD80 and DCs' maturation marker CD83 on the DCs from X-irradiated monocytes was lower than that of the DCs from non-irradiated monocytes. However, no decrease was observed in the DCs stimulated by MIX. When iDCs were stimulated by LPS, the MMP-9 activity in the culture supernatants was impaired in the DCs from the X-irradiated monocytes in comparison to DCs from non-irradiated monocytes. However, in the case of MIX stimulation, MMP-9 activity in the culture supernatants of DCs from X-irradiated monocytes was largely maintained in comparison to non-irradiation. The intracellular ROS is an important mediator of TLR4-mediated signal transduction, which is receptor for LPS. Therefore, the behavior of intracellular ROS after LPS stimulation was measured. As a result, the intracellular ROS level of DCs from X-irradiated monocytes was lower than that from non-irradiated monocytes. *Conclusions*. These results suggest that the influence of X-irradiation on monocytes in the maturation of DCs depends on the type of maturation stimulus, and that MIX stimulation may therefore be preferable to immunotherapy using monocyte-derived DCs after radiotherapy.

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POTENT HUMAN MONOCYTE-DERIVED DENDRITIC CELLS (DCS) CAN GENERATE BY MATURATION WITH NATURAL KILLER (NK) CELLS IN THE PRESENCE OF TOLL-LIKE RECEPTOR (TLR) AGONIST

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The interaction between DCs and NK cells can play a key role in inducing the maturation of DCs for subsequent T cells priming *in vivo*. DCs play an additional role in NK cell activation and NK cells are also able to activate DCs leading to maturation. TLRs on the surface of immature DCs (imDCs) and NK cells induce the maturation and activation of these cells. We investigated what kind way of culture condition *in vitro* was more effective for generation of potent mature DCs by activate these DCs with activated or inactivated NK cells. Immature DCs differentiated form monocytes ($CD14^+$ cells) of healthy donor was maturated by coculturing directly or indirectly with NK cells that were activated with various cytokine combination. Direct contact between imma-

ture DCs and NK cells showed more potent DCs, based on the higher expression of several molecules and more IL-12 production, than indirect culture using filter membrane. Immature DCs stimulated with NK cells in the presence of cytokine could induce more potent DCs than those stimulated with activated NK cells that were previously activated with cytokines. Among the various combination of cytokines, the stimulation of IL-2, poly I:C (TLR 3 agonist) and IFN-α was the most useful combination to induce potent DCs, in terms of phenotypic expression on DCs, production of IL-12p40 and IL-12p70 by DCs, IFN-α production by NK cells, allogeneic T cell stimulatory capacities, and CTL assay using IFN-α ELISPOT assay with standard chromium release or DNA fragmentation assay, and recently new CD38 molecule expression on DCs. These studies suggest that potent DCs differentiated from human monocytes can generate by the direct contact culture between human monocytes-derived DCs and NK cells in the presence of combination of IL-2, TLR 3 agonist and IFN-α.

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THE STUDY ON THE POTENCY TEST FOR HUMAN IMMUNOGLOBULIN PREPARATIONS

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Backgrounds. Immunoglobulin manufactured by using normal plasma currently applies the anti-measles assay using the cell culture as lot release potency test in Korea. It is difficult to obtain the international reference standard (NIBSC 97/648, 3rd) for the anti-measles because of limited quantity. The European Pharmacopoeia is already adopted the anti-HBs assay. This is why the demands for improving or substituting the test method instead of the anti-measles assay. *Aims*. Through this study, first, it makes the domestic manufacturers reduce the dependence of the international reference standard for the anti-measles assay. Second, the national standard (KFDA 08/026) for the potency test of Human Hepatitis B Immunoglobulin can be utilized for the anti-HBs assay instead of the anti-measles assay. *Design and Methods*. In order to introduce the anti-HBs assay for Human Immunoglobulin preparations, the collaborative study was performed with four research laboratories including Korea Food and Drug Administration (KFDA) and 3 manufactures. We investigated to examine the validity for the introduction of the anti-HBs assay, to validate the test method, and to establish the specification. In preliminary examination, each laboratory performed tests two times, once a day, duplicate per one test in order to decide the dilution range. In main examination, the anti-HBs assay was performed three times, once a day, duplicate per one test, with 18 lots of pooled plasma, 10 lots of Human Normal Immunoglobulin, and 20 lots of Human Normal Immunoglobulin in Maltose (pH 4.25), respectively. *Results.* he anti-measles assay is set up in South Korea, Japan, and the United States (anti-diphtheria and anti-polio are also used in the US), and the anti-HBs assay is set up in Europe, Australia, and England. As a result of the anti-HBs assay, it is found that the anti-HBs titer is 0.343±0.093 IU/mL in pooled plasma, 4.731±0.687 IU/mL in Human Normal Immunoglobulin, and 2.363±0.722 IU/mL in Human Normal Immunoglobulin in Maltose (pH 4.25), respectively. *Conclusions*. Based on this study, the draft specification of the anti-HBs assay is established, and the potency is different depending on the source (domestic or import) and the type (pheresis or recovered) of plasma. Compared to the specification in Europe and England, it is over 32 times higher in Human Normal Immunoglobulin and over 7 times higher in Human Normal Immunoglobulin in Maltose (pH 4.25). In conclusion, for the potency test in Human Immunoglobulin preparations, it is suggested that the anti-HBs assay can be used as a potency test instead of the anti-measles assay in Korea. It is also expected that the anti-HBs assay is more reproducible than the anti-measles assay and that the national lot release test and the quality control of the manufacturer are internationally harmonized.

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HAPLOTYPE ANALYSIS OF β THALASSEMIA PATIENTS IN WESTERN IRAN

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Background and Aims. β-thalassemia (β-thal) is the most common single gene disorder in Iran. The aim of present study was to find the haplotype background of β thalassemia mutations in Western Iran. Patients and *Design and Methods*. We studied β-globin gene cluster haplotypes in 314 β-thal and 70 βA chromosomes with a Kurd ethnic background from the province of Kermanshah, Iran using PCR-RFLP. β-thal mutations were analyzed using PCR-ARMS, RFLP and direct genomic sequencing. Haplotypes were constructed by analyzing the pattern of seven restriction sites through the β-globin gene cluster. *Results*. Haplotype I was the most prevalent haplotype (35.7%) among β-thal chromosomes followed by haplotype III (28.6%). βA chromosomes similar to β-thal chromosomes were linked to diverse haplotypes but predominantly with haplotype I (42.9%). The predominant IVSII-1 (G'A) mutation in this population (33%) was strongly linked to haplotype III (66.1%) but was also found on chromosomes with haplotypes I, II, V, X and atypical. The second prevalent mutation was CD8/9 +G (13.5%) and showed a strong association with haplotype I (96.4%) and a weak association with haplotype V (3.6%). Haplotype background for Kurdish mutations among our studied population was similar to those among Kurdish Jews and people of Kurdistan of Iran. *Conclusions*. Identification of the most common mutations on different haplotype backgrounds can be explained by a variety of gene conversion and recombination events.

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A NEW INDEX FOR DISCRIMINATION BETWEEN IRON DEFICIENCY ANEMIA AND β-THALASSEMIA MINOR: RESULTS IN 284 PATIENTS

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Discrimination between β-thalassemia trait (β-TT) and iron deficiency anemia (IDA), the two most common causes of microcytic hypochromic anemias, is important. We recently proposed an easily calculable index, MCV - (10 - RBC), for this purpose, and studies have started to evaluate this index. We report our results in 284 carefully selected patients (130 patients with IDA and 154 with β-TT). Sensitivity, specificity and Youden's index were compared between the proposed index and four other indices, namely England-Fraser, Mentzer, Srivastava and RBC count. The new index correctly identified 263 (92.96%) patients, standing inferior only to Mentzer which correctly diagnosed 269 (94.71%) patients. The best discrimination index according to Youden's criteria was Mentzer (Youden's index = 90.1) followed by the new index (Youden's index = 85.5). Larger studies are needed to confirm the results obtained in the present study.

Table.

	IDA (n = 130)	β-TT (n = 154)	Total number of correctly diagnosed patients (n = 284)	Percentage correctly diagnosed (%)
England-Fraser IDA > 0 β-TT < 0	129 1	47 107	236 (129 + 47)	83.09
Mentzer IDA > 13 β-TT < 13	122 8	7 147	269 (122 +147)	94.71
Srivastava IDA > 3.8 β-TT < 3.8	115 15	22 132	247 (115 + 132)	86.97
RBC count (×106) IDA < 5 β-TT > 5	112 18	3 151	263 (112 + 151)	92.61
New index IDA > 15 β-TT < 15	117 13	7 147	264 (117 + 147)	92.96

1255

PHENOTYPIC AND FUNCTIONAL STUDIES OF NATURAL KILLER (NK) CELL SUBSETS IN β-THALASSEMIA MAJOR PATIENTS

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Background, β-thalassemia major are hereditary disease that character-

ized by absence of β-globin chain production. In most patients, multiple blood transfusions and iron overload can induce to immunomodifications in lymphocyte subsets and cytokine levels. NK cells are lymphocyte subpopulations that are important effectors of innate immune responses against infectious pathogens and tumor cells. The cytotoxic activity of NK cells is regulated by positive and negative signals from multiple receptors expressed on their cell surface and cytokines that are secreted from immune cells. *Aims*. The aim of this study was to evaluate factors influencing NK activity in β-thalassemia major patients. *Design and Methods*. This study was carried out to investigate details NK cell function of 27 patients with β-thalassemia and 18 controls. The mean age of patients and controls was 18,48±7,59 and 19,38±4,79, respectively. The female: male ratio was 2:1 for both patients and controls.We analyzed their cytolytic function against K562 cells in both pure NK cells and PBMC. Before and after the experiment, CD16, NKp30, NKp44, NKG2D receptors are examined in $CD56^{dim}$, $CD56^{bright}$ subsets by flow cytometry. The supernatants of NK cells inside PBMC were collected before and after induced K562 and were measured IL2, IL12, IFNγ, TNFα IL10, IL15, TGF-β1 levels by ELISA. *Results*. We observed that β-thalassaemia patients had lower activities of pure NK cells and inside PBMC than controls. While CD16 of $CD56^{dim}$ NK subsets in PMNC was significantly increased in controls after experiment on the contrary it was increased in CD56bright subset of β-thalassemia patients but was not changed in pure NK cells. These differences were lower in β-thalassemia patients than controls. NKG2D receptor was not changed in both groups. While NKp30 receptor of $CD56^{dim}$ subset in pure NK and inside PBMC was decreased in controls after experiment it was only decreased in pure NK cells of β-thalassemia patients and this difference was low compare with controls. NKp44 receptor of $CD56^{bright}$ subset in pure NK cells was significantly increased in β-thalassemia patients after experiment and it was not changed in controls however it was higher than patients as significantly. IL2, IL12, IFNγ, TNFaα levels were not changed but IL10, IL15, TGF- β1 levels were significantly high in patients compare to controls. *Conclusions*. These findings demonstrate that environmental factors such as ineffective cytokine production and defective function of monocytes and NK cells, may cause low NK activity in beta-thalassaemia patients.

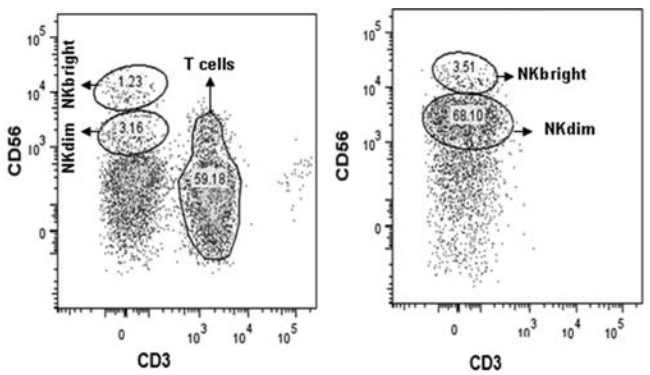

Figure 1. NK subsets in both pure NK cells and PBMC.

1256

PARTICULAR MANIFESTATIONS OF COBALAMINE DEFICIENCY: RETROSPECTIVE ANALYSIS

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Background. Anemia is the common manifestation of cobalamine (Cob)/vit B12 deficiency. However neuropsychiatric disturbance, thrombotic events, gonodal dysfunction may occur as the main or sole symptom in the absence of hematological manifestation. Aims: we analyzed retrospectively patients who were diagnosed in our center with the aim to determine the incidence atypical or no classic presentation of Cbl deficiency and to describe clinical manifestation and their evolution. *Patients and methods*. From 1994 to December 2008,575 were diagnosed, 340 M and 225 F, mean age 57±23 years. Diagnosis was suspected on abnormalities of blood numeration, as decrease of hemoglobin (Hb) and/or macrocytis, megaloblastosis at the myologram and confirmed by low B12 level and/or therapeutic test (IM injection of 1 micg B12, 3days, expect bust of reticulocytes). Homocystemia performed only in some patients. *Results*. among 575 patients, 47 had middle amemia (Hb>10 g /dL) and 13 without anemia (HB>12 g/dL) (2.3%). Forty five (8%) studied had atypical manifestations and hospitalized in neuropsychiatric, cardiologic ophthalmology department. Nine revealed by thrombotic events, peripheral vein thrombosis(3), ischemic stroke(1), myocardial infarction(1), central vein retina thrombosis (CVRT) (1), large vessel thrombosis (1), sever retinopathy in 2 sibling with congenital transcobalamine (TC II) deficiency, mean Hb was 7,6 g/dl(6,1'11,7). Ten patients oriented from neurology for severe peripheral neuropathy with combined degeneration of spinal cord, 4 of them had inadequate intake of folate or B12, average Hb 9(7,1'13,1). We reported 10 cases of young patients (mean age 43 years, 8M/2F), who presented psychotic symptoms: cognitive impairment (4), schizophrenia (2), depression (2), dementia (1), anemia was severe in those patients, Hb 4,4(3,9'5,6). In 3 women diagnosis was oriented by checkup of sterility (2 primary 1 secondary) mean Hb 10 g/dL. Atypical hematological expression reported in 11 patients with severe pancytopenia (Hb:4,3, WBC: 1300/μL, Platelet:19000), and in 3 others had hemolytic expression (enlarged splenomegaly). Biermer's disease was confirmed in 13 patients. All patients received parenteral B12 supplementation; 2 deaths (1 pulmonary embolism, 1 cardiogenic shock), 8 had partially reversible neuropsychiatric manifestations, 1acute blindness after CVRT. Newborn obtained only in 1 patient despite appropriate B12 supplementation. One patient died from gastric carcinoma. *Conclusions*. B12 deficiency can be revealed or complicated by various non hematological events, with risk of mortality and definitive sequels.

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REFRACTORY IDIOPATHIC PURE RED CELL APLASIA (PRCA) DARBEPOETIN DEPENDENT SUCCESSFULLY TREATED WITH ALEMTUZUMAB

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Idiopathic PRCA is a rare immune mediated haematological disease with unpredictable reponse to conventional therapy. Alemtuzumab has been shown to be effective in this condition although the experience is very limited. We report a woman wih refractory idiopathic PRCA darbepoetin dependent successfully treated with alemtuzumab. A 33 year old woman presented with PRCA in 2003. Exhaustive study of underlying causes including PCR for TCRγ gene rearrangement was negative. The patient was treated with prednisone without response and she required 2 units of red blood cells monthly with mean Hb 5-6 g/dl prior to transfusion. She received treatment with cyclosporine and antithymocyte globulin simultaneously with subcutaneus moderate-high doses of β epoetin without response. In 2005 a second course of ATG was administered and β epoetin was changed by darbepoetin with effective response but with recurrence of severe anaemia when tapering it, requiring the patient continuous every two weeks subcutaneous darbepoetin administration to mantain Hb about 10 g/dl. In June 2006 she received rituximab (375 mg/m^2 weekly x4) but darbepoetin coudn´t be discontinued. In April 2008 subcutaneous alemtuzumab was administered, to a total dose of 230 mg, and darbepoetin was given up with stable complete remission. In this patient the PRCA was not induced by erythropoiesis-stimulating agents, in contrast she had a very good reponse to subcutaneous darbepoetin. But in spite of inmunosuppressive treatments she became darbepoetin dependent and finally, after being treated with alentuzumab the darbepoetin could be discontinued. We conclude that alentuzumab is an effective treatment for PRCA and, should be considered as an option to treat refractoy patients.

1258

SIGNIFICANCE OF SERUM LEVELS OF LEPTIN IN PATIENTS WITH β THALASSEMIA INTERMEDIA AND SICKLE CELL ANEMIA. CORRELATION WITH BODY MASS INDEX (BMI) AND IRON METABOLISM

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The leptin is a protein of 167 amino acids transcribed by the gene 'ob' located on chromosome 7q31.It is a hormone produced by adipocytes. This protein acts as a signal molecule in the hypothalamus producing variable effects on appetite, on energy expenditure and on neuroendocrine axes. These different metabolic effects involve specif-

ic receptors located on the central nervous system and on the peripheral tissues. Such receptors belong to Class I cytokine receptors, to interferon receptor and to growth hormone receptor. Leptin receptors, expressed in the hypothalamus, have been recently identified. The leptin is able to overcome the blood-brain barrier through a saturable transport system. It was reported that the leptin-deficient mice have a complex phenotype including hypogonadism, delayed sexual maturity, and infertility due to the lack of hypothalamus-pituitary hormones. The exogenous administration of leptin will allow the recovery of reproduction. Experimental studies have shown that iron is well represented in adipocytes of the subcutaneous tissue and that iron ions can inhibit the activity of adipocytes. Based on this background, we conducted the research on the serum levels of leptin and correlates it with body-mass index (BMI) and iron metabolism in patients with β-thalassemia intermedia and sickle cell anemia (HbS) in order to verify whether it can occur a dysfunction of adipose tissue as a further endocrine disease. Our results demonstrate that even in patients with β-Th intermedia, serum levels of leptin are lower compared to healthy subjects as control. The mild high level of serum iron and the moderate iron overload present in patients with -Th-intermedia, all under regular transfusion and iron chelation therapy, are the major determinants of reduced leptinemia for altered activity of the adipocytes in the subcutaneous tissue and bone marrow mediate by iron ions. With regard to the BMI, we do not found changes in patients with β-Th-intermedia as compared to control groups. The male patients with β-Th intermedia show leptinemia values markedly reduced when compared to those of women with β-Th-intermedia and even more compared to healthy subjects with the same BMI. This finding confirms the data reported in the literature that serum concentrations of leptin are higher in women than men for the same BMI, percentage of body fat and age. It has in fact demonstrated *in vivo* the ability of estrogen, to increase the production of leptin, unlike testosterone, which inhibits the gene expression and the secretion of the adipose tissue. The increase in iron serum level and ferritin even if mild in patients with β-Th-intermedia would be involved in reduced secretion of leptin by the adipocyte for the inhibitory effect of iron ions on the activity of the adipocytes. Finally considering the endocrine mechanism of action of leptin, the present study on the levels of circulating leptin in patients with β-Th intermediate and βTh/HbS, shows that even a disfunction in the adipose tissue occur, as further endocrine disease, in these congenic hemolytic anemias.

1259

NATURAL KILLER ACTIVITY AND EFFECT OF IL-2 IN PATIENTS WITH β-THALASSEMIA MAJOR

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Background. A depressed level of natural killer (NK) activity is one of the various immunological abnormalities in patients with thalassemia major. NK cells play role in controlling infections and malignancy. Defective NK cell functions can be partially restored *in vitro* by interleukin IL-2. *Aim*. Aims of this study evaluate which factors effect on NK cytotoxicity and modulation of the defective natural killer activity. *Patients and Design and Methods*. The study includes 26 patients with thalassemia major (TM) with age ranged 7-35 (median age was 18, 17 of them were female and 9 male), and sex and and age matched 16 normal healthy subjects as controls. Fourteen of the patients were splenectomized. Patients regulary chelated with deferasirox (n:16), deferipron (n: 8) and desferroxamin (n: 2). NK cells were isolated from PBMC of subjects by using RosetteSep. The cytotoxic activity of NK cells and IL2-activated Killer cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We analyzed their cytolytic function against K562 cells. *Results*. NK cytotoxicity was found to be lower in patients with thalassemia major ($p<0.015$) when compared to controls. We have previously shown that thalassemia children have had decreased NK cells. Patients with above 18 years old had significantly lower NK activity than below 18 years old. Sex, presence of splenectomy and chelation type did not effect on NK activity (p>0.05). NK cytotoxicity significantly increased by IL-2 stimulation in 26 patients ($p<0.001$). *Conclusions*. Lymphokine activated killer cells activity was significantly higher in the patients. According to these results, IL-2 may have potential therapeutic effect to improve the defective NK activity in patients with thalassaemia major patients.

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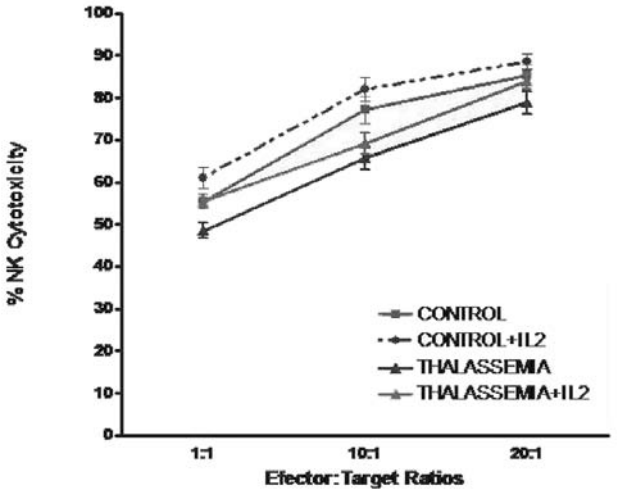

Figure 1. IL2-activated NK cells activity in TM patients

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SUCCESSFUL TREATMENT WITH THE COMPLEMENT INHIBITOR ECULIZUMAB OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)-ASSOCIATED BUDD-CHIARI SYNDROME

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Background. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disease caused by a mutation of the X-chromosome gene PIG-A, characterized by severe hemolytic anemia, peripheral blood cytopenia and an an increased risk of severe venous thrombosis, particularly in cerebral, hepatic, portal, mesenteric, splenic and renal veins. Budd-Chiari syndrome, a condition characterized by intrahepatic vein thrombosis with pronounced ascites and hepatomegaly, is one of the most serious and life-threatening thrombotic complications caused by PNH. Budd- Chiari syndrome can be treated by medical (thrombolysis, anticoagulation) and surgical (angioplasty, shunting) therapy, and may require liver transplantation (LT) in refractory cases, but thrombosis may recur after LT, especially if the underlying predisposing condition (PNH) is still active. Eculizumab, an anti-complement antibody targeting the C5 complement component, has proven effective not only in counteracting intravascular hemolysis, but also in preventing thrombotic complications. *Aims*. On the basis of these data, we administered eculizumab to a PNH patient with persistent symptoms of portal hypertension, caused by a previous Budd-Chiari syndrome, and with transfusion-dependent anemia. *Design and Methods*. The patient, a 40 yr-old male, was affected by PNH since 1995, and was receiving packed red cell transfusions (2-4 units/month) since 1999, because of severe hemolytic anemia, refractory to medical treatment (iron, folic acid and erythropoietin). On december 2007 he was admitted to hospital because of right upper quadrant abdominal pain, ascites and hepatomegaly: a diagnosis of Budd-Chiari syndrome was made, on the basis of Doppler ultrasound (US) and computer tomography scan of the abdomen, showing thrombosis of the hepatic veins (right, median and left), and enlargement of left and caudate lobe and splenomegaly. He was treated with anticoagulation, and came to our observation on February 2008. At that time persistent clinical signs ad symptoms of portal hypertension were present, in association with severe transfusion-dependent hemolytic anemia. Blood laboratory data were: hemoglobin (Hb): 8.8 g/dL (transfused), leukocytes: $6.5x10^9$/L, platelets: $164x10^9$/L, total bilirubin: 9.4 mg/dL, conjugated bilirubin: 5.0 mg/dL, LDH: 6.000 U/L, PNH clone (granulocytes): 84%.

Eculizumab treatment was started on april 2008, following the approved dosing schedule: 600 mg every week for the 1st 4 weeks, followed 1 week later by 900 mg, and then by a maintenance dose of 900 mg every 2 weeks. *Results*. Immediately after the 1st dose of therapy the patient experienced a brisk improvement of well-being and a decrease of urine hyperpigmentation and of LDH level (1130 U/L after 2 weeks, 443 U/L on January 2009). The transfusional need progressively decreased, and on july 2008 completely ceased. On may 2008 Doppler US documented a complete resolution of ascites and full restoration of previously impaired hepatic veins (median, left and right) flux. The patient is still maintaining a satisfactory clinical condition, under eculizumab therapy. *Conclusions*. To our knowledge, a complete recovery (clinical and echografic) of PNH-associated Budd-Chiari syndrome after eculizumab has not been reported previously. Successful treatment of PNH with eculizumab may therefore be followed by resolution of late consequences of this dangerous thrombotic complication.

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SUCCESFUL RESPONSE TO AUTOLOGOUS STEM CELL TRANSPLANTATION IN A PATIENT AFFECTED BY PRIMARY ACQUIRED PURE RED CELL APLASIA

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Background. Primary Acquired Pure Red Cell Aplasia (PAPRCA) is a rare disease characterized by anemia, reticulocytopenia and erythroid aplasia in bone marrow. This disease seems to be mediated by an inmunological mechanism. Thus, inmunosuppressive therapy has been widely used and generally induces hematologic responses. Despite this, about 30% of patients relapse and almost 25% are refractory. For these patients, allogenic stem cell transplantation has been ocassionally reported with some succes, but this treatment approach is associated with considerable morbidity and mortality. Interestingly, autologous stem cell transplantation (ASCT) has been reported in the literature in six cases, with succesful and durable response in only one case. *Aims*. Our objective is to report a case of PAPRCA refractory to different lines of inmunosuppressive/cytotoxic therapies who achieve a success and durable hematologic response after ASCT. *Design and Methods*. A 32-year old man, black race, was diagnosed as having PAPRCA in July 2006. At diagnosis, the hemoglobin was 7.3 g/dL; hematocrit 21.2%, reticulocytes 0.34%. Platelet and white blood cell counts were in normal range. The initial aproach was transfussion support with Red Blood Cell (RBC) units and inmunosuppressive therapy with high dose steroids. Different therapies were applied, including cyclosporine A, cyclophosphamide, azathioprine, anti-CD20 monoclonal antibody and anti-interleukin2 receptor antibody (Daclizumab). Other treatments used were danazol, subcutaneous erythropoietin and intravenous inmunoglobulins. Besides all of that, the patient did not reach hematological response and became transfussion dependent (two units of RBC every two weeks). Oral quelation therapy was started. There was no HLA-matched related donor available and ASCT was decided. Peripheral blood stem cell mobilization was acomplished with subcutaneous granulocyte-colony stimulating factor (10 ug/kg/day, 5 days). $CD34^+$ cells collected were $5.2x10^6$/Kg. Conditioning regimen consisted of intravenous cyclophosphamide 50 mg/Kg/day (days -6 through -3) and intravenous rabbit tymoglobulin 6 mg/kg/day (days -5 through -3). Complications post-ASCT were: grade 1 oral mucositis, asymptomatic synus bradicardia and one non-neutropenic febrile episode. Time to reach an ANC of 500 cells/mm3 was 11 days. Before ASCT106 RBC units were transfussed. After transplant procedure, patient only received four RBC units, all of them during the first two weeks post-ASCT. Since day +38, hematological response was observed and it is maintened and durable during 10 months of follow up. Last complete blood cells count is: hemoglobin 14 g/dL, reticulocytes 1.29%, platelets 162.000/mm^3, white blood cells 6.000/mm^3. *Conclusions*. In this case, ASCT was a feasible and safety procedure in a patient refractory to several inmunosuppressive/cytotoxic therapies. Furthermore, our patient achieved complete and durable (10 months of follow up) hematological response. This case is the second report of a successful result for ASCT in PAPRCA.

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GINKGO BILOBA EXTRACT DECREASES TERT-BUTYL HYDROPEROXIDE INDUCED HEMOLYSIS IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT ERYTHROCYTES

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Background. Glucose-6-phosphate dehydrogenase deficiency (G6PD) is the most common form of enzymopathy of red blood cells that affected more than 400 million people worldwide. G6PD deficient erythrocytes show decreased level of antioxidant capacity, therefore; they are more susceptible to oxidative stress and this predisposes them to chemical induced hemolysis following ingestion of Fava beans and drugs such as antimalarial drugs, antipyretics, and analgesics. Antioxidants play a pivotal role in the prevention of oxidative damage to the erythrocyte membrane. *Aim*. Since the incidence of G6PD deficiency is relatively high in our country, IRAN, and considering to the serious complications of G6PD deficiency and the debate on the curative effect of the investigated antioxidants, in this study, we evaluate the effect of Ginkgo biloba extract (EGb), a potent water-soluble antioxidant. *Design and Methods*. To assess the property of EGb to prevent hemolysis in G6PD deficient erythrocytes, tert-butyl hydroperoxide (t-BHP), an oxidant, was used to induce hemolysis. Different concentrations of EGb (100, 200, 300, 400 µg/mL) were used in this experiment. First, Aliquots of RBC suspensions of known G6PD deficient patients were challenged with t-BHP in the presence and absence of EGb and then the percent of hemolysis were calculated. Results: According to our data, EGb significantly decreased the percent of hemolysis at all investigated concentrations, compared to control group.This makes EGb good candidate for further *in vitro* and *in vivo* studies. *Conclusions*. We propose that EGb may decrease chemical induced hemolysis and the following complications in G6PD deficient patients .EGb exerts its effect through several mechanisms such as its antioxidant property, its increasing effect on poly unsaturated fatty acids (PUFAs), and finally by up regulating heme oxygenase-1 gene expression and activity.

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IRON DEFICIENCY ANEMIA AND LATENT IRON DEFICIENCY AMONG YOUNG FEMALE MEDICAL STUDENTS

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Background. Iron deficiency anemia has worldwide prevalence. Iron deficiency anemia has 5% prevalence in the world, but in developing countries it is 18% among adult women and 10% in adult men. The serum ferritin level correlate with total body iron stores, thus the serum ferritin is the most accurate laboratory parameter to estimate iron stores. Decreasing serum ferritin levels are regarded to be an important laboratory sign for negative balance although the hemoglobin may remain normal. Aims To study the frequency of Hypoferritinemia and iron deficiency anemia in fifth year female medical student according to eating habits and menstruation cycle. *Design and Methods*. 472 young normal female medical students were in fifth year of Medicine. Stratified random sample (sampling error=0.07) comprised 109 students were included in the study. A full blood count, serum iron concentration, Transferrin saturation and serum ferritin were measured. Iron deficiency anemia was diagnosed if hemoglobin <11.5 g/dL, Ferritin <15 ng/mL and saturation index <16%. Latent iron deficiency have ferritin <15 ng/mL and saturation index <16%. Exclusion criteria were pregnancy and iron therapy before and during study period and history of malabsorbation or congenital anemia. Accordingly 6 students were excluded. *Results*. 103 female students were studied. Mean age was 22.6 years, age range from 21-26 years. Only 41.7% of students were found to have normal ferritin. Iron deficiency anemia diagnosed in (12/103)11.7%. (48/103)46.6% have low ferritin level. Out of these students (21/48) 43.8%have normal saturation index, and 27/48 (56.2%) have low saturation index <16%. *Conclusions*. Iron deficiency anemia is common among female medical students, and latent iron deficiency is more common affecting more than 46% of female medical student.

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MYOCARDIAL VELOCITY AND SPEED DATA IN PATIENTS WITH HEMOLYTIC ANEMIA

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Background. Some cardiac complications have been reported in patients with some hemolytic anemias such as thalassemia and sickle cell anemia. Conventional two dimensional and Doppler echocardiography including tissue Doppler imaging have been used for detecting cardiac involvement in hemolytic anemia. However, angle dependency is a main limitation for Doppler-based echocardiographic techniques. Recently, speckle tracking on two-dimensional images has been introduced to clinical use with which myocardial velocities can also be measured without angle dependency. *Aims*. We aimed in this study to determine left ventricular myocardial velocities in apical and short axis views by using relatively new software (QLAB version 6.0) in patients with hemolytic anemia and healthy subjects. *Design and Methods*. Twelve male patients with hemolytic anemia (mean age: 22.3±1.2 years) and twelve healthy male subjects (23.1±1.5 years) were included in the study. Transthoracic echocardiography was performed with an I33 machine equipped with S5-1 transducer (Philips, USA). In addition to short axis images in mitral valve level, we obtained apical four and two chamber images. All recorded images were transferred to DVD media and evaluated with QLAB software (version 6.0) with six segment model in each view. Resulting data including speed and velocity data were exported to a dedicated excel spreadsheet for further analysis. Maximum speed, maximum velocity (radial in all planes) and percent time of these events were calculated. We used SPSS 15.0 for statistical analysis. Continuous data showing normal distribution was compared with Student t test, otherwise, Mann Whitney U test was used. *Results*. Myocardial speed and velocity data are presented in Table 1. The patient group had significantly higher myocardial maximum speed (5.1±1.1 vs 3.8±1.1 cm/sec, $p<0.001$) and radial velocity (4.0±0.8 vs. 2.5±0.2, $p<0.001$) than control subjects at short axis plane. Moreover, maximum speed time percent was significantly lower in the patients compared to the controls at short axis plane (40.6±21.3 vs. 58.9±11.9, $p=0.049$). Conversely, time percent of maximum speed was significantly longer in the patient group than the control group (Table 1). *Conclusions*. Increased myocardial speed and radial velocity in only short axis may reflect altered left ventricular mechanics in patients with hemolytic anemia. We thought that increased time for reaching maximum speed or velocity at short axis plane also points to possible alterations in left ventricular mechanics.

Table 1.

	Maximum Speed (cm/sec)			Maximum Velocity (cm/sec			Maximum speed time percent (%)			Maximum velocity time percent (%)		
	Patient	Control	P value	Patient	Control	P value	Patient	Control	P value	Patient	Control	P value
SAX at mitral valve level	5.1±1.1	3.8±1.1	**<0.001**	4.0±0.8	2.5±0.2	**<0.001**	40.6±21.3	58.9±11.9	**0.049***	17.8±4.3	18.6±6.4	0.86*
Apical 4ch	7.1±1.6	6.0±1.9	0.18*	2.8±0.6	2.4±0.6	0.12	59.7±6.7	50.2±13.9	**0.03***	21.8±5.7	16.5±6.3	0.09
Apical 2ch	6.7±1.1	6.0±2.0	0.46	2.9±0.6	2.5±0.3	0.11	62.8±4.9	56.7±7.4	0.10*	24.5±6.6	19.9±7.8	0.18*

*Manny Whiney U test

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MALIGNANT LYMPHOMA AND ACUTE LYMPHOBLASTIC LEUKEMIA AFTER APLASTIC ANEMIA: TWO CASE REPORTS AND REVIEW OF JAPANESE LITERATURE. IS IT UNDERESTIMATED?

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Background. Aplastic anemia (AA) is characterized by diminished or absent hematopoietic precursors in the bone marrow, most often due to injury to the pluripotent stem cell. There is an appreciable risk of progression to a clonal disorder (myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), following treatment for AA. However; lymphoid clonal diseases like malignant lymphoma (ML) and acute lymphoblastic lymphoma (ALL) are very rare. We have two cases of malignant lymphoma after AA. We've already reported one case (Acta hematologica, in press). *Aim.* We conduct this study to clarify the type of lymphoid clonal disease, cause of the lymphoid malignancy, and duration after AA therapy. *Design and Methods*. We show our two cases. And we conducted literature review by Pubmed and Japanese Centra Revuo Medicina. *Results*. Case1-1: 61 years old Japanese man suffered from Non-Hodgkin's Lymphoma 24 month after Antithymocyteglobulin (ATG) and CyclosporinA (CyA) therapy for AA. Case 1-2(same with 1-1): 66 years old Japanese man developed NHL of left thigh four months after the second ATG/CyA. He needs to take chemotherapy and radiation (RT). Case 2: 67 years old Japanese man developed chronic lymphocytic lymphoma (CLL) twenty years after methyl predonisolone(mPSL) therapy for AA. We also show the result of literature review (Table). Sixteen patients are reported. All B-NHL patients developed lesion in extranodal site (GI tract 3, tonsil 1, skin 1, soft tissue 1). Eight out of sixteen patients had history of using CyA, five cases had history of using ATG, and four cases had history of using mPSL. The median duration until onset of ML and LL from AA treatment was 16(1.5-120) month. *Conclusions*. We speculated about the onset of ML and ALL. 1) Small malignant clone may have existed at the onset of aplastic anemia. 2) Certain virus like EB virus may've been activated by ATG or CyA, and it caused the ML. 3) It was just an accident. As far as we searched, 1) and 2) are suspected to be the main reason of these ML and ALL. Although, they are very rare complication for aplastic anemia, it may be serious and fatal.

Table.

Lymphoma and acute lymphoblastic leukemia after aplastic anemia: Japanese case reports

Case(case)	16
Age(y.o)	58(3-91)
Gender(M:F)	11 : 5
Duration(M)	
AA->L/L	42(1.5-360)
treatment of AA->L/L	16(1.5-120)
Type (case)	
ALL	6
B	5
T	1
Lymphoma	11
HL	1
NHL	
B	5
T	2
?	3
Resion of NHL (except for two CLL)	
Extranodal	7
Nodal	1

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ETIOLOGY OF COBALAMIN DEFICIENCY IN TUNISIANS PATIENTS

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Background. Vitamin B12 or cobalamin deficiency occurs frequently (> 20%) among elderly people, but it is often unrecognized because the clinical manifestations may be subtle. They are also potentially serious (neuropsychiatric and hematological perspective). Causes of the deficiency include, most frequently, food cobalamin malabsorption syndrome (>60% of all cases), pernicious anemia (20% of all cases), insufficient dietary intake (<5% of all cases) and other malabsorption. Aims: The aim of this work was to establish the etiology of cobalamin B12 deficiency. *Materials*. Data were collected prospectively in 70 Tunisians patients with established cobalamin deficiency. An exhaustive exploration was conducted in this cohort of patients (intrinsic factor and gastric parietal cells antibodies, digestive endoscopy, and dietary intake). *Results*. The median patient age was 52,9 years; 40 patients were women. The most common clinical manifestations were physical asthenia (88, 5%), glossitis (82,8%), neurologic (paresthesia (55,7%), sensory polyneuropathy (21.4%), confusion (7.1%)). Hematologic abnormalities were reported in all patients: anemia (98.5%), leucopenia (40%), thrombopenia (44,3%), and pancytopenia (27.1%). The mean hemoglobin level was 7.13 g/dL and the mean erythrocyte cell volume 111.17 fl. All patients had low serum vitamin B12 levels (<180 pg/mL), and a mean total serum homocysteine level of 65, 74 µmol/l. Intrinsic factor antibodies were detected in 32 patients (45.7%), and gastric parietal cells antibodies in 59 patients (84, 2%). Atrophic gastritis was described in 51/53 patients (96.2%). The etiology of cobalamin deficiency included pernicious anemia in 57 patients (81.4%), Food cobalamin malabsorption in 9 patients (12.8%) and nutritional deficiency in 3 patients (4.3%) and undetermined in one patient (1.4%). Correction of the serum vita-

min B12 levels and hematologic abnormalities was achieved in patients treated with intramuscular cyanocobalamin. *Conclusions*. This study suggests that etiologies of cobalamin deficiency are multiple. They are dominated by pernicious anemia in our patients contrarily to the literature.

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COMPLETE HAEMATOLOGICAL RESPONSE AFTER LOW DOSE RITUXIMAB IN A PATIENT WITH REFRACTORY WARM-TYPE AUTOIMMUNE HAEMOLYTIC ANAEMIA

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Background. The use of rituximab administered with the same schedule used for treatment of B-cell non Hodgkin's lymphomas (375 mg/sqm weekly for 4 weeks) has been extended to the management of autoimmune cytopenias, such as the idiopathic thrombocytopenic purpura (ITP), the cold agglutinin disease and the warm-type autoimmune haemolytic anaemia (AIHA). The optimal schedule has not been established yet and standard dose might be an overtreatment. Low dose rituximab (100 mg weekly for 4 weeks) has been recently tested in patients with ITP and in a case with AIHA. In that latter case, a partial response was reported but no further details on degree and duration of response has been described. *Aims*. We report here the effects of low dose rituximab (LD-R) in a patient with relapsed/refractory AIHA. The absence of an underlying lymphoproliferative disease and the purpose to reduce the immunosuppressive effects of rituximab were the reasons of the use of LD-R. *Design and Methods*. LD-R (100 mg weekly for 4 weeks) was administered in a 78-year old man with a long lasting and symptomatic IgG warm-type idiopathic AIHA who was ineligible to splenectomy and resistant to steroids, immunosuppressive agents and high-dose intravenous immunoglobulins. The haematological and laboratory data before rituximab therapy were the following: haemoglobin (Hb) 8,9 g/dl (supported with 4 packed red-cell transfusions in the previous 2 months), lactate dehydrogenase (LDH) 250 U/L, indirect bilirubin 3,8 mg/dl, haptoglobin 0 mg/dL, reticulocyte count 84". According to the criteria reported by D'Arena *et al.*, complete remission (CR) was defined as stable Hb level > 12 g/dL, transfusion independence and absence of clinical and laboratory signs of haemolysis for at least 4 weeks after rituximab treatment, irrespective of direct antiglobulin test positivity. *Results*. CR was rapidly achieved at the 6th week and maintained until the 29th week. After 6 months of CR, AIHA relapsed (Hb 8,3 gr/dL, LDH 170 U/L, indirect bilirubin 2,27 mg/dl, haptoglobin 24 mg/dl and reticulocyte count 35") and a second course of LD-R (100 mg weekly for 4 weeks) was administered. A second CR was documented after 15 weeks and maintained until 24th week. After 6 months from the second course, a maintenance therapy with 100 mg of rituximab every 2 months was started and up to now (17 months from the first course of therapy and 11 months from the second course) the patient is still in CR. A deep B-cell depletion was documented during all the period of the first and second course of treatment but neither infections nor other toxicities were observed. *Conclusions.* Our report suggests that LD-R may induce a CR in refractory/relapsed warm AIHA as well as the standard dose. A new CR can be obtained in case of relapse. In our case, we observed a slower time of response than the one observed during the first course (6th vs 15th week). Considering the efficacy and the good safety profile, LD-R could be tested in a larger series of patients with warm AIHA.

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PRODUCTION OF ENDOGENOUS ERYTHROPOIETIN AND EXPRESSION OF RECEPTORS TO ERYTHROPOIETIN AT THE PATIENTS WITH LYMPHOPROLIFERATIVE DISEASES WITH THE ANAEMIC SYNDROM

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Background. Erythropoietin (EPO) plays a key role in the adaptation of erythropoiesis to metabolic requirements for oxygen. Inadequate production EPO is one of the reasons of development of an anaemia at patients with haemoblastosis and solid tumors. Aims. To investigate level of erythropoietin of blood whey (s-EPO), receptors to EPO on a surface of erythrokariocytes of a bone marrow and to estimate efficiency of use of recombinant erythropoietin (rch-EpO) at patients with lymphoproliferative diseases and anaemia. *Design and Methods*. It is surveyed 109 patients with lymphoproliferative diseases and anaemia (indolent and aggressive lymphomas, Hodgkin,s disease and plural myeloma). Level of serum erythropoietin (s-EPO) was investigated at all patients. Level of an expression of receptors to erythropoietin has been researched on a surface erythropoietin - sensory cells of a bone marrow for 23 persons. *Results*. The average level s-EPO has made 46.8±8,16 mIU/mL at patients with indolent lymphomas. It is reduced (16.8±2.55 mIU/mL) at 24 person (60%). The average level s-EPO has made 36.9±7.97 mIU/mL at patients with aggressive lymphomas. It is decreased (16.4±2,35 mIU/mL) at 31 patients (79.5%). The average level s-EPO has made 30.6±8,07 mIU/mL at patients with Hodgkin,s disease, is reduced (20.9±2.44 mIU/mL) at 14 person (82,3%). The average level s-EPO has made 38.8±12.42 mIU/mL at patients with plural myeloma, is decreased (17.6±3.91 mIU/mL) at 8 patients (61,5%). Thus, EPO production was inadequate at 70,7% of the surveyed patients in all groups of patients. Average quantity of EPO-R-positive (EPO-R +) cells 22.2±4.23% in a bone marrow. Average quantity of erythrokariocytes has made 24.1±4.06% in a bone marrow. Quantity of EPO-R-positive cells (26.4±4.81%) corresponded to quantity of erythrokariocytes in a bone marrow (26.4±4.75%) only at 73,9% of patients. Quantity of EPO-R-positive cells (10.2±3.93%) was much lower than quantity of erythrokariocytes in a bone marrow (17.7±4.26%) at 26.1% of the surveyed patients (p<0.0001). *Conclusions*. Thus, the received data specify a significant role inadequate production of erythropoietin in pathogenesis of anaemias at patients with lymphomas. Reduction expression of receptors to erythropoietin on surface erythroid cells of a bone marrow is one of the reasons of development of anaemia at a part of patients with lymphomas and anaemic syndrome. It can cause resistance of erythrokariocytes a bone marrow to endogenous erythropoietin and inefficiencies of therapy by preparations recombinant human erythropoietin.

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ACQUIRED PURE RED CELL APLASIA (APRC) IN CHURG-STRAUSS SYNDROME; REVERSAL WITH CORTICOSTEROID THERAPY.

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We report a case of 17 years old woman, with known β thalassemia minor evaluated for severe anemia: 5.2 g%. She presented with prolonged febrile syndrome, recurrent Quincke edema, lymph node enlargement, splenomegaly, recurrent dysphonia, papillomatous laryngeal lesions; CT imaging shows alveolar infiltration, mediastinal and abdominal lymph nodes enlargement, normal Thymus and hepatosplenomegaly. Biologically associated to anemia which has the characteristics of β thalassemia we observe low serum iron, neutrophilia, lymphopenia with severely low number of CD 4 lymphocytes, negative HIV serology, thrombocytosis, severe reticulocytopenia (0%); we consider necessary IHC and histopathological examination of the lymph node indicating reagent and HP and IHC examination of papillomatous laryngeal lesions showing granulomatous lesions and perivascular inflammatory infiltrated. The immunologic evaluation results indicate anti pANCA antibody and APL / LA antibody mostly positive, maximal IgE concentration, C1q inhibitor with normal values. The bone morrow was hypercellular, with a small numbers of dysmorphic vacuolated proerythroblasts (1.5%), increased numbers of plasmocytes (18%), aggregated and diffusely scattered, increased granulopoiesis and megakariocytopoiesis. Data presented support the diagnosis of Churg-Strauss syndrome complicated with pure red cell aplasia; It initiates corticosteroid therapy: Solumedrol pulse therapy with 3g spout corticosteroid therapy followed by 60 mg / day; revaluation at 10 and 21 days indicate reticulocytes 15%, correction of anemia, inflammation regression with normal biological VSH, PCR, fibrinogen, disappearance of symptoms. We highlight that before diagnosis the patient had been in the care of infectious diseases, pneumology clinics etc. *Conclusions*. Along with another malignant lymphomas and hematologic malignancies, thymoma, nonhematologic solid tumors, systemic lupus erythematosus, rheumatoid arthritis, infections, intoxication, severe kidney failure, hemolytic anemia we can state that Churg-Strauss syndrome is a potential cause of APRC.

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HYDROXYUREA-INDUCED HEMATOLOGICAL RESPONSE IN TRANSFUSION-INDEPENDENT β-THALASSEMIA INTERMEDIA

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The efficacy of hydroxyurea (HU) in β-thalassemia intermedia (TI) is unclear. We treated 16 transfusion-independent TI patients (8 males,8 female) aged 10.7±5.0 years with HU, 20 mg/kg/day four days per week, for 6 months. Treatment was well tolerated, there was a significant increase in both Hb and HbF (p<0.001), and the increments were strongly correlated (r=0.94; p<0.001). XmnI polymorphism was not correlated with hematological response. Hb (p=0.026) and HbF (p=0.046) showed a more significant rise in patients with a Fr8/9 allele than those with one or two IVS-II-1 alleles.

Table 1. Hematological response to HU in TI patients.

Study	No.	Response	Comments
Ehsani *et al.*, 2008	16	Hb: rise 1.6 g/dL HbF: rise 1.7 g/dL	- Duration of treatment: 6 months- No correlation between response and Xmnl polymorphism- Fr8/9 allele predicted good response- No correlation between response and age or sex- Hb rise and HbF rise strongly correlated
Koren *et al.*, 2008	7	Hb: rise 2 g/dL	- Mean duration of treatment: 46 months - Xmnl polymorphism predicted good response - No correlation between response and beta mutation
Bradai *et al.*, 2007	9	Hb: rise 3.7 g/dL	- Duration of treatment: 1year- Xmnl polymorphism predicted good response- No correlation between response and beta mutation
Dixit *et al.*, 2005	37	Hb: rise > 2 g/dL in 17 patients; 1-2 g/dL in 9 patients	-No correlation between response and beta mutation - No correlation between response and Xmnl polymorphism - Older age and low baseline HbF predicted poor response
Karimi *et al.*, 2004	16 13	Hb: rise ~1 g/dL HbF: rise ~1.5 g/dL	- Duration of treatment: 1 year
Hoppe *et al.*, 1999	5	Hb: rise 2.2 g/dL HbF: rise 2.6 g/dL	- No correlation between Hb rise and HbF rise

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ANAEMIA AND SERUM ERITROPOETIN LEVELS IN THE INFLAMMATORY BOWEL DISEASES

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Background and Aim. In chronic diseases, anaemia is a seriuos problem. Inflammatory bowel disease (IBD) which is involving the gastroentestinal system primarly,is also a . Chronic disease but the etyology is not known yet. In IBD, refracter anaemia affects the prognosis and the quality of patient's life negatively. The main reason of refracter anaemia in chronic inflammation, is the inhibitor effect of proinflammatuar citokines on erythropoetin (EPO) which is a growth hormon, control the erythropoesis. Erythropoesis is a process which is controled by the end point feedback mechanism between hemoglobin and the erythropoetin levels. In this study, as IBD is a chronic inflammatuar ,we searched the reason of chronic anemia in these group of patients and try to examine the role of supressed EPO although there is anaemia. *Material and Method*. 145 patients (55 patients with ulcerative colitis (UC), 51with Crohn's disease (CD) and 39 with gastrointestinal bleeding-GIB) and 16 healty controls were included in the study. UC was diagnosed with clinic findings, endoscopic processes and laboratory findings. Patients and controls both gave permission for the study. 11 gr/dL of hemoglobin level was accepted for the anaemia in women with UC and CD and 12 gr/dl for men. The anaemia in patients were grouped as ıron deficiency and chronic disease anaemia. All the blood sample were taken from brachial vein after 12 hours hungry. Hemogram parameters were studied in automatic blood counters(in coulter LH 750); serum ıron, iron binding capacity and ferritin levels were measured in autoanalizators (Roche System, Germany).Eritropoetin levels were studied with ELSA (Bender Med System,Viana, Australia). *Results*. The reverse corelation between Hb and Log EPO was found in CD with IDA and GIB group. But this corelation was not found in CD with chronic disease anaemia that showed there was no answer for EPO. n groups which UC and CD patients were divided according to the activation and remission, no difference was found in Hb and EPO levels between two groups but high EPO levels were found in CD patients who were in remission. These results showed that when remission was occured ,the inhibitor effect of citokins on EPO was disappered and EPO levels began to raise. When the UC patients were grouped according to the localization of the disease, only reverse correlation between Hb and Log EPO was found in women patients with pancolit . The 85 percent of these women were under 50 years old and during the premenapose period. The 26 percent of this group women was diagnosed as IDA and 13 percent as CDA. *Conclusions.* When the anaemia in patients with IBD was investigated, we found that there was an inhibition on Hb levels which caused the CDA. But ın patients with UC, this result was not seen so more investigations must done on this subject.

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INVESTIGATION OF ANEMIA BASED ON MCV AND MCH IN CHILDREN

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Background. Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) constitute useful erythrocytic indices of significant clinical value. MCV and MCH obtained by hematological analyzers therefore are the first step in the investigation of anemia. Based upon MCV and MCH, anemia is divided into micro-, normo- and macrocytic anemia, as well as into hypochromic and normochromic anemia. *Aims*. The aim of the study is to register and investigate children with anemia based on MCV and MCH. *Design and Methods*. Complete Blood Counts (CBCs) of healthy children aged 2-12 that attended our department for regular check up during 2007-2008 were studied. SYSMEX XE-2100 was the hematological analyzer in use, that provided Hemoglobin (Hb), Hematocrit (Hct), MCV and MCH. *Results*. Among 2758 CBCs, 2113 were normal concerning anemia. Hb was higher than 11,5g/dl and Hct was higher than 34-35%. 645 counts were ubnormal, presenting anemia with Hb<11,5g/dl and Hct<34-35%. Among these 645 children with low Hb and Hct, 320 were aged 2-6 years and the remaining 325 were 6-12. In the 2-6 age group 183 children were boys and 137 were girls. Among children with 6-12 years of age 154 were boys and the rest 171 were girls. The frequency of micro-, normo- and macrocytosis as well as normo- and hypochromia according to sex and age are displayed at the table below. *Conclusions*. The classic values given by all hematologic analyzers are useful, easy to obtain and constitute the first step in the algorithm of anemia's investigation. In our study most common type of anemia in 2-12 years old children is hypochromic microcytic anemia, which in Greece is usually due to iron deficiency or Thalassaimia's syndromes. Macrocytic anemia on the grounds of hypothrepsia is extraordinary.

Table.

CHILDREN WITH ANEMIA		2-6 YEARS Hb<11,5g/dl and Hct<34%		6-12 YEARS Hb< 11,5g/dl and Hct<35%	
MCV (fl)	MCH(pg)	MALES	FEMALES	MALES	FEMALES
<80	27-32	14	2	2	1
<80	<27	137	104	130	136
80-100	27-32	20	28	22	35
80-100	<27	9	3	0	0
>100	27-32	3	0	0	0
>100	<27	0	0	0	0
TOTAL		183	137	154	171

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REFLECTION OF HEAMATOCRIT CHANGES TO LIPIDEMIC PROFILE

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Background and Aims. To study the possible variation of the lipidemic profile as a result of heamatocrit and heamoglobin chagnges. *Design and Methods*. We studied 61 patient cases (27 men and 34 women) mean averege age of 71.8±11.2 years, to whom their lipidemic profile was calculated, before and after the correction of heamatocrit. The study included hospitalised patients of our hospital and others in outpatient basis to whom though their initial hematocrit value was measured low (due to prior heamorrage or other past conditions), it was restored to a normal value. It is noted that all the tests for all the patients took place in the same laboratory, -so that the results could be compared-, while the statistical analysis was performed with the SPSS package. *Result*. As you can see at the Table. *Conclusions*. It is therefore proven that, an increse to a patients heamoglobin and heamatocrit, leads to statistically significat raise to most of the lipidemic profile variables, like the value of triglycerides and cholesterol (total, HDL and LDL). Hence, these values should be accounted for only when the blood heamatocrit and heamoglobin are stable. Otherwise, before the "correction" of the patients heamatocrit the measurement is of no clinical value and in contrast might lead to wrongful conclusions.

Table.

PARAMETERS	PERIOD I	PERIOD II	p
Heamatocrit (%)	25,2 +/- 5,5	36,3 +/- 2,8	0,18
Heamoglobin (g/dl)	7,9 +/- 2	10,5 +/- 1,9	0,45
Total Cholesterol (mg/dl)	146 +/- 47	163 +/- 46	0,001
HDL - Cholesterol (mg/dl)	11,8 +/- 7,1	41,5 +/- 10,2	0,001
LDL - Cholesterol (mg/dl)	82 +/- 50	94 +/- 38	0,001
Triglycerides (mg/dl)	110+/- 40	128 +/- 43	0,006

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THE CASE OF HEMOPHAGOCYTIC SYNDROME IN 24 YEARS OLD WOMAN

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The hemophagocytic syndrome (hemophagocytic lymphohistiocytosis) is a rare clinical condition included fever, splenomegaly, jaundice and pancytopenia as a sign of hemophagocytosis. This disease while not hereditary is usually followed by viral infections like EBV, HHV6, HHV8, CMV or some tropical diseases and malignancies like T-cell lymphomas. The aim of this paper is to demonstrate a severe case of probably viral infection associated hemophagocytic syndrome in young woman very successfully treated in our hospital. 24-years old woman was admitted to hematological ward in very severe clinical condition with signs of extended haemolytic anemia, jaundice and fever, also with slight hepatosplenomegaly. Initially haemoglobin level was 3.8 g/L, platelets 177 G/L, total bilirubine 51 mg%, ferritine 5987 ng/mL, triglyceride 414 mg% .Other biochemical signs of internal organs damage including liver disfunction with markedly enzymes elevating and transient renal insufficiency with plasma creatinine level up to 2 mg%. In bone marrow aspirate smear we found a signs of hemophagocytosis. Then despite of initial therapy with high dose of steroids (5 times 1g i.v.) clinical status and morphological and biochemical parameters were down to nadir of hemoglobin 2.2 g/L and platelets 9 G/L. Then we introduced immunoglobulins 1 g/kg and therapeutic plasma exchange (TPE) that was made 9 times and further simultaneously rituximab (3 times 600 mg i.v.). In the clinical course there was a severe complication after TPE probably central venous catheter triggered massive bilateral lower extremities deep vein thrombosis successfully treated with e.g. small moleculed heparins which forced us to stop this procedure. After those procedures there was a slight both clinical and biochemical improvement. Then the clinical course was complicated with the splenic abscess that was treated by an urgent splenectomy. After that the therapy was continued by steroids and new introduced mofetile mycofenolate 1500 mg/day and also still keeping an intensive supportive therapy as erythrocyte substitution and some antibiotics and intravenous fluids. In the end our young lady left the hospital after 7 weeks therapy within excellent condition with all normal values of prior extremely disordered parameters shown above. Our patient despite of initial critical clinical status after a long therapy achieves a long lasting complete remission. Present immunosupressive maintenance therapy with methyloprednisolone and mofetile mycofenolate allows to keeping her in perfect clinical condition without signs of disease for already one year from a disease onset. Finally we would like to discuss over used treatment options in this case especially in the situation of lacking the international treatment standards in this rare although difficult to diagnosing and treating clinical syndrome. The open question remains how long we should continue our present maintenace therapy or we have to use some other treatment options like alloBMT considered as a therapy of choice in hereditary hemophagocytic syndrome. We also would like to propose our therapeutic schedule in this case as a standard in severe viral infection associated or unclear origin hemophagocytic syndrome.

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STEPS TOWARDS THE DEVELOPMENT OF A SPECIFIC SANDWICH ELISA FOR MEASURING HEPCIDIN IN SERUM

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Background. Hepcidin is the key regulator of iron homeostasis in humans and it has been implicated in the pathogenesis of several iron disorders. Therefore, hepcidin quantification in human blood or urine is expected to provide further insight in the pathogenesis of anaemia in various diseases and might also prove to be a valuable tool for its differential diagnosis. Several methods for hepcidin quantification, mostly based on mass spectrometry, have been published, during the past few years and are still undergoing standardization. Our group has also recently reported the development of an immunochemical method for hepcidin quantification in human serum, based on the use of a recombinant hepcidin peptide and a polyclonal antibody against it. This assay had specificity, accuracy, reproducibility and provided biologically significant results. *Aim*. The aim of this study was to provide tools in order to further improve our previously reported quantification technique. For this purpose we developed a sandwich ELISA by using the previously reported polyclonal antibody and a new monoclonal antibody against the recombinant hepcidin peptide. The clinical value of the method is under investigation. *Design and Methods*. The recombinant hepcidin25-His was expressed in yeast P. pastoris, purified by Ni2-NTA affinity and size exclusion chromatography and the isolated monomer was tested for its biological activity (*Koliaraki et al., Biochimie, 2008*). New Zealand white rabbits were immunized subcutaneously with recombinant hepcidin and after three re-immunizations, polyclonal antiserum was obtained and extensively purified by affinity chromatography (*Koliaraki et al., PLoS ONE, 2009*). Furthermore, C57/BL6 mice were also immunized with the recombinant peptide and spleen cells were fused with the sp2/0 myeloma cell line in order to produce anti-hepcidin hybridoma clones. The monoclonal antibody was purified from the culture supernatant by protein-G affinity chromatography. *Result*s. From the fusion selection procedure one clone was finally chosen, which had both high affinity and specificity for the antigen and was also stable during its production. The purified monoclonal antibody was then used, along with the previously described anti-hepcidin polyclonal antibody, for the development of a sandwich ELISA. The optimal concentration of both monoclonal and polyclonal antibodies were determined as well as the analytical characteristics of the sandwich ELISA. The recombinant hepcidin25-His peptide was used for the construction of the calibration curve. First results show that this new method can identify between serums coming from patients with different iron disorders (such as juvenile hemochromatosis and Hodgkin's lymphoma) and healthy volunteers. *Conclusions*. In this study we described the development of a sandwich ELISA for hepcidin quantification in human serum. This ELISA assay displays sufficient accuracy and reproducibility using a minimal amount of serum and therefore is potentially a valuable tool for research and clinical applications.

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A NOVEL DELETION IN UDP-GLUCURONOSYLTRANSFERASE GENE IN A PATIENT PRESENTING CRIGLER-NAJJAR SYNDROME TYPE I

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Hepatic glucuronidation of bilirubin is catalyzed by isoenzyme 1A1 of uridine 5'-diphosphate-(UDP-) glucuronosyltransferase (UGT1A1), which is essential for its efficient biliary excretion. Genetic alterations causing absence, or severe reduction, of UGT1A1 enzymatic activity result respectively in Crigler-Najjar syndrome (CNS) type I and type II (MIM #218800 and MIM #606785). Diagnosis of CNS type I patients is of major relevance because presently liver transplantation is the sole curative treatment. We report the molecular characterization of a Portuguese patient presenting clinical and biochemical features of CNS type I since the neonatal period. The infant was born at full term after an uneventful pregnancy, by eutocic delivery, from non-consanguineous parents and without family history of jaundice. Serum total bilirubin was 15 mg/dL on the second day of life. The child was exposed to intensive phototherapy on the 15th day, when she presented 28.8 mg/dL of serum total bilirubin levels. At 5 weeks of age, total/conjugated bilirubin in serum was 34.7/0.9 mg/dL. Enzymatic induction by phenobarbital (20 mg/kg followed by daily administration of 5 mg/kg) was inefficient in significantly reducing the serum unconjugated bilirubin concentration. Bile profile of bilirubin species showed 90% of unconjugated bilirubin, 6% of bilirubin monoglucuronide and 4% of bilirubin diglucuronide, supporting the diagnosis of CNS type I. Genetic analysis of the patient and her parents was performed on genomic DNA, isolated from blood samples using standard methods. The three individuals were screened for the TA duplication in the promoter region of UGT1A1 gene. Direct sequencing of the five exons and of the phenobarbital-responsive enhancer sequence (c.-3576 to c.-3209) was also performed. Sequencing of the five exons of the UGT1A1 gene from the patient revealed the presence of a novel 24 bp deletion (c.609_632del) encompassing the first exon. Both parents were found to be carriers, thereby confirming homozygosity in the patient. Screening for the TA duplication in the promoter region of UGT1A1 showed a normal number of repeats in the patient and in her parents. None of the three presented the c.-3279T>G polymorphism in the phenobarbital-responsive enhancer module of UGT1A1 gene, known to be associated with Gilbert syndrome. This novel gross mutation predictably gives rise to an internally deleted polypeptide (p.His203_Lys211delinsGln). Besides its use in antenatal screening and in extending our knowledge of the spectrum of disease variants, the identification of this deletion in exon 1 of the UGT1A1 gene, which is known to encode the substrate specific region of bilirubin-UDP-glucuronosyltransferase, may contribute towards a better understanding of the molecular pathology of the disorders characterized by severe unconjugated hyperbilirubinemia.

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CONTRIBUTION OF RED CELL MASS AND UGT1A1 ALLELES IN SERUM BILIRUBIN LEVELS OF THE PORTUGUESE POPULATION

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Hepatic glucuronization of insoluble bilirubin is catalyzed by isoenzyme 1A1 of UDP-glucuronosyltransferase (UGT1A1), which is essential for efficient biliary excretion of bilirubin. The main cause of Gilbert syndrome (GS) in all populations studied to date is a TA duplication [(TA)7 allele] in the repetitive TATA-box sequence of the gene promoter, which normally consists of six TA repeats. However, this genetic polymorphism is not sufficient for the clinical phenotype of GS. By this reason, some studies have been performed to provide information about additional factors that could contribute to the pathogenesis of this disease. Recently, it was described that increased red cell mass probably plays a role in the pathogenesis of GS (*Buyukasik et al. 2008 Am J Med Sci. 335,115-119*). The aim of this work is to investigate the putative role of increased red cell mass and the (TA)7 allele in bilirubin serum levels, in the Portuguese population. This study was performed in 109 volunteer healthy young adults (20.3±1.9 years) without liver and/or hematological disorders, chronic infection, recent inflammation, malignancy, hemorrhage and medication. Blood samples were collected and processed in order to determine bilirubin serum levels, complete blood cells count, and DNA extraction. The TATA-box region was analyzed by PCR amplification followed by subsequent analysis by automated capillary electrophoresis. Among our population, 6 were homozygous for the (TA)7 allele, 55 were heterozygous and 48 were homozygous for the normal allele. One of the subjects was a compound heterozygous for the (TA)5 and (TA)7 alleles. Comparing the blood cells counts and the bilirubin serum levels according to the UGT1A1 genotype, we found statistically differences only in bilirubin levels [(TA)6/(TA)6: 0.49±0.20 mg/dL; (TA)6/(TA)7: 0.70±0.32 mg/dL; (TA)7/(TA)7: 1.10±0.74 mg/dL, $p<0.05$]. A positive statistically significant correlation ($p<0.05$) were found between bilirubin serum levels and haematocrit and mean cell volume. Our work showed that higher bilirubin serum levels are correlated with an increase red blood mass. However, no association was found between higher red blood mass and abnormal number of TA repeats in the promoter of UGT1A1 gene. This data suggests that in our population the presence of abnormal number of TA repeats in the UGT1A1 gene is associated with increased bilirubin levels but not with higher red blood mass, as previously described for GS patients.

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LONG TERM SAFETY AND EFFICACY OF IRON CHELATION WITH DESFERRIOXAMINE(DFO) AND DEFERIPRONE(DFP) IN MULTITRANSFUSED THALLASSAEMICS NINE YEARS EXPERIENCE

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Introduction. Transfusion dependent thalassaemia patients are at risk of developing iron overload leading to serious organ damage and premature death. Optimal chelation therapy can prevent iron accumulation, improves organ function and quality of life resulting in extended survival. Co-administration of two chelators , DFP and DFO, in adequate doses, appears to produce an additive chelating effect and minimize the toxicity of chelators. Objective: Estimation of effectiveness and safety of combination regimen with two chelating agents (DFP and DFO) in severely iron loaded thalassaemia patients with multiorgan complications and the possibility of reversing organ damage, mainly heart and liver, in a nine-year follow-up. *Patients and methods*. We selected 14 multitransfused heavily iron loaded thalassaemics, mean age 32 years, 7 males and 7 females switching from DFO/DFP monotherapy to combined chelation therapy with oral DFP and subcutaneous (sc) DFO. Group I. 4ps with established cardiomyopathy were treated with DFP 75 mg/kg/day and DFO 40 mg/kg x24hours, 6-7 days per week . Group II. 10ps asymptomatic referred to cardiac disease, received DFP 75 mg/kg/day and DFO 40 mg/kg x 24hours, 2 days per week. Four patients, 2 of each group, had medical history of diabetes mellitus type II and another 2p one of each group, HCV-infected, progressed to chronic hepatitis C. Measurements included: White blood cells weekly, compliance estimated by compliance index, biochemical markers (ALT, AST and γ-GT) and serum ferritin monthly. ECHO cardiography focusing on myocardial contractility markers including Fraction Shortening (FS), Left Ventricular Ejection Fraction (LVEF) and End Systolic Diameter (ESD) as well as Heart/Liver iron assessed by Magnetic Resonance Imaging (MRI T2, T2*) were annually performed. *Results*. Compliance with treatment was good (medium to high) and no side-effects were noticed. Overall patients' serum ferritin dramatically decreased ($p<0.0001$) and liver enzymes in non HCV infected patients were reduced and normalized 5 years after beginning of therapy (p: 0.010). Patients with preexisting cardiac dysfunction completely recovered and clinical symptoms improved relatively shortly after initiation of chelation (3 to 4 months). Contractility markers significantly improved (almost normalized after 5-6 years) as well as heart and liver MRI T2 and T2*.In asymptomatic patients with normal LVEF, it improved even more during treatment. Heart T2/T2* and liver T/T2* increased significantly after 4 to 5 years ($p=0.001$ and $p=0.010$ respectively). *Conclusions*. Combined chelation therapy with DFO and DFP is effective and preferred treatment in removing cardiac iron and reversing cardiac damage in severely iron loaded thalassaemic patients without serious side effects. Reduction of liver iron and

improvement of liver function in this cohort of patients, achieved after prolonged treatment, should be attributed to serious liver damage by long-lasting iron deposition while hepatic viruses (HBV, HCV) constitute an additive harmful factor in infected patients.

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TREATMENT WITH DEFERASIROX FOR NON-TRANSFUSIONAL IRON OVERLOAD IN PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Patients with thalassaemia intermedia (TI), though rarely transfused, may develop iron overload. The mechanism of iron loading is similar to that observed in hereditary hemochromatosis, with inappropriately low hepcidin levels due to enhanced erythropoiesis, leading to increase intestinal iron absorption. *Aims*. In this study, we evaluated the efficacy and safety of treatment of iron overload with deferasirox in patients with TI. P*atients and Methods*. Eleven patients (6 male, 5 female, mean age: 31.7 years, age range: 24-40 years) with TI and iron overload were included in this study. Two patients had never been transfused, six had received <3 lifetime transfusions and the rest <20. All patients but one, were splenectomised. Patients were treated with deferasirox for a period of 1 year, while 4 for 1 additional year. Seven patients initiated treatment with 10 mg/kg/day and 4 with 20 mg/kg/day. Dose adjustment was implemented only after completing one year of treatment according to the severity of iron burden. Efficacy of treatment was evaluated with ferritin levels and degree of hepatic and cardiac iron overload, which were estimated by MRI (T2* sequence). Safety parameters included transaminases, creatinine and cystatin C levels. *Results*. Mean, SD and range of the baseline evaluation showed: ferritin: 1356±1039 ng/mL (536-4086 ng/mL), liver iron concentration (LIC): 17.6±9.9 mgFe/g d.w. (2.1-31.8 mgFe/g d.w.), cardiac T2*: 35.6 ms (28.9-40.2ms), and left ventricular ejection fraction (LVEF): 66.7±4.5 ms (59.6-73.2%). Ferritin levels correlated well with LIC (R=0.84, $p<0.005$), but with a different slope that the one observed in transfusion-dependent thalassemic patients. No cardiac siderosis was observed. After 1 year of treatment, only one patient, who was non-compliant, did not show decrease of iron overload. Mean serum ferritin and LIC decreased significantly ($p<0.05$) to 914 ng/mL (range: 127 - 4400 ng/mL) and to 10.7 mgFe/g d.w. (range: 1.2 - 32 ng/mL), respectively. Three patients reached levels of ferritin <250 ng/mL and LIC < 4 mgFe/g d.w. Changes in LIC and ferritin levels were related to deferasirox dose, but even patients with severe iron load, treated with 10 mg/Kg/day responded well. There were no changes in cardiac T2* and in LVEF. Improvement continued on the 2nd year of treatment, but it did not reach statistical significance. Treatment was well tolerated and no serious adverse events were noted. Creatinine and cystatine C levels did not change during the treatment. Significant decrease in transaminases levels was noticed during both the 1st ($p=0.0002$) and the 2nd year of treatment ($p=0.024$). This improvement is probably due to decreased hepatic siderosis. *Conclusions*. Rarely or non-transfused patients with TI can present with significant iron overload, which predominantly affect the liver. Treatment with 10 mg/Kg/day of deferasirox is effective in chelating this iron excess. Dose of 20 mg/Kg/day in severely iron-loaded patients achieves increased iron excretion and is not associated with side affects.

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CORRELATION OF BCL11A SNPS AND FETAL HEMOGLOBIN LEVELS IN SICKLE CELL PATIENTS FROM OMAN

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Background. Recently, genetic association studies have shown several single nucleotide polymorphisms(SNPs) that are linked to expression of HbF into adulthood. These SNPs are located in the gene BCL11A on chromosome 2 and HBSIL-MYB on chromosome 6. *Aims.* To assess the BCL11A SNPs associated with high HbF in Sickle cell disease (SCD) patients from Oman. *Design and Methods*. Genomic DNA was isolated using the semi-automated 6100 nucleic acid extractor. Sickle mutation was defined by direct sequencing of the β globin gene (ABI 3100 genetic analyzer). Sickle haplotypes were defined using previously described techniques by RFLP using specific restriction enzymes(HincII5'to e, XmnI 5' to Gg, Hind III within Gg and Ag, Taq1 within Gg and Ag, Hinc II within and 3'to b and Hinf I and RsaI5' to b) Direct sequencing of intron 2 of BCL11A gene was performed and three BCL11A haplotypes were constructed as A, B and C using five SNPs (rs7557939, rs7584113, rs4671393, rs34211119 & rs11886868). *Results*. 160 SCD patients (SS) aged between 7-41 years, with a mean age 21.5 + 7.2(SD) were consecutively enrolled. There were 85 males. bs haplotyping revealed that 54,22, and 31cases were homozygous Benin, Bantu and Arab-Indian(AI) haplotypes respectively, with the remaining 53 being mixed heterozygous. BCL11A haplotyping with regards to the 5 SNPs revealed that 32(20%), 82 (51.25%) and 46(28.75%) showed AA(Wild/Wild), AC or AB(Wild/Mutant) and BC,BB,CC(Mutant/Mutant) haplotypes respectively. There was a significant difference in the HbF amongst the wild v/s mutant haplotypes in the SS group overall as well as in the bs haplotypes namely Ben/Ben; Ban/Ban & AI/AI homozygotes (Figure). *Conclusions*. The study reveals a good correlation of the BCL11A SNPs with fetal hemoglobin levels in SCD patients. The same results within the SS SCD bs haplotypes indicate that BCL11A SNPs independently regulate the fetal hemoglobin levels in adulthood. rs 4671393 and BCL11A B haplotype were significantly associated with raised fetal hemoglobin levels in this patient population.

Table 1.

BCL11A Haplotypes	AA [Wild/ Wild]	AB [Wild/ Mutant]	AC [Wild/ Mutant]	BC [Mutant/ Mutant]	BB [Mutant/ Mutant]	CC [Mutant/ Mutant]
HbF [Mean± SD]	8.34 ± 6.5	11.54 ± 8.2	9.97 ± 6.7	12.84 ± 6.3	14.53 ± 6.5 AA v/s p=0. 007	9.58 ± 8.9
Arab-Indian SS SCD			AA	AB		BB/BC/CC
HbF [Mean±SD]			13.08 + 6.7	21.1 + 6.0 AA v/s p=0.02		16.9 + 2.8 AA v/s p=0.8
Ban/Ban SS SCD			AA	AB		BB/BC/CC
HbF [Mean±SD]			3.37 + 4.7	8.22 + 9.0 AA v/s p=0.1		8.13 + 9.5 AA v/s P =0.1
Ben/Ben SS SCD			AA	AB		BB/BC/CC
HbF [Mean±SD]			5.06 + 1.8	9.55 + 7.0 AA v/s p=0.5		14.4 + 6.2 AA v/s P <0.004

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PANCREATIC AMYLASE, A POTENTIAL INDICATOR OF CARDIAC IRON OVERLOAD

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Regular transfusion therapy in thalassemia major (TM) is improving patients quality of live, but creates a state of iron overload with the consequence of iron deposition in parenchymal tissues such as endocrine glands, hepatocytes and myocardium. Cardiac iron in TM patients is nowadays assessed by fast MRI-R2* methods. However, TM patients will not be measured before the age of 10 years due to compliance problems with MRI measurements. Unfortunately do some TM patients exhibit R2* (1/T2*) relaxation rates >50s^{-1} corresponding with increased cardiac iron concentrations already at that age. The underlying mechanisms of iron accumulation into cardiomyocytes may also be involved in other cells such as pancreatic cells, anterior pituitary gland cells, and neurons. Especially, calcium channels offer a pathway for ferrous iron, which is not down regulated in iron overload conditions. A significant but loose correlation was found between cardiac and pancreatic T2* relaxation times [*Au et al, 2008*], however, the association with endocrine function tests is spoiled by fatty infiltration of the pancreas in thalassemia patients with impaired function [*Papakonstantinou et al, 2007*]. A decreased pancreatic exocrine function with subnormal serum amylase levels was found to be associated with pancreatic iron index measured by MRI in TM patients [*Midiri et al, 1999*]. In a clinical setting of 20 transfusion dependent TM patients (age 11 - 41 years, 9 female), we measured cardiac iron by MRI-R2*, liver iron by biosusceptometry and pancreatic exocrine function by serum amylase. ROC analysis revealed a high specificity (78%) for elevated cardiac iron (R2* > 50 s-1 or T2* < 20 ms) at amylase levels ≤13 U/L ($p=0.02$). In contrast, liver iron concentration could not predict cardiac iron ($p=0.9$). Using pancreatic serum amylase as an indicator for exocrine pancreatic function may help to

indicate patients at risk of cardiac iron overload and could lead to an intensified iron chelation therapy in these patients. TM patients with low pancreatic serum amylase levels should undergo a more investigative diagnosis by MRI.

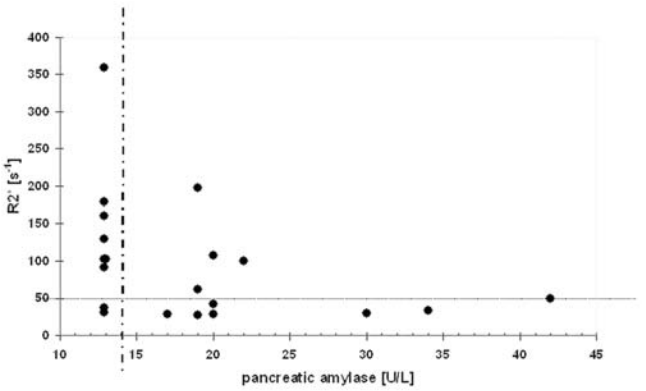

Figure 1. Relationship between cardiac mid-papillary.

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THE PLASMA HYPERCOAGULABILITY AND VASCULAR ENDOTHELIUM ABNORMAL CYTOKINES' RELEASE MAY INFLUENCE THE PROGRESSION OF THE OSTEOPATHY IN HOMOZYGOUS β-THALASSEMIA PATIENTS

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Background. It is well known that a chronic hypercoagulation and vascular endothelium dysfunction are responsible for thromboembolic events in homozygous β-thalassemia(β-Th). *Aim*. In this scenario, an other severe complication such as the osteopathy,which becomes certain in adult β-Th patients, has recently evidenced and mainly referred to the several endocrine deficiencies,while the contribution of the hypercoagulation and abnormal vascular endothelium cytokines' release has not been considered. *Design and Methods*. 11 Sicilian homozygous β-Th patients(6 females and 5 males),aging 24-66 yrs, were studied. 11 sicilian heterozygous β-Th subjects and 10 healthy individuals of comparable age served as controls. Bone density scans showed severely low bone mass in 7/11 and low bone mass in 4/11 respect to the control groups. The osteoblastic cytokines' net-work as the Platelet-derived growth factor(PDGF),Transforming growth factor-β (TGF-β)and Interferon-α (IFN-α)together with the osteoclastic cytokines such as the Interleukin-1(IL-1), Interleukin-6 (IL-6) and Tumor necrosis factor-β (TNF-β)were determined by ELISA. *Results*. Our results showed a significant (p<0.001) increase of the osteoclast cytokines in homozygous β-Th respect to those observed in the controls groups.The osteoblastic cytokines were in normal range in all groups. *Conclusions*. From these observations we suggest that the chronic red blood cells haemolysis, blood transfusions and iron chelation 'quoad vitam' would determine continuously biochemical changes in β-Th leading also to an abnormal release of several cytokines from injured vascular endothelium of the bone microenvironment. The osteoclastic cytokines' formation is dominant over the osteoblastic one and by reducing bone mineral density so potentiating the osteoporosis as observed in adult homozygous β-Th patients by clinical diagnostic findings. In this context, the chronic vascular endothelium suffering of the bone microenvironment together with the hypercoagulability,leukocytes' and platelet hyper-activation could enhance further the osteoclast cells functions in β-Th.

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HYDROXYUREA EXPERIENCE IN THE MENAGMENT OF ALGERIAN PATIENTS AFFECTED WITH SEVERE HEMOGLOBINOPATHIES

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Background. Conventional treatment of severe hemoglobinopaties is limited in the many countries. Hydroxyurea (HU) is the first widely used treatment ,as an alternative to blood transfusions, to have an impact on severity of sickle cell disease (SCD).Its effects in thalassemia (Thl) are controversial, particularly in Thl major (TM). *Aims*. to assess long-term use of HU in the management of Algerian patients with severe SCD and Thl, previously treated with blood transfusion (BT) by the evaluation of hematological response, hemochromatosis, growth velocity, sexual maturation, compliance side effects and also the coast of this approach. Patients and methods: since 2000 total of 123 patients were enrolled in HU protocol. Forty six with SCD (33 SS, 14 Sβ Thl, and 1SC), medium age at the initiation of treatment was 15 years (3 to 25yrs), 38 with history of >vaso-occlusive-crisis and/or >1 acute chest syndrome, 6 with severe anemia (Hb <6 g/dL), 2 priapism, 1 nephropathy. The main daily dose of HU was 19,5 mg/kg/d(14 to 24) . Seventy nine were THL patients, 69 TM and 10 Intermediate (TI), median age was 11 yrs (3 to 21), 13 firsts patients were included for major BT complication. Mean daily dose was 16,1 mg/kg(13 to 21) We defined good response when decrease in annual BT requirement greater 70% with sustained Hb above 7 g/dl. Management of hemochromatosis in both groups was based on Deferoxamine (DFO) administered subcutaneous bolus infusion (2 injections 4 to 5 d/w) and phlebotomy performed when Hb level≥8 g/dL. Serum ferritin was evaluated every 3 months, cardiac function every year, growth and sexual development by measurement of high, weigh and Tanner stage. We also compared the coast per year of this approach and an optimal conventional treatment by assessment of coast of blood units, DFO, day hospitalisations (DH). *Results*. Median follow-up for SCD was 82 months, significant decrease of annual rate of VOC, ACS, BT and DH. Stopped BT obtained in 33 patients, mean gain Hb was 1,4g/dl (0,8 to 3,3), 1 had stroke, 1 recurrent priapism, 4 developed hip osteonecrosis 2 patient died(1 sepsis, 1 renal deficiency). For7patients with initial ferritin>2000, level fell from 3500 to 870ng/mL with DFO (32 mg/kg/d) and phlebotomy (7ml/kg every 3 week). Only 5 patients have growth retardation. The annual coast was reduced around 85 percent. In the group of THL good response was obtained in 36 (9 TI and 27 TM), no response in 31, and partial response in 11 cases (reduction 50% of BT need) Response is associated with older age at the first TS(p=0.02) ,higher preHU Hb(p=0.0004),codon6(-A)mutation (p=0.002), TI(p=0.03), history of splenectomy(p=0,05), Xmn1 polymorphism (p=0,008) ((*Bradai M et al. Transfusion 2007; 47:1830-36*). Portal venous thrombosis PVT occurred in 4 patients and 3 others had autoimmune anemia, treated with steroids and Immunoglobulin. In group of good responders mean ferritin level fell from 4100 to 2100ng/ml, with DFO 35mg kg/d and phlebotomy performed in 14 patients (5 ml/kg/3w). Growth retardation is observed in 7 patients, Cardiac function is conserved. Six patients died, 3 from cardiac deficiency and 1 after PVT, 1 sepsis and 1 acute leukaemia. Compliance was excellent, No serious acute toxicity was seen. In this group the annual coast is reduced around 75 percent. *Conclusion*. HU appear a good alternative of conventional treatment and seem have favourable cost effectiveness in Algerian SCD and THL patients, particularly when blood supplies are limited.

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ROLE OF αα/αααANTI-3.7 TRIPLICATIONS ON THE PHENOTYPE OF SICKLE β THALASSEMIA PATIENTS

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Background. HbS occurs due to A - T substitution at codon 6 of the β globin gene leading to the nonsynchronous replacement of valine for glutamic acid. Sickle patients have a very variable clinical phenotype. Some of the possible explanations for the observed variable clinical severity are coinheritance of α-deletions and triplications. *Objective*. To determine the frequency of α deletion and triplication in Sickle β thal patients and to study their effect on the phenotype of Patients. *Design and Methods.* Subjects were 25 Sickle cell patients who attended the Hematology out patient department of the institute diagnosed by HPLC.Patients were divided into three subgroups according to a scoring system based on seven clinical criteria as mild (score 0-3.5), moderate (score 4-7) and severe (score7.5-10). α deletions and triplication were studied by GAP-PCR

and multiplex-PCR respectively. *Results*. α deletion was found in 8 (32%) out of these 8 patients 6 (αα/-ααα$^{3.7}$) were from Gp1 and 2 (αα/-ααα$^{3.7}$) were from Gp2.β triplication was found in 2 (8%) both of these were (αα/-ααα$^{3.7}$) from Gp3. *Conclusions*. Phenotype of Sβ^{thal} largely depends on coexisting β gene abnormality. α$^{3.7}$deletion showed Milder phenotype while β triplication showed severe phenotype.

Table. Effect of a globin gene numbers on the phenotype of sβ patients.

	Gp1		Gp2		Gp3	
	α del αα/ αα		α del αα / αα		αα / αα	αα / ααα
	N=6	N=6	N= 2	N=6	N=3	N=2
Mean Hb	10.4±2.2	7.9±.56	7.5±1.9	4.8±.14	4±2.1	3.5±1.9
Mean BT	**not req.**	4.8±1.4	**not req.**	11.2±2.1	20.5±3.2	26.5±2.6
Episode of pain	**None**	2.1±1.1	1.5±0.7	2.8±1.4	3.3±1.5	12±2.1
Splenectomy	**None**	**None**	**None**	**1**	**none**	**1**

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MAGNETIC RESONANCE IMAGING R2 MAPPING ACCURATELY ESTIMATES IRON OVERLOAD IN SICKLE CELL DISEASE PATIENTS

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Background. The majority of patients with sickle cell disease (SCD) require episodic blood transfusions on a chronic basis which makes them at risk for developing iron overload. The principal methods of determining body iron levels are measurement of serum ferritin levels and assessment of liver iron concentration (LIC) from biopsy tissue. Non-invasive approaches for determining liver iron concentration are increasingly used as an alternative to biopsy. R2 magnetic resonance imaging (MRI) is currently the only validated approach. A significant correlation between serum ferritin and liver iron concentration has been established in regularly transfused patients with thalassemia major. Data on patients with SCD is limited. *Aims*. To study the correlation between LIC determined by R2 MRI and serum ferritin levels in chelation-naïve SCD patients. Correlation between LIC and total number of lifetime transfusions, patient- and disease-related characteristics will be made. The data reported here represents the largest investigation of this correlation in SCD patients, and thus, provides valuable information on the relationship between these parameters in this specific patient population. *Design and Methods*. This was a cross-sectional study of randomly selected patients with SCD treated at two comprehensive Lebanese SCD centers: Rafik Hariri University Hospital (RHUH) and Nini hospital. The sampling frame consisted of 200 chelation-naïve SCD patients >2 years of age and a simple random sample was obtained. Data on patient demographics, transfusional history, status of the spleen, and comorbid illnesses or infections was obtained. Blood samples were obtained for assessment of steady state serum ferritin, hemoglobin and liver enzyme levels. Direct determination of LIC was performed using R2 magnetic resonance imaging. Written informed consent was provided by all patients. LIC values <3, 3-7, >7 mg Fe/g dry weight and serum ferritin levels <1000, 1000-2500, >2500 ng/mL were considered normal, mildly overloaded, and severely overloaded; respectively. *Results*. Data from 52 SCD patients was included in the analysis (Table 1). None of the patients had evidence of hepatitis C or B infection, or elevation in liver enzymes. A significant positive correlation was observed between LIC and serum ferritin (Pearson correlation 0.874; $p<0.0001$). Moreover, 20.0 vs. 15.4% had severe, 12.0 vs. 15.4% had mild, and 68.0 vs. 69.2 had absence of iron overload by LIC and serum ferritin measurement; respectively. Neither LIC nor serum ferritin had a significant correlation with age or hemoglobin level; however both correlated positively with the total number of lifetime transfusions ($p<0.001$). There was no statistically significant difference in mean LIC and serum ferritin levels between males and females, splenectomized and non-splenectomized patients, or patients with or without persistent splenomegaly (>6 years). *Conclusions*. Hepatic R2 MRI values correlate well with serum ferritin level and the total number of lifetime transfusions in SCD patients, thus, confirming the value of this technique for assessing iron overload in this patient population. Moreover, iron levels in the study population demonstrate that many chelation-naïve patients with SCD have serum ferritin and LIC levels above the recommended threshold levels identified in patients with thalassemia major, indicating a risk of significant morbidity and mortality.

Table 1. Patient, disease and iron overload characteristics.

Parameter	Value
Mean age ± SD, years (range)	18.54 ± 9.0 (4-49)
Male/Female	5/8
Splenectomized, (%)	16 (30.8)
Persistent splenomegaly[a], (%)	21 (42)
Mean transfusions[b] ± SD, n (range)	50.9 ± 61.4 (0-300)
Mean hemoglobin ± SD, g/dL (range)	8.6 ± 1.4 (5.6-11.9)
Mean SF ± SD, ng/mL (range)	987.5 ± 1230.3(16-6190)
Mean LIC ± SD, mg Fe/g dw (range)	5.9 ± 9.4 (0.3-50.0)

SF = serum ferritin at steady state; LIC = liver iron concentration; dw = dry weight.

[a]For more than 6 years.

[b]Total number of lifetime transfusions.

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MAGNETIC RESONANCE IMAGING T2* IN THE EVALUATION OF CARDIAC IRON OVERLOAD IN PATIENTS WITH SICKLE CELL DISEASE

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Background. In patients with thalassemia major, cardiac dysfunction resulting from iron overload is the leading cause of morbidity and mortality. Although systemic and hepatic iron overload have been recognized in patients with sickle cell disease (SCD), data on iron overload associated cardiac dysfunction is quite limited. Few studies have reported absence of cardiac involvement. Therefore, monitoring cardiac iron levels in such patients is justified. The use of magnetic resonance imaging (MRI) T2* to detect cardiac iron overload is becoming increasingly common because of its non-invasive and highly reproducible nature. AIMS: To use MRI T2* to measure myocardial iron overload in patients with SCD; and to establish its correlation with echocardiographic findings and body iron parameters. *Design and Methods*. This was a cross-sectional study of randomly selected patients with SCD treated at two comprehensive Lebanese SCD centers: Rafik Hariri University Hospital (RHUH) and Nini hospital. The sampling frame consisted of 200 non-chelated SCD patients > 2 years of age and a simple random sample

was obtained. Patient charts were reviewed for drug use, transfusional history, status of the spleen, and comorbid illnesses or infections. Blood samples were obtained for assessment of steady state serum ferritin, and liver enzyme levels. Doppler echocardiography to detect pulmonary hypertension (PHT) and assess left ventricular ejection fraction (LVEF) was done. Cardiac iron levels were measured by MRI T2*. Direct determination of liver iron concentration (LIC) was performed using R2 magnetic resonance imaging. In this study, cardiac T2* >20 ms was considered normal. Written informed consent was provided by all patients. *Results*. Data from 23 patients were included in this analysis. The mean age was 24.4±9.7 years (range: 4-49 years) with a male to female ratio of 15:8. None of the patients had a history of iron chelation therapy, evidence of hepatitis C or B infection, or elevation in liver enzymes. Six patients (26.1%) were splenectomized, and 9 patients (40.9%) had evidence of persistent (>6 years) splenomegaly. The mean total number of lifetime transfusions was 73.8±80.6 (range: 1-300). The mean LVEF was 64.6±5.3% (range: 58.0-76.0%). Six patients (27.3%) had evidence of PHT. Mean steady state serum ferritin level was 997.7±1110.9 ng/mL (range: 41-3430 ng/mL) and mean LIC value was 4.6±7.0 mg Fe/g dry weight (range: 0.3-21.7 mg Fe/g dry weight). None of the patients had evidence of cardiac iron overload (mean cardiac T2*=37.3±6.2.3 ms; range: 21.9-46.8 ms). Moreover, cardiac T2* did not correlate with either serum ferritin (p=0.75) or LIC (p=0.97). There was no correlation between LVEF or presence of PHT and cardiac T2* values. All remaining study variables (age, gender, splenectomy, persistent splenomegaly, total number of lifetime transfusions, and hemoglobin level) showed no correlation with cardiac T2* values. *Conclusions*. The results of this study show that SCD patients do not have evidence of cardiac iron overload as measured by T2* MRI. This is consistent with previous data showing that patients with SCD are generally less prone to cardiac iron overload associated morbidity and mortality.

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IRON CHELATION IN PATIENTS WITH THALASSEMIA INTERMEDIA AND OTHER NON TRANSFUSION DEPENDENT CONGENITAL HEMOLYTIC ANEMIAS

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Iron overload is one of the main causes of morbimortality in patients with congenital anaemias. Erythroid hyperplasia and chronic tissue hypoxia increase iron absorption in non transfusion dependent anaemic patients, most of them requiring iron overload monitoring and, eventually, iron chelation. Cardiac disease and pulmonary hypertension are the leading cause of death among these patients, nevertheless myocardial iron is not usually significantly increased. The aim of this study was to analyse iron chelation data on 22 non transfusion dependent patients with congenital anaemias. In a group of 22 patients (18 β-thalassemia intermedia, 2 piruvate kinase deficiency, 1 pyrimidine 5' nucleotidase deficiency and 1 hereditary xerocytosis), ALT, AST, serum ferritin level and liver iron concentration (LIC) by MRI were determined before and 42±5 weeks after iron chelation therapy. Cardiac iron overload was evaluated by MRI T2* only at the end of the observational study (Table 1).

Table 1. Evaluation of patients with congenital hemolytic anemias, not transfusion dependent, before and after 42±5 weeks of iron chelation therapy. DFO: deferoxamine; DFP: deferiprone. *Patient with HCV.

Age	Sex	Pathology	Hb g/dL	Initial evaluation		Re-evaluation		Myocardial T2* ms	Treatment
				Serum ferritin ng/mL	LIC µmol/g	Serum ferritin ng/mL	LIC µmol/g		
18	M	β-TI	8	859		69	55	75	DFO
27	M	β-TI	8,5	1138	230	108	45	59	DFP
29	M	β-TI	7	302		295	210	82	None
34	F	β-TI	7,5	1158	340	110	45	39	DFP
35	F	β-TI	7,5	1500	> 360	2315	> 360	38	STOP DFP
38	F	β-TI	8,5	415	250	369	270	25	STOP DFP
39	M	β-TI	9,5	869	160				STOP DFP
40	F	β-TI	7,5	1535	350	436	260	23	DFO + DFP
40	M	β-TI	9	349	180	235	240	52	STOP DFP
42*	M	β-TI	7	1988	340	92	70	49	DFO + DFP
43	M	β-TI	7	1909	330	293	150	37	DFO
43	M	β-TI	7,5	653	250	2261	330	35	STOP DFP
44	M	β-TI	8,5	876	220	558	260	95	STOP DFP
45	M	β-TI	11	1340	310	313	220	47	DFP
54	F	β-TI	10	1047	95	274	70	26	DFP
54	F	β-TI	8	806	280	363	95	37	DFP
59	F	β-TI	8	648	190	87	60	51	DFP
59	M	β-TI	7	1087	290	170	180	36	DFO + DFP
18	F	PK deficiency	8	723		543	250	33	DFO
18	M	PK deficiency	8,5	1206		679	230	31	STOP DFP
56	M	PSN deficiency	10,5	1219	220	183	90	34	DFP
55	M	Hereditary xerocytosis	14	936		210	220	30	Phlebotomy

All the patients had normal liver enzymes, except one with active hepatitis C, and most of them presented high initial serum ferritin levels and significant hepatic iron overload. Patients with good therapeutical compliance had a very significant serum ferritin decrease, with a less significant decrease on LIC. There was no consistent correlation between serum ferritin levels and LIC especially after iron chelation. Myocardial T2* was above 20 ms (normal) in all the patients. In conclusion: according to the present data, iron overload in patients with non transfusion dependent congenital anaemias may be underestimated by serum ferritin levels alone. Assessment of LIC in these patients may establish the need for iron chelation to prevent liver dysfunction, and to monitor the treatment

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BONE MARROW MITOGEN-STIMULATED DIRECT ANTIGLOBULIN TEST IN A CASE OF ERYTHROBLASTIC SYNARTESIS

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Background. Erythroblastic synartesis is a rare disease in which an autoimmune phenomena against erythroblasts has been hypothesised. Recently, anti-erythroblast autoimmunity has been demonstrated in half patients with early MDS (RA or RARS) by means of mitogen-stimulated direct antiglobulin test (MS-DAT) performed on bone marrow (BM) aspirates. *Aims*. To report a case initially diagnosed as unclassified myelodisplastic syndrome characterized by hyperplastic erythropoiesis and haemolytic anemia, who showed the presence of anti-erythroblast autoimmunity (positive BM MS-DAT), together with morphological features of erythroblastic synartesis. *Design and Methods*. MS-DAT was performed by stimulating BM with PMA and PHA and antibodies were detected in supernatants by competitive solid phase ELISA. Control BM cells were separated by magnetic beads in $CD45^+$ (myeloid cells) and CD45- cells (erythroblasts) and patient's BM culture supernatant was tested on both BM populations. The growth of normal BM progenitors on methylcellulose medium was evaluated in the presence (or not) of patient's BM culture supernatant. Smears were obtained from CFU after 14 days of culture. *Results*. The propositus is a 44 years old male, with a history of anemia since 1998 and numerous BM aspirates showing hyperplastic and dysplastic erythropoiesis, T and NK infiltrate, increased iron deposition and various non conclusive diagnoses (initial pure red cell aplasia, T-large granular lymphocyte lymphoma, myelofibrosis), and the patient was treated with steroids and cyclosporin with some response. After admission to our Hospital, the patient's records showed Hb 9 g/dL, reduced reticulocytes ($20x10^9$/L), and slightly alteration of hemolytic markers (unconjugated bilirubin 1.8 mg/dL, LDH 550 U/L, haptoglobin <20 mg/dL); the study of red cell enzyme activity, membrane proteins, and autoimmune tests were negative. BM aspirate showed hyperplastic erythropoiesis with some dysplastic features, mostly binuclear erythroblasts; in addition, clusters of closely linked erythroid cells at different maturation stages and small lymphocyte aggregates were observed. These features resembled those of the uncommon disorder named erythroblastic synartesis, first described in 1973, in which an autoimmune phenomena against erythroblasts was hypothesised. Thus, MS-DAT was performed on BM samples, giving positive results (389 ng/mL BM mononuclear cell-bound IgG in unstimulated cultures, 408 ng/mL in PHA-, and 534 ng/mL in PMA-stimulated cultures). Patient's BM culture supernatant showed greater levels of IgG binding to normal erythroblast cells (CD45-) than to myeloid ones (CD45+); moreover it induced dyserythropoietic signs (nuclear atypia i.e. multiple nuclei, nuclear inclusions, and intercellular bridges) and erythroblastic clustering of CFU growing of normal BM samples. The patient was treated with steroids (prednisone 50 mg/die followed by slowly tapering) achieving clinical and hematological response. The patient is still on regular follow-up without further relapse. *Conclusions*. In this case characterised by morphological features of the erythroblastic synartesis we demonstrated by MS-DAT an autoimmune reaction against erythroid precursors resulting in erythroid hyperplasia/dysplasia.

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EFFICACY, EFFICIENCY AND SAFETY OF DEFERASIROX FOR TREATING TRANSFUSIONAL IRON OVERLOAD IN NON-ACTIVELY TRANSFUSED PATIENTS

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Background. Deferasirox has been approved for the treatment of transfusional iron overload in patients with various types of anemias. The studies on deferasirox, reported so far, focused mainly on patients with a continuing need of transfusion therapy, in whom efficiency of deferasirox was estimated to be around 27%. Herein, we evaluated treatment of iron overload with deferasirox in non-transfusion-dependent patients. *Patients and Methods*. Eleven patients (9 males, 2 female, mean age: 31.7 years, age range: 21.1-44.8 years) with iron overload were included in this study. Eight patients had thalassemia intemedia, 2 sickle cell disease, and 1 congenital dyserythropoetic anemia II. Patients had been previously regularly transfused, usually until splenectomy, but had not had any transfusions for >3 years. Lifetime transfusions ranged from 50-350. Mean dose at the 1st year of treatment was 16 mg/kg/day (range: 10-20 mg/kg/day, 11 patients) and 17.9 mg/kg/day (range: 10-20 mg/kg/day, 7 patients) at the 2nd year. Efficacy was evaluated with ferritin levels and degree of hepatic and cardiac iron overload, which were estimated by MRI (T2* sequence) (9 patients). Safety parameters included transaminases, creatinine and cystatin C levels. Chelation efficiency was determined as the% iron excretion versus the theoretical iron binding capacity of the chelator. Iron excretion was calculated based on the changes of liver iron concentration (LIC) and the variation in body iron load according to Angelucci et al. (N Engl J Med 2000;343:327). RESULTS: Mean, SD and range at baseline showed: ferritin: 1634±903 ng/mL (352-3176 ng/mL), LIC: 20.6±13.6 mgFe/g d.w. (4.7-39.1mgFe/g d.w.), cardiac T2*: 34.3±4.7ms (25.6-40ms) and left ventricular ejection fraction (LVEF): 66.7±4.5 ms (63.1-70.3%). All patients showed a steady decrease in iron overload. Mean serum ferritin decreased significantly to 1061±570 ng/mL (range: 125-2014 ng/mL) after 1 year and to 504±391 ng/mL (range: 159-1058 ng/mL, 7 patients) after 2 years (p<0.05). Mean LIC decreased significantly (p<0.05) to 13.6±7.79 mgFe/g d.w. (range: 2.6 - 28.9 ng/mL) after 1 year (p<0.05) and to 8.06±6.6mgFe/g d.w. (range: 2.1 - 16.1 ng/mL, 3 patients), after 2 years. There were no changes in cardiac T2* and LVEF. Treatment was well tolerated. No serious adverse events were noted, including hematological toxicity, even though in 7 patients concomitant medications included hydroxyurea. Creatinine and cystatine C levels did not change. Significant improvement in transaminases levels was noticed, probably due to decreased hepatic siderosis. Chelator efficiency ranged considerable in between patients (median:20.4%, range:3.5-42.3%). It did not changed significantly in the 2nd year of treatment. This calculated chelator efficiency is different than previously described, either because deferasirox achieves slower iron excretion, or because LIC underestimates total iron body stores in these patients. *Conclusions*. Treatment with deferasirox in non-actively-transfused patients is effective and well tolerated in chelating excess iron, which has accumulated from previous transfusions. The rate of iron overload improvement may be lower than previously observed. Larger studies are required to verify these conclusions.

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CROSS-TALK BETWEEN INFLAMMATION AND IRON STATUS, AND ITS ASSOCIATION WITH RESISTANCE TO RECOMBINANT HUMAN ERYTHROPOIETIN THERAPY IN HAEMODIALYSIS PATIENTS

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It is known that the inflammatory stimulus has an important impact in iron metabolism, by mobilizing iron from erythropoiesis traffic to storage sites within the reticuloendothelial system, inhibiting erythroid progenitor proliferation and differentiation. This change in iron mobilization way lead to an iron depleted erythropoiesis. Furthermore, recently, a complex regulatory network that governs iron traffic emerged, and points to hepcidin as a major evolutionary conserved regulator of iron distribution. The synthesis of hepcidin is stimulated by inflammation, and has been reported to be increased in haemodialysis (HD) patients. Given the importance of iron mobilization for erythropoiesis, we hypothesized that alterations in iron traffic could be related to resistance to recombinant human erythropoietin (rhEPO) therapy in HD patients. The aim of our study was to assess possible relations between iron status and inflammatory markers in HD patients, as well as its association with resistance to rhEPO therapy. Sixty-tree HD patients and 26 healthy controls were enrolled in the study. Among HD patients, 31 were non-responders and 32 were responders to (rhEPO) therapy. Red blood cell and reticulocyte counts, haemoglobin (Hb) concentration and circulating levels of ferritin, iron, transferrin, C-reactive protein (CRP), soluble interleukin 2 receptor (s-IL2R), soluble transferrin receptor (s-TfR), interleukin 6 (IL-6) and prohepcidin were measured in all patients and controls. HD patients showed statistically significant higher reticulocyte count and circulating levels of ferritin, s-TfR, CRP, IL-6, s-IL2R and prohepcidin. Lower number of erythrocyte count, and decrease levels of Hb and transferrin were also found in HD patients when compared to healthy controls. Higher levels of s-TfR and CRP, and lower levels of Hb and prohepcidin were observed among non-responders compared to responders. Prohepcidin levels correlated negatively with s-TfR and reticulocyte count. The weekly rhEPO/Kg dose was found to be positively correlated with CRP, Hb and s-TfR. In conclusion, our data show that a close interaction exists between inflammation, iron status and prohepcidin serum levels that ultimately regulate intracellular iron availability. Prohepcidin and s-TfR, together with CRP, may prove to be good markers of resistance to rhEPO therapy in HD patients. Moreover, HD non-responders patients to rhEPO therapy seem to present a "functional" iron deficiency, characterized by the presence of adequate iron stores as defined by conventional criteria, but apparently with an inability to sufficiently mobilize iron to adequately support erythropoiesis.

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INTERACTION OF AZOTOBACTIN WITH BLOCKING AND MOBILIZING AGENTS IN THE MEASUREMENT OF NON TRANSFERRIN BOUND IRON IN HEMATOLOGICAL DISORDERS

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Background. Hematological disorders involving non transferrin bound iron result in multiple organ damage, caused due to its potentially toxic nature which can be attributed to free radical generation. However NTBI is not employed as a routine parameter to diagnose iron overload particularly in India, because of lack of a direct and reliable assay. Accurate estimation of NTBI requires two key issues to be addressed efficiently; complete mobilization of the available NTBI from all putative ligands in serum and blocking of endogenously present apotransferrin, which could scavenge the mobilized NTBI. These two critical conditions if not optimized may lead to underestimation or false estimation of NTBI concentration. Simultaneously the selected blocking and mobilizing agents should also be compatible with the fluorophore in the given experimental conditions of a fluorescence assay. *Aims.* In this study we therefore sought to examine the interaction of different blocking (Co3+ and Ga3+) and mobilizing agents (EDTA, sodium oxalate and nitrilotriacetate) with azotobactin, the bacterial siderophore utilized in the developed assay. The main aim was to study the spectroscopic characteristics of azotobactin in the presence of these agents and also the effect of these agents on the binding kinetics of azotobactin and Fe3+ under defined experimental conditions. *Design and Methods*. A very simple methodology involving UV-Visible spectrophotometry and fluorescence spectrometry was adopted to study the interaction of above agents with the probe molecule azotobactin. The nature and degree of interference offered by the blocking and mobilizing agents was studied for selection of the suitable agent. We have also investigated the competitive complex formation of Co3+ / Ga3+and Fe3+ for azotobactin in aqueous medium at pH 4.4. Association kinetics of scavenged serum NTBI with different mobilizing agents and then the displacement of scavenged NTBI by azotobactin from mobilizing agents were also established. Results: Results indicate that Co3+ in specific anionic complexes is effective in blocking apotransferrin- Fe3+ binding sites. Even at concentrations as high as 0.5mM there is no shift or decrease in the fluorescence emission spectrum of azotobactin as compared to Ga3+ which significantly affects the fluorescence properties. Competitive binding experiments also indicate that Co3+ does not interfere in the binding of Fe3+ to azotobactin.

Among the mobilizing agents studied, nitrilotriacetate being a mild chelating agent than the rest, efficiently scavenges all the available NTBI in serum and easily donates to azotobactin for its accurate quantification. The threshold concentration for NTA was found to be 80 mM. *Conclusions.* The present study shows that Co3+ and nitrilotriacetate are suitable intermediate agents for blocking apotransferrin and mobilizing NTBI respectively, since they do not affect either the spectroscopic properties of azotobactin or the binding kinetics of azotobactin and Fe3+.

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THE METHODOLOGY OF THE 'IRON ABSORPTION TEST'; ITS IMPORTANCE IN THE DIAGNOSIS OF IRON DEFICIENCY ANEMIA SECONDARY TO IRON MALABSORPTION

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We have performed the iron absorption curve with the examination of the serum iron concentration after 30, 60, 90, 120 and 180 minutes, subsequent to the administration of 325 mg ferrous sulfate, on a hundred normal subjects. The curves obtained show 50% - 200% increases of the iron level, (iron concentration) with the peak plasma level's values recorded after 90-120 minutes subsequent to the administration of the ferrous sulfate (after ingestion of iron salts). In this context, the methodology applied in the diagnosis of iron malabsorption has the following steps: Time 0: we take the sample for the basal iron level (concentration) and the immediate administration of 325 mg ferrous sulfate. Time 90 minutes: we take the sample for the second determining of (serum) iron concentration. Interpretation: an increase greater than 50% indicates a perfectly normal absorption of iron; increases lower than 20% indicate the iron malabsorption; increases between 20% and 50% require monitoring and reevaluating the subject. By applying this methodology, we have identified the frequency of occurrence of anemia through the malabsorption of iron as 3% of a sample consisting of 1323 subjects presenting the anemic syndrome, a frequency close to that of the occurrence of megaloblastic anemia (4%) and anemia caused by hemoglobinopathies (3.25%) in our sample. If we refer only to the 444 subjects with iron deficiency anemia the frequency of anemia through the malabsorption of iron amounts to 9% of the investigated subjects. We stress the fact that 25% of the subjects diagnosed with the iron malabsorption syndrome had previously been diagnosed as MDS-RA. The etiology of iron malabsorption identified in the 40 subjects has been represented by: Celiac sprue: 30%, Subtotal gastrectomy with Billroth anastomosis: 17.5%, Atrophic gastritis: 15%, Achlohydria: 7.5%, Exudative gastroduodenitis: 7.5%, Progressive systemic sclerosis: 5%, Polypoid lesion: 2.5%, erosive gastritis: 2.5%, subtotal pancreatectomy: 2.5%, Crohn disease: 2.5% exudative enteropathy: 2.5%, retroperitoneal lymphadenopathy: 2.5%, celiac sprue associated to subtotal gastrectomy: 2.5%. We stress the possibility of transitory iron malabsorption, as observed in 15% of the monitored subjects. *Conclusions*. The iron absorption test is indicated for subjects with iron deficiency anemia with unidentified etiology and that do not respond to the iron oral therapy. We underline the frequency of diagnostic error in these cases labeled as: MDS-RA, neoplastic disease, etc. The test will not be performed before PNH and the presence of gastric lesions with hemorrhagic potential has been excluded (ruled out). The methodology is simple, non-invasive, and the cost is reasonable.

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TREATMENT SATISFACTION (TS) OF THALASSAEMIA PATIENTS WITH IRON CHELATION THERAPY IN RELATION TO COMPLIANCE WITH CHELATING AGENTS AND DEPRESSION

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Introduction. Treatment satisfaction was referred to play a significant role regarding compliance with treatment followed by patients with a chronic disease. Also compliance with iron chelation therapy in thalassaemia patients was well documented as a prerequisite for patients' survival and was negatively affected by depression. *Objective*. To investigate patients' treatment satisfaction with different chelation regimens during one year period, in relation to compliance with different iron chelators such as Desferrioxamine (DFO), Deferiprone (DFP) alone or in combination, Deferasirox (DFX),as well as depression. *Design and Methods.* We studied 45 thalassaemics,21 males, 24 females, mean age 39,2 years, divided according to iron chelation into four groups. Group A: 14p on DFO(40 mg/kgx24 hsx6 days/week s.c.), Group B:10p switched to DFP(75 mg/kg/day p.o.), Group C:11p on combination therapy, Group D: 10p switched from DFO to DFX(30 mg/kg once daily p.o.). Every patient was privately interviewed exactly before starting new chelation scheme, and 1 year later . Treatment Satisfaction rating scale (TS-rs), Version 1.0, graded from 1 to 7, was used as adapted for Greek population. Compliance was estimated by Compliance Index. Depression level was determined by Hamilton rating scale (HAM-D). HAM-D score=>20 indicates depression. Psychological support applied for all patients and psychiatric treatment protocols for depressed patients did not change during the study period. *Results*. According to baseline data low TS, grade 3, in overall patients was detected, associated with increased HAM-D score>20 (80.2%), with medium and poor compliance. One year later no significant change in TS-rs was unexpectedly observed for patients either on DFO (from 3 to 4) or DFP (from 3 to 4-5), while encouraging was the improvement of HAM-D score=19 in depressed patients on DFP (25%). Although TS-rs increased for patients on combination therapy (Group C) from 3 to 5 and on DFX (Group D) from 3 to 6-7 , all patients in Group C remained depressed (HAM-D >20) while 80% of patients on DFX were detected with HAM-D=15 (p<0.001). *Conclusions*. In our series of patients depression was detected in a high percentage (80.2%). Treatment satisfaction was a very important factor associated directly with the route and frequency of chelator's administration, while TS improved significantly depression, as well as compliance with certain chelation regimens.

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EVALUATION OF IRON STATUS IN PATIENTS WITH RHEUMATOID ARTHRITIS (RA)

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Background. Anemia is the most common extraarticular manifestation of RA and is virtually always the result of either iron deficiency, active inflammation, or both. *Aim*. of the study was to evaluate the diagnostic efficiency of laboratory tests including serum transferring receptor and prohepcidin measurements in the diagnosis and differentiation of anemia in RA patients. *Materials and methods*. Blood samples were obtained from 32 RA patients. The samples were analysed for full blood count, iron, ferritin, transferrin, sTfR (serum soluble transferrin receptor) and prohepcidin. The TfR-F index (sTfR-log ferritin index) was also calculated. Results: Anemia was observed in 17 patients(53.1%), 8 of which had anemia of chronic disease(ACD) and 5 patients had iron deficiency anemia(IDA). The mean MCV, MCH, iron and Hb level was significantly lower in anemia RA patients compared with no anemia patients (p<0.001). The prohepcidin level was significantly lower in IDA patients compared with ACD and ACD+IDA patients(124.5 ng/mL versus 324.68ng/mL and 454 ng/mL; p<0.001). sTfR levels in IDA group was significantly higher than the ACD patients(4.49±0.95µg/mL versus 1.25±0.75µg/ml). We have found a high TfR-F index(3.9±0.82) in the IDA patients compared with ACD group(0.599±0.648). ACD+IDA patients had high sTfR and high TfR-F index compared with ACD patients(p<0.001). *Conclusions*. We conclude that the sTfR, TfR-F index and prohepcidin measurements represent efficient tests for the clinical evaluation and differentiation of anemia in RA patients.

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THE CARDIAC EFFECTS OF DESFEROXAMINE DEFERIPRONE COMBINATION THERAPY AND DESFEROXAMINE MONOTHERAPY IN THALASSEMIC PATIENTS

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Background. In the absence of adequate chelation therapy, cardiomyopathy due to iron overload and heart failure remains the leading cause of death in patients with β-thalassemia major (β-TM). The use of iron chelators is the mainstay of treatment in β-TM patients in order to ameliorate the inevitable complications of iron overload due to regular transfusions. *Aim*. The efficacy in reduction of myocardial iron and maintaining left ventricular functions of combinational therapy with deferoxamine and deferiprone was compared with single deferoxamine group using the gold standard measurement method of cardiac iron, T2* MRI. *Design and Methods*. The inclusion criteria for patient screening with cardiac T2* MRI were diagnosis of β-TM currently maintained on subcutaneous deferoxamine monotherapy; age ≥10 years and maintaining pre-transfusion hemoglobin > 9 g/dL. The patients were either initiated

combined therapy (n=26, Group 1) with oral chelator deferiprone 20-25 mg/kg/dose, tid in addition to subcutaneous deferoxamine 3-5 times a week 30-40 mg/kg/day or continued previous deferoxamine therapy as a single agent (n=13, Group 2). Exclusion criteria were previous initiation of deferiprone, neutropenia (ANC <1.5x10^9/L) or thrombocytopenia (<50 x109/L) at screening and liver enzymes above three times upper limit of normal. All patients were scanned on baseline and prospectively continued to be scanned every six months. The changes between initial and last MRI screenings' T2*, ejection fraction (EF) values and serum ferritin levels at these two points were compared with Wilcoxan Signed Rank for individual drug and for both drugs Mann Whitney U Test. Results The mean age of patients in Group 1 and Group 2 were 18.1±5.2 and 17.6±7.2 years, respectively. The groups were similar in terms of age and gender. The groups were also similar in terms of splenectomy (76.9% and 53.8%, respectively) and Hepatitis C virus (positive in 19.2% and 7.6%, respectively) status. The mean duration of treatments were similar (22.2±7.8 vs 19.6±7.5 months). There was no significant difference between groups in terms of initial serum ferritin levels (3357±2038 vs 2557±1105 ng/ml, respectively). The initial baseline T2* value of Group 1 was significantly lower compared to Group 2 (8.8±4.1 vs 23.7± 6.9 ms, p<0.001) and initial EF was significantly lower in Group 1, as well (48.7±9.2 vs 58.1±3.6%, p<0.001). In group 1, T2* value and EF measured at last MR significantly increased (12.8±7.3 ms, p<0.001 and 56.6±8.6%, p=0.001) compared to initial value of the same group. Whereas, T2* change was insignificant in Group 2 (24.4±5.6 ms, p 0.807), but EF increased to 61.5±4.9 and significant (p 0.039). When the T2* and EF changes compared between two study groups, the differences between T2* and EF values between groups were comparable (p>0.05). *Conclusions*. The results indicate that combinational therapy is effective in decreasing the iron overload in patients with β-TM, in terms of increasing T2* and EF values, compatible with the previously reported literature data.

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DEFERASIROX MONOTHERAPY MAINTAINS THE GOOD CARDIAC IRON STATUS IN THALASSEMIC PATIENTS

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Background. In the absence of adequate chelation therapy, cardiomyopathy due to iron overload and heart failure remains the leading cause of death in patients with β-thalassemia major (β-TM). The use of iron chelators is the mainstay of treatment in β-TM patients in order to ameliorate the inevitable complications of iron overload due to regular transfusions. Aim The effects of deferasirox on cardiac iron load are limited mostly to the gerbil models and unfortunately there is limited data on its effects in humans. The efficacy in reduction of myocardial iron and maintaining left ventricular functions of deferasirox was investigated using the gold standard measurement method of cardiac iron, T2* MRI. *Design and Methods*. The inclusion criteria for patient screening with cardiac T2* MRI were diagnosis of β-TM a wash-out period from the previous chelation for 24 hours; age ≥10 years and maintaining pre-transfusion hemoglobin > 9 g/dL. The patients were initiated deferasirox therapy as a single agent in a dose of 32±5.9 mg/kg/day (n=17). Exclusion criteria were neutropenia (ANC <1.5x10^9/L), thrombocytopenia (<50 x10^9/L) at screening, serum creatinin exceeding the normal upper limit for age, liver enzymes above three times upper limit of normal. 10 mg/kg dose reduction in deferasirox dose was established if serum creatinin increases more than 50% of the baseline value, but still within normal limits. All patients were scanned on baseline and prospectively continued to be scanned every six months. The changes between initial and last MRI screenings' T2*, ejection fraction (EF) values and serum ferritin levels at these two points were compared with Wilcoxan Signed Rank. Results The mean age of patients were 18.1±7 years (10-33). The male to female ratio 9/8. All of the patients were HBsAg negative and Anti-HBs positive, whereas 1 (5.8%) patient was HCV positive. The splenectomy was performed in 43.7% of the patients. The mean duration of treatment was 11.9±5.9 months. The initial serum ferritin level was 2630±1430 ng/mL (917-7293). The initial baseline T2* and EF values were 25.8±6.4 ms (15.4-39.5) and EF 61.4±5.1% (43.4-73.2), respectively. By the end of the follow-up T2* and EF measured as 24.6±5.2 ms (18.3-36.3) and 63.3±6.8% (50.8-72), respectively; the differences being insignificant. *Conclusions.* Our data on cardiac efficacy of deferasirox is limited, although the initial gerbil studies reported suggest promising data. The initial T2* values of all of the patients in study were above 15 ms, indicating a mild cardiac iron accumulation. But these results may indicate the maintenance of good iron status be deferasirox in at least mildly affected patients. The most important limitation in our knowledge of deferasirox is its being a new drug and the time and further prospective studies will enlighten the efficacy better, especially in the patients with lower intial T2* values.

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A RARE HEMOCHROMATOSIS TRANSFERRIN RECEPTOR 2 MUTATION

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Background. Hereditary hemochromatosis, a disorder of iron metabolism causes iron overload due to high intestinal absorption with several clinical complications and is prevalent in whites. Usually hemochromatosis presents with mutations in the HFE gene on chromosome 6. About 85% of persons with the established disease are homozygous for the C282Y mutation. The H63D mutation may contribute to the development of hemochromatosis (1.5%), together with the S65C. Other gene mutations include the transferring receptor 2 and the ferroprotein. *Aims*. To establish the genetic basis of a patient with clinical presentation suspicious of hemochromatosis. A 70-year old Caucasian woman from Epirus, Greece with previous history of arterial hypertension, coronary heart disease and a permenant pacemaker, type II diabetes mellitus, and chronic renal failure presented to the outpatient clinic with severe dyspnea and vomiting. *Design and Methods*. General blood analysis, biochemichal liver and iron related tests and genetic testing with Haemochromatosis Strip Assay A (TM) (VIENNA LAB, AUSTRIA). *Results*. On clinical examination orthopnoia, bilateral pleural effusion, enlarged liver size, splenomegaly and ascites were detected. General blood analysis showed leucocytosis (19.72x10^9/L) and mild thrombocytopenia (102x10^9/L). The biochemical tests revealed markedly increased liver function tests ALT 925 IU/L, AST 286 IU/L, GT 397 IU/L, total bilirubin 2.8 mg/dL, direct bilirubin 1.2 mg/dl, serum ferritin 17709 ng/mL and normal TIBC and iron. HBV, HCV and HIV serology was negative. Based on the clinical features and laboratory tests an abdomen ultrasound was performed which confirmed the enlarged liver without the presence of any intra- or extrahepatic findings. Due to the excessively high serum ferritin levels, the clinical findings and the patient history, genetic testing for the presence of hemochromatosis was ordered. The analysis showed wild-type HFE gene for hemochromatosis and a T-->A transversion (T515A), which causes a Methionine-->Lysine substitution at position 172 of the protein (M172K) on TFR2 gene. M172K has a complex effect: it causes a missense in the α-form, but it may also prevent the β-form production because it affects its putative initiation codon. *Conclusions*. The mutation has a variable severity, from asymptomatic to severe clinical expressions. The mutated TFR2 gene detected in the patient implicates that all patients with suspected hemochromatosis should be tested for TFR2 mutation in addition, if an HFE genotype is not mutated or has the heterozygote form. Last, there is a current attempt to approach 1st degree relatives of the patient in order to establish a family pedigree for the rare TFR2 mutation in our region.

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MTHFR MUTATION IN PATIENTS WITH SICKLE CELL ASSOCIATED OSTEONECROSIS OF THE FEMORAL HEAD

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Sickle cell disease is commonly associated with Osteonecrosis (Avascular Necrosis) of the head of femur even in patients with mild sickle cell disease. Suspected risk factors include associated MTHFR gene mutation, α thalassemia trait, elevated HbF level and elevated hematocrit. We studied 55 patients with severe sickle cell disease (32 males and 23 females,(aged 18-43 years), 31 with Osteonecrosis of the femoral head (ON group, 24 males and 7 females) and 24 without Osteonecrosis of the femoral head (Control group, 8 males and 16 females). Each patient gave a consent and had a complete medical history and physical examination,examination,complete blood count, magnetic resonance imaging (MRI) of both hip joints, Hb electrophoresis, molecular diagno-

sis of MTHFR mutation by PCR-RFLP method, Hb F and Hb A2 level by column chromatography Results MTHFR mutation was found in 7 ON patients (3 males & 4 females=0.22) and 5 patients in the control group (one male & 4 females =0.21) without any significant difference between the two groups. Similarly, high Hb F level (>10%), and high Hb A2 (> 3.5%) were similar in both groups. *Conclusions*. There was no evidence that MTHFR mutation, elevated HbF or HbA2 (β Thalassemia) are risk factors or associated with Osteonecrosisof the femoral head in patients with sickle cell disease.

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PREDICTIVE VALUE OF HDL & CHOLESTEROL/HDL RATIO IN THALASSAEMIA MAJOR PATIENTS

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Background. A low level of high density lipoprotein (HDL<35 mg/dL) is an important risk factor for atherosclerosis and overt Coronary Heart Disease. Total cholesterol to HDL ratio is also suggested to have greater predictive value for CHD than serum total or LDL-cholesterol. Although the prolonged survival in thalassaemia major, heart mortality remains the major cause of death. These patients are characterized by a hypercoagulability state and a high incidence of thromboembolic events. Aim: Investigate the influence of standard serum lipid profile in cardiac and other co-morbidities in β-Thalassemia major patients (TMps). *Design and Methods*. 46 TMps, 23 males, mean age 33.9±1.8, and 23 females, mean age 37.6±0.5 were assessed. -Lipid profile included measurements of total cholesterol, triglycerides, LDL and HDL-cholesterol -Genetic thrombophilia mutations were investigated by PCR analysis of a sample of DNA for Factor V Leiden G1691A, Factor II G20210A, MTHFR C677T and PAI 4G/5G. -Cardiac function was assessed by echo-Doppler - Endocrine function by dynamic tests (OGTT) and hormonal screening (TSH, FT4) -Statistical analyses were performed using SPSS (p<0.05 was considered significant). *Results*. 20/23 (87%) males, had HDL<35mg/dL but only 8/23 (35%) an abnormal LDL>80 mg/dL and 9/23 (39%) high triglyceride concentration >150 mg/dL. All of them had normal cholesterol=120.3±22.5mg/dL. Inversely total cholesterol/HDL ratio was abnormal >5 in 15/23 (65%). The low levels of HDL and high cholesterol/HDL ratio in males TMps were correlated with: cardiac dysfunction: mean LVEF=60.6±6.9% (normal values for TMps >63%); Glucose metabolism disturbances (r=0.629, p<0.01); 7/15 (47%) were hypothyroid (TSH 5-10mU/mL and normal or decreased FT4) ; 13/15 (87%) carried abnormal (homozygous or heterozygous) MTHFR or/and PAI. 12/23 (52%) females had HDL<35 mg/dL but only 9/23 (39%) an abnormal LDL>80 mg/dL and 3/23 (13%) high triglyceride concentration >150mg/dL. All of them had normal cholesterol=130.5±36.2 mg/dL. Inversely total cholesterol/HDL ratio was abnormal >5 in 7/23 (30%). The low levels of HDL and high cholesterol/HDL ratio in females TMps were correlated with: Glucose metabolism disturbances (r=0.568 p<0.02); 2/7 (29%) had compensated hypothyroidism; 6/7 (86%) carried abnormal (homozygous or heterozygous) MTHFR or/and PAI; Normal cardiac function: mean LVEF=65.3±3.8%. In TMps with low levels of HDL and high cholesterol/HDL ratio mean Body Mass Index (BMI) was greater 23.6±3.1 than in TMps with normal HDL and cholesterol/HDL ratio (22.3±1.8). (iii) no correlation was found with mean Ferritin levels and Liver Iron Concentration or liver function. *Conclusions*. The predictive value of HDL & Cholesterol/HDL ratio appears to be high in Thalassemia major patients for whom we must exercise vigilance for close follow up of diabetes (as the lipid pattern is largely related to the glycemic control because is be due in part to hyperinsulinemia and can be detected before the onset of overt hyperglycemia), thyroid and cardiac function mainly in male TMps. Among thrombophilic mutations no prevalence of factor V Leiden and prothrombin G20210A mutations were found in TMps with low levels of HDL and high cholesterol/HDL ratio, but 86-87% had abnormal (homozygous or heterozygous) MTHFR or/and PAI.

1300

EFFECT OF IRON FORTIFIED MILK ON HEMATOLOGICAL CHANGES IN NON-IRON DEFICIENT THAI-SCHOOL CHILDREN

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Background. Iron deficiency has been known as a major cause of anemia in school aged children. To prevent iron deficiency anemia in this group, implementation of iron fortified milk has been proposed among other strategies. *Aims*. To determine the short term effect of iron fortified milk on hematological parameters of 4thgrade Thai-school children. *Design and Methods*. One-hundred and fifty-five 4th-grade apparently healthy Thai-school children were recruited. Written informedconsent was obtained from their parents. Subjects were divided into two groups; i.e. group 1 (intervention group) comprised 76 children who received 200 mL milk fortified with 5 mg iron (NaFeEDTA) daily, and group 2 (control group) comprised 78 children who received 200 mL of non-fortified milk daily. Blood samples were taken before starting the implementation and after three months when the implementation was terminated. Hematological parameters and ferritin were determined from all blood samples taken. Hb and DNA analyses were performed to detect thalassemia and hemoglobinopathies. *Results*. Based on ferritin level less than 20 ng/mL, none of the subjects had iron deficiency. No significant difference in the proportion of thalassemia trait between the two groups was observed. Hematological analysis in the intervention group revealed no significant difference in mean Hb levels before and after implementation (12.2 g/dL vs. 12.3 g/dL). For control group, a significant reduction in mean Hb levels was observed (from 12.6 g/dL to 12.3 g/dL). Although the MCV and MCH values significantly increased for both groups, the increment (2.6 fl for MCV and 0.5 pg for MCH) was significantly higher for the intervention group in comparison to the control group (2.1 fl for MCV and 0.2 pg for MCH). *Conclusions*. Although Hb concentrations in children who received iron fortified milk did not increase, a constant Hb level and a significantly higher MCV and MCH values suggest that iron fortified milk may be one of the strategies to maintain Hb level in school children living in areas with a well known high prevalence of iron deficiency.

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SAFETY, EFFICACY AND EFFECT ON QUALITY OF LIFE OF PARENTERAL IRON SUCROSE INFUSION IN PEDIATRIC PATIENTS

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Backround. Intravenous (iv) iron administration is used as a blood transfusion alternative in cases where blood parameters must be raised rapidly or for patients where oral administration is not indicated. However, experience on parenteral iron therapy is based mainly on adult patients receiving hemodialysis. Data regarding children, including iron pharmacokinetics and maximum tolerated doses of iv infusion, is limited. *Aims*. We aimed to determine the effects of iv iron therapy on blood parameters in pediatric patients who do not tolerate or fail to respond to oral iron therapy for various reasons; also, to define adverse events following iron infusion and to correlate possible side effects to infusion dose; finally to assess the effect of multiple parenteral infusions with regards to both safety as well as to quality of life of children receiving serial iv iron administration. *Design and Methods*. Iv iron was administered to 17 children, aged 14 months to 15 years (mean 6.5±4.3 years). Children received parenteral iron due to malabsorbtion, gastrointestinal blood loss, oral supplementation intolerance or bad compliance. In detail, 2 patients were diagnosed with celiac disease, 2 with allergy to cow's milk proteins, 1 with ulcerative colitis, 4 with cerebral palsy, 1 with autism, 1 with Aicardi syndrome, 1 with mental retardation, 1 with Down syndrome, 1 with immune deficiency, 2 with refractory anemia of unknown cause and 1 with inadequate iron intake due to sole breast-feeding (at the age of 14 months). Blood samples were taken before infusion (day 1), as well as 15 and 45 days following infusion. Hemoglobin values, erythrocyte indices and serum ferritin levels were measured. Iron sucrose was used as an intravenous iron preparation and a test dose, although not required, was used. Total iron to be infused was

determined by the formula: *total iron [mg] = weight [kg] x (expected Hb - patient Hb) [g/dl] x 3, where expected Hb = 12.5 g/dl.* In total 55 infusions were administered (1-12 infusions per patient), with a mean dose of 8.76±4.47 mg/kg (max 17.7 mg/kg) and the total single infusion dose not exceeding 200 mg. Results: A statistically significant elevation occurred between the time of diagnosis and the time of follow-up in all parameters. No adverse events were noted and no features of systemic iron toxicity encountered, even when large doses or multiple infusions were used. Children receiving multiple infusions remained free of oral iron during follow-up, both children and their parents reporting a significantly improved quality of life during the period of infusions with regards to school performance, physical activity and sense of general well being. *Conclusions*. Parenteral iron therapy in children is a rapid, easy and definitive solution and can replace oral therapy in a variety of pediatric diseases which lead to severe iron deficiency and where oral iron administration is contra-indicated. Intravenous iron seems to be safe even when administered to very young children or infants. Multiple infusions can be administered when required, offering a better quality of life to children suffering from chronic diseases.

1302

EFFICACY AND SAFETY OF PREOPERATIVE RECOMBINANT HUMAN ERYTHROPOIETIN ADMINISTRATION IN PATIENTS UNDERGOING SURGERY FOR HIP FRACTURE REPAIR. A PROSPECTIVE OBSERVATIONAL STUDY

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Background. Preoperative anaemia is a major risk factor for allogeneic blood transfusion (ABT) in patients undergoing hip fracture repair. Aims. We prospectively investigated the effect of a blood conservation protocol on ABT requirements in 196 consecutive hip fracture patients presenting with haemoglobin (Hb) between 10 g/dL and 13 g/dL. *Design and Methods*. The blood conservation protocol consisted of the application of a restrictive transfusion trigger (Hb<8 g/dL) and the peri-operative administration of IV iron sucrose (3x200 mg/48 h) (Group 1, n=115). Additionally, 81 patients received recombinant human erythropoietin (rHuEPO, 40,000 IU sc) on admission to the orthopedic ward. *Results*. Overall, 103 out of 196 patients (52.5%) received at least one ABT unit (2.1±1.0 U/patient). However, there were significant differences in perioperative ABT rates between groups (60% vs 42%, for groups 1 and 2, respectively; p=0.013). Postoperative Hb on postoperative days 7 and 30 were higher in Group 2 than in Group 1. In addition, in Group 2, Hb levels were higher on postoperative day 30 than on admission (12.7±1.0 g/dL vs. 11.9±0.8/dL, respectively; p=0.030). ABT, but not rHuEPO, increased postoperative complication and 30d mortality rates. Only 3 mild IV iron adverse effects were witnessed. *Conclusions*. Peri-operative IV iron and single doses of rHuEPO plus restrictive transfusion trigger seem to be safe and effective in reducing ABT in anaemic hip fracture patients. However, appropriate diffusion and a persisting awareness is needed to avoid protocol violations and to limit further the exposure to ABT and ABT-related risks.

1303

EVALUATION OF DIAGNOSTIC RELIABILITY OF DIFFERENT RBC INDICES IN THE DIFFERENTIATION OF MICROCYTIC HYPOCHROMIC ANAEMIAS

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Background. Iron deficiency anaemia (IDA) and β-thalassemia trait (βTT) are the most common causes of hypochromia and microcytosis in adults as well as in children. *Aim*. This study evaluates the diagnostic reliability of various red blood cell (RBC) indices and formulas in the differentiation of IDA and βTT. *Design and Methods*. In 458 children of both sexes (260 males, 198 females) aged between 1.8 and 7.5 years (mean age 5.6±1.7 yrs) with hypochromia and microcytosis certainly diagnosed on the bases of blood count, iron assessment and quantitative identification of HbA2 by HPLC (253 IDA, 105 βTT), we have evaluated the diagnostic reliability of the following indices and formulas: *Red Distribution Width index (RDWI), Mentzer index (MI), Green & King index (G&K), England & Fraser index (E&F)*. The discrimination indices incorporate MCV, MCH, RBC, RDW and Hb in various combinations. The Sensitivity (SENS), Specificity (SPEC), Positive and Negative Prognostic Value (PPV and PNV), Efficiency (EFF) and Youden's Index (YI) were calculated. Gauss curves were also constructed to evaluate the reliability of each index and formula. *Results*. In children with IDA haematological data were: RBC 4.75±0.35x10^{12}/L, Hb 10.2±1.7 g/dl, MCV 71.7±3.1 fl, RDW 17.3±3.1%; in children with β-TT mean values of RBC were 5.5±0.9x10^{12}/L, Hb 11.3±1.5 g/dl, MCV 63.2±2.6 fl, RDW 16.2±3.4%. The G&K shows the highest reliability, with SENS 78%, SPEC 97%, PPV 99, NPV 56, E&F 82, YI 71. The other calculated indices show lower reliability. *Conclusions*. Our study shows that all discrimination indices that were examined cannot be relied on for a safe differentiation between IDA and β-TT. The G&K Index results highly specific but its sensitivity is not satisfactory. We conclude that the certainly differential diagnosis between IDA and β-TT must be performed by blood count, iron assessment and quantitative identification of HbA2.

1304

SCREENING OF HIGH RISK ETHENIC GROUP FOR HEMOGLOBINOPATHIES: IS IT MORE COST EFFECTIVE THAN NO SCREENING

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Background. Egypt is an African country and at the same time is one of the Mediterranean area countries. Consequently, mutations of ß-globin and a-globin genes can occur resulting in high incidence of ß-thalassemias and a-thalassemias among children. Similarly, sickle haemoglobin is common in African ethnic group and in association to a-thalassemia with proposed increase in incidence of sickle cell disease (SCD) in Egypt. In this analysis we examined blood samples from Egyptian newborns in a trial to determine whether screening for hemoglobinopathies is more cost effective than no screening. Aim. Our primary goal is to identify diseased phenotypes. *Design and Methods*. Peripheral blood samples were collected from 2000 Egyptian newborns, after taking written consents from parents, and examined for hemoglobinopathies using hemoglobin electrophoreses. Haemoglobin (Hb) phenotype nomenclature follows a standardized format in which the order of the letters indicates a relative quantity of Hb present (i.e. HbFSA indicates HbF>HbS>HbA in the sample). High performance liquid chromatography was used to determine the variant β-globins (e.g. D, E, G). *Results*. 280 neonates are HbF phenotype, of them 230 are thalassemia major and the remaining 50 are extreme premature. 150 neonates are HbAF phenotype (transfused phenotype). 34 neonates are HbFA2 phenotype (thalassemia trait). 3 neonats are HbFS phenotype (SCD) and 6 neonates are HbFAS, HbFAC or HbFAD phenotype (trait phenotype). 1527 neonates are HbFA phenotype (normal phenotype). Identification of homozygous βs (HbSS or SCD) or Thalassemia major (HbF only) at birth allows a comprehensive clinical care and decreases mortality. Identification of trait phenotypes requires no specific hematologic follow up but family studies with genetic counselling can clarify the risks of disease in future children, especially in populations with high carrier rates. *Conclusions*. Screening of Egyptian newborns as a high risk ethnic group is more cost-effective than no screening. Practitioners should be familiar with the screening programs in Egypt and similar areas of high risk ethnic group.

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CEREBROVASCULAR ACCIDENT ASSESSMENT IN A POPULATION OF HBS/B-THALASSAEMIA PATIENTS

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Background. Cerebrovascular accident (CVA) is a potential complication of sickle cell anemia, which ranges from overt stroke (with abrupt onset of neurological deficit) to silent infarcts, which are not acutely clinically apparent but are associated with cognitive impairment and increased risk of secondary overt stroke. In populations of mostly HbSS patients, the prevalence of silent infarcts in children with sickle cell disease has ranged from 17% to 22-35%, while 11% will have a clinically apparent stroke by age 20 years and 24% by age 45 years. *Aims*. We assessed the incidence of CVA in a population of exclusively HbS/β-thalassaemia patients. *Design and Methods*. Brain MRI was obtained from each patient in a population of 20 HbS/β-thalassemia patients and was analyzed for evidence of CVA. Their age ranged between 5 and 33 years old (mean age = 17.7 years). All patients were in steady state with no neurological signs. *Results*. Seven patients had prior brain MRI 8 years

before, with silent infarcts having been identified in two of them. In the follow up, one of these two presented the same MRI condition, with no new findings added up, and the second presented a normal MRI. While the study was in progress one patient with a prior normal scan, underwent a clinically overt stroke confirmed by a new brain MRI. All other patients scanned had normal findings. Transfusion regimens have shown to reduce both primary and secondary risk for CVA. In our cohort four patients were under transfusion regimen at some point before the scan, for a period of 3 to 12 months (median = 4 months), 3 while treated for Avascular Necrosis of the Femoral Head and one during a period of excess spleen enlargement. Also hydroxyurea as an alternative to transfusion therapy for secondary CVA prevention was proposed since a decade ago. Four of our patients are under hydroxyurea treatment for a period of 1.5 to 8 years (median = 2 years). Notably the patient showing improvement in the follow up underwent both treatments. *Conclusions*. One overt stroke was noted (5%).The incidence of silent infarcts in the population of our study was 5%(1 of 20), which is slightly lower from that noted by other researchers, and can be attributed to the lighter clinical course of HbS/β-thalassaemia patients. More studies are needed to establish new therapeutic protocols in order to further reduce CVA risk in sickle cell disease.

1306

PARAOXONASE AND ARYLESTERASE ACTIVITY WITH OXIDATIVE STATUS IN CHILDREN WITH THALASSEMIA MAJOR

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Background. Paraoxonase deficiency is related to increased susceptibility to low density lipoprotein oxidation and development of atherosclerosis. *Aim*. The aim of this study was to study paraoxonase and arylesterase activities along with oxidative status parameters, and to find out if there is any increased susceptibility to atherogenesis, which might be reflected with increased oxidative stress and decreased serum Paraoxonase/arylesterase activity in β-thalassemia major (BTM) patients. *Design and Methods*. Eighty-seven subjects with BTM and 33 healthy subjects were enrolled in the study. Results: Paraoxonase and arylesterase activities were significantly lower in BTM subjects than controls (for all $p<0.0001$), while total oxidant status, total peroxide concentration levels and oxidative stress index were significantly higher ($p<0.0001$, $p<0.0001$ and $p<0.001$; respectively). In BTM subjects were correlations found between serum iron and ferritin and levels of total oxidant status, oxidative stress index, total antioxidant capacity. Significant correlation were found with serum total peroxide concentration levels and paraoxonase and arylesterase activities in patients with BTM. *Conclusions*. It was seen that oxidative stress increases, while serum paraoxonase activity is decreased in BTM subjects. Decrease in paraoxonase activity seems to be associated with both the degree of oxidative stress and anemia. BTM subjects may be more prone to development of atherogenesis due to low serum Paraoxonase/arylesterase activity.

1307

ERYTHROCYTES ACID AND ALKALINE FRAGILITY AS A CHARACTERISTIC OF THE MEMBRANE-ACTING PROPERTIES OF A CU/CD ACETATE COMPLEX WITH ETHYLENEDIAMINE - A POTENTIAL THERAPEUTIC AGENT

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Background. In previous study was shown bactericidal, fungicidal and antitumor (cytotoxicity effect on human erythroleukemia cell (K562), breast cancer cell (MCF-7) and Lewis lung carcinoma cell (LLC)) activities of a novel Cu/Cd acetate complex with ethylenediamine (code PO244). The aim is to study of Cu/Cd acetate complex with ethylenediamine cells memrane effects. *Method.* Erythrocytes fragility under action of PO244 [Cu(en)2][Cd2(CH3COO)6] and 0.75 mM or 7.5 mM HCl (acid fragility), or 7.5 mM NaOH (alkaline fragility) has been studied. *Results.* The Cu/Cd acetate complex with ethylenediamine interacts with membranes of erythrocytes what is manifested by increasing of the fragility of these cells to acid or alkaline action. PO244 increases the fragility of "old" and middle life cycle erythrocytes. The most active component of the complex towards membranes of erythrocytes must be copper (II) what is supported by hemolysis of 100% erythrocytes immediately after an injection of acid or alkaline after incubation of the cells with 10-4 M CuCl2. The influence of lower concentrations (10-6, 10-7 M) of these ions gives evidence that acid resistance of both "old" and "young" erythrocytes is decreased. The harmful influence of copper(II) exceeds that of PO244 more than four times at the same concentration of copper. Cadmium(II) ions (10-4 M) do not change acid fragility but decrease alkaline fragility of erythrocytes. Ethylenediamine increases fragility of "old" and middle life cycle erythrocytes. Sodium acetate increased in fragility of "young" and the middle life cycle erythrocytes. *Conclusions*. The influence of PO244 on erythrocytes is a result of the action of the initial compound and not the products of its decomposition what is confirmed by the different influence on the erythrocytes stability of the complex investigated itself and its "components": salts of copper (II) and cadmium, ethylenediamine and sodium acetate.

1308

IRON CHELATION THERAPY WITH DEFERASIROX IN ADOLESCENTS AND YOUNG ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Deferasirox is one of the main iron chelation agents for patients with hemoglobinopathies and myelodysplastic syndromes. Its role as iron chelation therapy has not been studied in patients with acute lymphoblastic leukemia (ALL). *Aim.* The investigation of the safety and effectiveness of Deferasirox as iron chelation therapy in young patients with acute lymphoblastic leukemia (ALL) who were over transfused with packed red cells during intensive chemotherapy. *Design and Methods*. Two patients with ALL aged 17 and 19 years old at diagnosis who were treated with intensive chemotherapy according to the high risk arm of the ALL-BFM 2000 protocol, were studied regarding the iron overload due to blood transfusions during their treatment and deferasirox administration. *Results*. Both patients started on deferasirox in a mean dose of 10 mg/kg, after the completion of intensive chemotherapy, during maintenance phase therapy with daily oral 6-Mercaptopurine and oral Methotrexate once weekly. The first patient had serum ferritin levels 8798 ng/mL after he had received 280mg iron/kg in an11month period (0.84 mg iron/kg/day) while the second one had ferritin levels 3533ng/mL following the transfusion of 338mg iron/kg during 2 years period (0.45mg iron/kg/day). The second patient has already finished his treatment protocol and is currently on follow up. Both patients had good compliance with deferasirox without any adverse effect. Temporary elevation of serum creatinine was reduced to normal with deferasirox dose adjustment. Six months after deferasirox initiation the first patient had serum ferritin levels 3100 ng/mL and the second one 2445ng/mL respectively. Both are still on iron chelation therapy and on follow up by cardiac T2* and liver iron concentration (LIC) measurements. *Conclusions*. Iron chelation therapy probably should be considered for high risk adolescents and young adults with ALL who are treated with very intensive protocols essential for cure to be achieved. The dose of deferasirox is under consideration.

1309

DETERMINATION OF β-GLOBIN GEN HAPLOTYPE IN SICKLE CELL ANEMIA PATIENTS IN AHWAZ AT 2007

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Introduction. The hemoglobinopathies affect the blood red cells are the most common monogenic disease world wide. The high frequency and clinical severity of hemoglobinopathies make them a major public health problem. Sickle cell disease is the most common hemoglobinopathy in the world. Clinical manifestation in some patients are severe and in some are mild. It is suggested that β-globin gene haplotype influence the clinical presentation of sickle cell disease. *Aim of study.* In this study the aim was to determine the origin of sickle cell mutation by determination of common haplotypes of βS gene cluster in sickle cell patients from Ahwaz ; a city in Southwest province of Iran. Method: Molecular genetic studies were undertaken to determine the haplotypes of chromosomes carrying the sickle-cell allele. From each SS patient 5 mL EDTA blood was taken and stored at -20. DNA prepared from the subjected leukocytes by DNA extraction kit. Haplotype analysis of the b-globin like gene cluster was performed by using the following restriction endonucleases: Hind II, Hind III, Xmn1, Ava II. Agarose gel electrophoresis of PCR and enzyme digested products were performed using 2%

agarose. *Results.* All seven haplotypes were found in our study patients. Arab-Indian haplotype was found to be the most prevalent haplotypes in this study population. This haplotype accounted for 12.9% as the homozygous form in total SS patients, while 51.8% of the chromosomes had Arab-Indian haplotype. Benin haplotype with prevalent of 12.9% and BantueA2 with prevalent of 11.1% were the second and third prevalent haplotype among all patients. Incidence of homozygote haplotype was 44.4%.. *Discussion and Conclusion*. The presence of the Arab-Indian haplotype as the predominant haplotypes might be suggestive of a gene flow from Saudi Arabia or India to this area and presence of Benin , Bantue A2 and Senegal haplotypes in this area must have occurred during the Arab slave and trade with Arabian countries.

1310

USEFULNESS OF THE PARAMETER RET-HE IN THE INITIAL DIAGNOSIS APPROACH OF THE ANEMIA DUE TO CHANGES OF THE IRON METABOLISM IN SUBSTITUTION OF THE STANDARD BIOCHEMISTRY DETERMINATION (SERUM FERRITIN)

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Background. Recently, there have appeared new generation autoanalyzers which have brought new parameters to the semiology of the red series such as the 'Content of Haemoglobin Reticulocytes'. This series is a marker capable of providing information about the contribution of iron to erythropoieisis, allowing the detection of iron deficiency in the synthesis of haemoglobin. Among these are the 'Chr' of Advia autoanalyzer and the "Ret-He' of the Sysmex 5000. Several groups have reported the presence of statistically significant differences between mean values of Content of Hemoglobin reticulocytes in Chronic Diseases Anemia (CDA) regarding to the Ferropenic Anemia (FA) and health control. These findings appeared at the same time with the contribution of knowledge to the CDA pathophysiology that became hepcidin. They also define the mechanism by which inflammation is an iron sequestration at the mononuclear phagocytic system, and would be consistent with values "Ret-He" intermediate between the FA and the healthy controls. *Aims*. 1-Describe the middle values of Ret-He in the patients diagnosed of FA and CDA during 2006-2007 in our service, as well as control group. 2-Analyze the usefulness of the parameter Ret-He in the initial diagnosis approach of the anemia due to changes of the iron metabolism in substitution of the standard biochemistry determination (level of serum ferritin). *Design and Methods*. A total of 672 samples were processed with the autoanalyzer Sysmex 5000, of which 385 were FA, CDA 130, and 157 normal controls. Statistical analysis was performed with SPSS 14.0 software. *Results*. Statistical analysis using the Student t for samples with different population variances showed that the difference in the mean value of "Ret He" was statistically significant (p<0001) To test the potential usefulness in the initial approach to study the probability of diagnosis by the design of contingency tables that cover the following variables: FA if Hb<10 gr/dl , MCV <78 fl and ferritin <20 mg/dl, FA if Hb<10 gr/dl , MCV<78 fl and Ret-He <23 pg, CDA if Hb<10 gr/dl , MCV 80-100 fl and Ferritin >200 mg/dl, CDA if Hb<10 gr/dl, MCV 80-100 fl and Ret-He 26-30pg. In FA found in a similar probability for the diagnosis (169 cases with Ferritin and 160 in Ret-He group) with an advantage for the reason 'Diagnostico FA Ret-he <23' of 49x (95% CI 19,8-121, 5) against not. For the group of CDA, we found a difference in value against the Ret-He versus standard ferritin (33 of 17 cases) with an advantage for the reason, 'Diagnosis of CDA Ret-He26-30 'only 3.1 x (IC 95% 1,63-5,89) against not. *Conclusions.* In this work, we found significantly different statistical Ret-He values for the different groups of patients (CDA, FA, normal) coherent with present phisiopatologic knowledge. We found similar probability for the diagnosis of FA to replace the standard "Ferritin" with this new parameter, however this is not reproducible advantages for patients in group CDA.

Table.

	Average	Median
FA (n=385)	22,41 pg/dL	21,82
CDA (n=130)	28,41 pg/dL	28,45
NORMAL (n=157)	33,29 pg/dL	33,40

1311

PROMPT RECOVERY OF END STAGE ACUTE HEART FAILURE IN β-THALASSAEMIA MAJOR AFTER INTENSIVE COMBINED CHELATION: A CASE REPORT

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Background. Heart failure (HF) caused by myocardial iron overload, is the leading cause of mortality in Thalassaemia major patients (TMps). Continuous IV Deferoxamine is considered to be the standard of care in these cases. Although combined chelation has been demonstrated to be of clear benefit in the treatment of cardiac siderosis in TMp, its role in the management of severe end-stage HF has not been specifically addressed. Aims: To evaluate the effectiveness of an intensive combined chelation protocol in the case of an end-stage acute HF of a 28 year-old male TMp. *Patient*.A transfusion dependent splenectomised TMp, non-compliant to chelation therapy who the last 3-5 years developed non insulin-dependent diabetes, subclinical hypothyroidism (reduced FT4, FT3), hypoparathyroidism (PTH=5.2 pg/mL), hypocalcemia=6.3 mg/dL and Hypogonadism (reduced LH & Testosterone). He was presented with acute decompensate HF (NYHA) class IV: fatigue, weakness, inability to perform usual activities, weight gain, abdominal distention, generalized edema and dyspnoea. *Design and Methods*. Echo-Doppler: dilated cardiomyopathy, Left Ventricular (LV) systolic & diastolic dysfunction with concomitant Right Ventricular (RV) enlargement & systolic dysfunction and dilatation of vein cave, abdominal ultrasound: hepatomegaly (22 cm) & abundant ascetic collection. Laboratory tests: BNp=414 pg/mL, important increase of ALT, AST. Iron load was evaluated by Ferritin (MEIA), Signa-MRI 1.5 Tesla T2* sequences for the heart & the liver and Liver Iron Concentration (LIC) calculated by Ferriscan. He was hospitalized in a specialist center for close monitoring. The treatment focused on two goals: intensive combined chelation with Deferiprone 120 mg/kg/d & IV Deferoxamine 60 mg/kg/d in order to decrease heart hemosiderosis and management of heart dysfunction (IV diuretics, Sodium & fluid restriction, hemodynamic stabilization, support of oxygenation and symptom relief). *Results.* After 6 months of outcome treatment with diuretics, β blockers, Calcium, vit D and intensive combined chelation (Deferiprone 120 mg/kg/d & SC Deferoxamine 60 mg/kg/d): serum Ferritin decreased dramatically from 6.839 μg/L to 632 μg/L, MRI T2*H increased from 1.6 ms (very severe hemosiderosis) to 3 ms (severe hemosiderosis) and T2*L improved significantly from 0.5 ms (severe hemosiderosis) to 5.5ms (mild iron load), LIC decreased significantly from 52 mg/g/ dwt (very severe hemosiderosis) to 4.8 mg/g/dwt (mild iron load), progression from HF NYHA class IV (severe) to class II (mild) with substantial regression of symptoms: weight loss (25Kg), no edemas, no dyspnoea and a slight limitation of physical activity. Echo-Doppler: improvement of systolic & diastolic LV function and LVEF from 27% to 43% and regression of RV systolic dysfunction. Regression of hepatomegaly and normalization of ALT, AST. No more ascetic collection. No adverse events were observed from intensive combined chelation. *Conclusions*. This case report demonstrates that intensive combined chelation with Deferiprone 120 mg/kg/d & Deferoxamine 60 mg/kg/d along with symptomatic treatment could improve end-stage acute HF promptly and preserve TMp life. It is the first time that Deferiprone was used in the dose 120mg/kg/d without any side effects and so tremendous and rapid results. The cardio-protective effect of Deferiprone, as reported in many publications, give us the expectation of complete restoration of cardiac function in short term.

1312

CHELATION THERAPY IN MAJOR β THALASSEMIA

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Background. Progressive iron overload is the life-limiting complication of transfusion therapy.Effective and convenient iron chelation remains one of the main targets of clinical management of thalassemia major. The combined treatment with deferoxamine (DFX) and Deferiprone (DFO) could have an increased chelation efficacy. Aims: Deferiprone may play a role in shuttling iron within membranes and within intracellular pools.We compare DFX alone with combined therapy with DFX and DFO in β thalassemia patients with iron overload. *Design and Methods*. We studied 28 patients with β thalassemia major in two group (mean age±SD, 20.02±5.3; range 10-34 years) attending the day care unit for regular transfusional support. They received packed red

cells every 3-4 weeks to maintain pretransfusion hemoglobin major concentration above 9 g/dl. They had been receiving DFX at a daily dose of 40 mg/kg/d by subcutaneous infusion for 8-10 h on 5-6 nights each week for the past several years. (DFX) alone (40-50 mg/kg/d S.C. over 8-12 h, five times weekly) was compared with combined DFX 40 mg/kg/d (3times a week) and DFO 75 mg/kg/d daily over 6 months period. Serum ferritin level and side effects were monitored over a 6 month period. *Results*. Serum ferritin fell from98/3434±83/7539 µg/L (mean±SD) to 26/2706±66/4848 µg/L(p<0.001) and to75/2308±33/4338 (p<0.001) after three and six month in the combined group and raised from83/3613±00/5668 µg/L to 93/3940±81/6210 µg/L (P153/0≤) and to99/3205±07/5742 (p/0≤902) after three and six month in the DFX group. The most common adverse events were gastrointestinal symptoms (8.25%) and joint pain (8.25%) and transaminasemia (21%) in combined therapy. *Conclusions*. The availability of effective oral chelators would be a major clinical advance.Acording our study combined therapy with DFX and DFO is a practical and effective procedure to decrease severe iron overload in patients with thalassemia major.

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ANTIOXIDANTS SUPPLEMENTATION DURING PREGNANCY PREVENT PRE-ECLAMPSIA AND PROVIDE FETAL ERYTHROCYTE STABILITY

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Background. Pre-eclampsia affects 2.8% of all pregnancies, characterized by vasoconstriction, cell damage and coagulation disorders. About 30% of maternal deaths are due to pre-eclampsia. Also, environmental, nutritional status, genetic factors all are predisposing factors lead to placental ischaemia endothelial cells dysfunction and decreasd blood flow. Free radicals play a part in the genesis of the disease Natural antioxidant have antioxidative, antiproliferative and anti-inflammatory effects. Newborns, especially the preterm ones have a limited antioxidant protective capacity. The protective system against free radicals on red blood cell membrane includes vitamins A,E,C ,trace element selenium(Se) and enzymes as glutathione peroxidase (GSH-Px). *Design*. Aim of the study : To investigate the possibility of preventing pre-eclampsia and protect feotal erythrocytes from haemolysis through antioxidant therapy during late pregnancy. *Design and Methods*. The study was done on the peroid from December (2006) to December (2008) on one handered and thirty pregnant females , group I (18-40 year old) whome are risky for developing pre-eclampsia and had received antioxidant therapy in the early pregnancy (first trimester), this patients repeat their hospital visit for follow up, and other one handered pregnant females, group II (of the same age and risk factors), that had not been received antioxidants as a control group. Both group were subjected for lab. investigation (C.B.C on H-Max Coulter system , coagulation profile : PT, aPTT, Fibrinogen, TT, D-dimer on Sysmex system, vWF by latex agglutination method , Platelet activation by ADP/Collagen on PFA-100 , blood chemicistry as uric acid, total cholesterol, low density lipoprotein, liver and renal function also blood glucose). The neonates of both groups were subjected to complete blood count & reticulocytes, total and direct serum billirubin, RBCs antioxidant enzymes (GSH) & (G6PDase) . Samples from all females and babies of both group were subjected to measurement the level of antioxidant (Vitamines: C, E, A, D) in the circulation by high performance liquid chromatography HPLC method. Results: Antioxidant therapy during pregnancy in high risk female can improve the clinical condition of pre-eclampsia to minimal degree, the Bl.P is not affected, L.L. oedema is non significantly improved,only the platelate activity, D-dimer and vWF are significantly decreased (p<0.05), C.B.C including platelet count is not affected. As regard coagulation parameters P.T and a.P.T.T are not significantly changed, T.T and plasma fibrinogen is significantly decreased in the treated group, renal function, liver function- s.albumin and lipogram are non significantly affected. Neonatal improvement by maternal antioxidant therapy is noticed mainly on indirect s.billirubin level which is highly significantly decreased (p<0.005), RBCs and reticulocytes count are significantly improved (p<0.05), As regard WBCs ,the neutrophiles count is not significantly changed, the staff(band) is significantly decreased (p<0.05). Birth wt is significantly increased (p<0.05). neonatal deaths is significantly decreased (p<0.05), still birth is not affected. *Conclusions*. Antioxidant therapy during pregnancy in high risk females for preeclampsia is penificial in improvement neonatal hyperbilirubinaemia and to some degree improved clinical condition to the mothers

1314

IN VITRO STUDY OF NEUTROPHIL APOPTOSIS IN CHRONIC HEPATITIS C PATIENTS

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The apoptotic process appears to be a host defence mechanism against viral infections and tumourigenesis. However, many viral genomes encode proteins which repress apoptosis so as to escape from immune attack by the host. Apoptosis of liver cells may play a significant role in the pathogenesis of hepatitis C. The hepatitis C(HCV) core protein exhibits both proapoptotic or antiapoptotic actions. This study aimed to detect the role of neutrophil apoptosis as a mechanism of liver cell injury in chronic hepatitis C in order to determine its relation to shortened neutrophil survival in HCV patients, accounting in part for the mechanism of neutropenia as a trials to use therapeutic inhibitors of apoptosis in HCV patients, which might be an effective cure of the associated neutropenia. Thirty patients with chronic HCV infection and 15 normal controls were included in this study. HCV patients classified into 2 groups (Group I) 15 patients with chronic HCV without neutropenia, (Group II) 15 patients with HCV with neutropenia. They were subjected to full clinical examination ,abdominal ultrasono- graphy, haematological profile, liver and kidney function tests, hepatitis markers for HBsAg and HCV AB , qualitative HCV-RNA detection by PCR in patient groups and parasitological examination. Neutrophils were separated using Percoll density gradient and cultured , then neutrophil viability was assessed by the trypan blue dye exclusion test where viability was >98%. For detection of apoptosis , quantitative assay using flow cytometry to measure Annexin V as a marker of early apoptosis and a semiquantitative assay using terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling test (TUNEL) system which measures nuclear DNA fragmentation as well as the level of soluble Fas (sFas) on neutrophils in culture supernatant by enzyme linked immunosorbent assay(ELISA) in addition to the detection of the morphological features of apoptosis by light and electron microscopy. The results showed that the level of annexin V by flow cytometery decreased with the progression of the hepatitis in relation to the control group because annexin V is a marker of early apoptosis. We noticed that with the progression of the hepatitis C there was increase in the neutrophil apoptosis with DNA fragmentation , that is why the cells which uptook PI dye (necrotic cells) increased with the progression of the disease in relation to the control group.TUNEL test revealed a significant increase in group I in comparison to the control group and to group II but there was no significant difference between group II in comparison to the control group . Soluble Fas (sFas) showed a significant increase between group II in comparison to the control group but there was no significant difference between group I in comparison to the control or in comparison to group II. LM & EM examination revealed that apoptotic cells were detected in 100% of neutropenic HCV patients while it was 30% in HCV patients alone and we could observe the sequence and steps of apoptosis represented by intense perinuclear chromatin condensation , fragmented nucleus , vacuolated cytoplasm , apoptotic bodies with intact cell membrane and finally release of apoptotic bodies.We concluded that sFas, tunnel tests and EM examination are more significant in detecting apoptosis in HCV patients rather than annexin V. We can use some inflammatory mediators for delaying neutrophil apoptosis in HCV patients to give chance for neutrophils to interrupt viral replication and elimination of viral infected cells by the host.

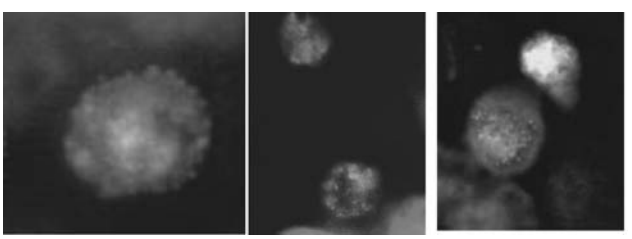

Figure 1. The nuclei with fragmented DNA were labeled.

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INCIDENCE OF CHEMOTHERAPY-INDUCED NEUTROPENIA IN LYMPHOMA PATIENTS AND USE OF PROPHYLAXIS WITH GRANULOCYTE COLONY-STIMULATING FACTORS IN CLINICAL PRACTICE

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Background. Lymphoma patients receiving chemotherapy (CT) frequently develop neutropenic complications. Subsequent dose delays and/or dose reductions may reduce CT efficacy. Current European and US guidelines (2006) recommend primary prophylaxis with granulocyte colony-stimulating factors (G-CSF) for patients at ≥20% risk of febrile neutropenia (FN) due to CT and patient-related factors. *Aims*. To evaluate the incidence of grade 3-4 neutropenia (G3-4N) and FN over the first four cycles of CT regimens with high or intermediate FN risk (FN risk ≥20% or 10-20%, respectively) in lymphoma patients, and to describe the use of G-CSF prophylaxis in clinical practice. *Design and Methods*. This multicentre, prospective, observational, single-cohort study, included adult patients with lymphoma initiating a new CT regimen (≥10% FN risk), with at least four planned cycles and a minimum expected survival time of 3 months. Patients initiating daily CT or with active infection were excluded. The primary endpoint was incidence of G3-4N, defined as absolute neutrophil count $<1.0x10^9$/L. Secondary endpoints included incidence of FN, percentage of patients receiving full dose on schedule (FDOS, defined as ≤15% dose reduction and ≤3 days dose delay) and number and duration of hospitalizations. Patients were recruited consecutively from 21 Spanish centres between Nov-2005 and Dec-2008. Each patient was followed-up until the end of CT or for a maximum of 8 cycles. Descriptive analyses were mainly centered in the subgroup of patients receiving G-CSF. *Results*. The study included 294 patients (96.6% with non-Hodgkin lymphoma and 3.4% with Hodgkin lymphoma), with a median age of 58.0 years (range:19-85), 52.4% male, 87.4% with ECOG 0-1 and 61.7% at stage III-IV (44.5% at stage IV). At least four CT cycles were completed by 88.8% of patients (median cycle duration of 21 days, range:14-42), and the most common CT regimen was CHOP±rituximab-based (75.7%). G-CSF prophylaxis was used in 83.8% of pts [of which 76.6% was primary prophylaxis (PP) and 23.4% secondary prophylaxis (SP); 48.3% filgrastim (70.3% PP) and 51.7% pegfilgrastim (78.8% PP)]. The incidence of G3-4N over the first four cycles was 40.4% in the overall group. The incidence of G3-4N in patients receiving filgrastim was 50.0% vs 41.1% with pegfilgrastim. Patients treated with PP showed a trend to lower incidence of FN (15.8%) than patients with SP (21.8%). In total, 30 out of 244 (12%) patients treated with G-CSF were hospitalized due to FN. The mean (SD) duration of hospitalization due to FN was shorter for patients receiving pegfilgrastim (5.9[5.8] days versus 12.4[11.1] days in patients receiving filgrastim. FDOS was achieved in 67.8% and 66.1% of patients with PP and SP, and 61.2% and 72.1% of patients with filgrastim and pegfilgrastim. *Conclusions*. In this study of Spanish clinical practice, more than one third of lymphoma patients at high or intermediate FN risk developed grade 3-4 CT-induced neutropenia. The use of primary prophylaxis with G-CSF is becoming widely adopted in Spain. Our descriptive data suggest that, relative to filgrastim, use of pegfilgrastim prophylaxis may reduce the duration of neutropenia-related hospitalization and allow better CT delivery. Study sponsored by Amgen S.A.

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ASSOCIATION OF NEUTROPHIL MYELOPEROXIDASE INDEX (MPXI) WITH SUBSETS OF BACTERIAL INFECTIONS

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Background. Mobilization of neutrophils in inflammatory conditions may be associated with an increased myeloperoxidase level or, as a result of azurophil degranulation, decreased level. [Aims] This study was aimed to elucidate the pattern of changes in neutrophil myeloperoxidase levels in specific subsets of infectious and non-infectious inflammatory diseases. *Design and Methods.* The mean myeloperoxidase index (MPXI) produced as part of a routine complete blood count (CBC) performed by a flow cytochemistry blood autoanalyser was used as a parameter in this study. The study was performed at the regional central hospital of Awaji Island in central Japan, where most of the island's 150,000 population were referred. Hence the spectrum of diseases treated in this hospital represented the general health status of typical Japanese regional population. *Results.* The average MPXI levels among healthy adults (n=50) and children (n=40) were 0.05 and -0.40 with the standard deviations of 2.74 and 2.95, respectively. There were no sex or age-related differences. In patients with bacteremia documented by positive blood culture tests (n=51), the mean WBC and CRP were elevated to 15050/μL and 10.87 mg/dL, respectively, while the mean MPXI was significantly lowered to -2.10, at the date of culture. Moreover, in most of the cases, the MPXI values further decreased during the septic courses. By contrast, among patients with bacterial infections (n=40), where bacteremia and tuberculosis were excluded, the mean WBC, CRP and MPXI were all significantly elevated to 10850/μL, 11.47 mg/dl and 5.50, respectively. Intriguingly, the MPXI values paralleled specifically with CRP during the individual courses. Tuberculous patients (n=37) showed the elevated WBC and CRP levels (means 7520/μL and 5.05 mg/dL, respectively), but the MPXI was unchanged (mean -.050), at the presentation. Similarly, patients with viral diseases (n=60) exhibited the elevated WBC and CRP levels (means 10740/μL and 2.17, respectively) but the MPXI level was unchanged (mean -0.50). Non-infectious inflammatory diseases, such as rheumatoid arthritis, showed that the mean MPXI was unchanged and no correlation of MPXI with CRP or other disease parameters was demonstrated. Next, the correlation of MPXI with other laboratory data was assessed. Among parameters of inflammation, solely CRP was very weakly correlated with MPXI levels ($y=0.7505x+3.3434$; $r=0.58$). The correlation was stronger when the MPXI and CRP in non-septic and non-tuberculous bacterial infections were extracted and assessed, but vanished when non-septic and non-tuberculous bacterial infections were excluded. In contrast, there was no correlation between MPXI and other laboratory tests including WBC and neutrophil counts. Thus, MPXI appeared to be a candidate biomarker that was independent of other markers of infectious diseases. *Conclusions*. These results indicate that MPXI is specifically correlated with some specific infectious states, namely, the low MPXI level in bacteremia and the high MPXI level in bacterial infections excluding sepsis and tuberculosis. MPXI appears to be an independent parameter of bacterial infection and might be a useful biomarker for the diagnosis (and follow up) of some inflammatory diseases, especially when serial MPXI levels are evaluated in the disease courses of individual patients.

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IMPROVING CONGENITAL NEUTROPENIA DIAGNOSIS: MUTATIONAL ANALYSIS OF ELA2 AND HAX1 GENES. A SINGLE CENTRE EXPERIENCE

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Severe chronic neutropenias are represented by a heterogeneous group of disorders with very different clinica outcomes. Recently, genetic mutations causing many of these diseases have been identified. Mutations in the ELA2 gene represent the most common cause of congenital neutropenia. They are responsible for familial and sporadic cases of cyclic (CN) and severe congenital neutropenia (SCN) whereas HAX1 mutations cause autosomal recessive congenital severe neutropenia (Kostmann disease). *Aims*. To evaluate mutations in the ELA2 and HAX1 genes to highlight their use as a new tool in the diagnosis of severe neutropenia. *Design and Methods*. Since February 2007 we have evaluated the mutational status of both genes in every child with severe chronic neutropenia, either by specific amplification of known mutations, or by gene sequencing. *Results*. Twenty-one patients were evaluated. The mutated cases are shown in theTable. *Conclusions*. All our patients affected of NC or NCS had ELA2 gene mutated. The study of the mutational status is been of great help as a new diagnostic tool at our hospital. It should be implemented in the routine study of severe chronic neutropenia. Although further work is needed, recent studies were able to relate certain ELA2 mutations with more severe phenotype and increased risk of developing leukemia/myelodisplasia. Such patients could benefit

from a more exhaustive follow up or even early bone marrow transplantation.

Table. (*): not reported, (+): possible polymorphism, OP: osteopenia, LPT:liver pseudotumor AI: autoimmune neutropenia.

n	age diagnosis	diagnosis	gene mutation	familiar study	severe infections	G-CSF	out come	follow up
1	2y4m	CN	*ELA2*	+	no	yes	1500 ANC	8y
2	10m	SCN	*ELA2*	-	no	yes	1000 ANC OP/ LPT	10y
3	5m	AI	*HAX1*+*	+	no	no	3000 ANC	2y
4	7m	SCN	*ELA2*	-	no	yes	1000 ANC OP	14y
5	2m	SCN	*ELA2**	-	yes	yes	1000 ANC	1y
6	3y	CN	*ELA2**	-	no	yes	1500 ANC	1,5y

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LEUCOCYTOSIS AS PARANEOPLASTIC SYNDROME OF HEPATOCELLULAR CARCINOMA

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Background. Paraneoplastic syndromes are diseases of predominantly solid tumours, which can precede or accompany malignancies. Most common are thrombosis, endocrinological disorders (e.g. hypercalcemia, hyperglycemia), myopathies (e.g. Lambert-Eaton-Syndrome, polymyositis or dermatomyositis), polyneuropathy and osteo-arthropathy (Pierre-Marie-Bamberger-Syndrome). We report about a case of pronounced leucocytosis due to hepatocellular carcinoma. *Case report*. A 50-year-old male was admitted to hospital with oedema of the lower legs, jaundice, night sweat and reduced general state of health. 15 years before he had undergone sinistral nephrectomy due to Wilms' tumour. Laboratory tests showed a marked leucocytosis of 91.000/µL accompanied by a mild anaemia and thrombocytopenia. γ-GT was increased to 1300 U/l and bilirubin to 7 mg/dL. The main results of laboratory tests on admission are shown in Table 1.

Table 1.

Parameter	[reference range]	Finding
leukocytes	[4.0000-10.000/µL]	91.000
hemoglobin	[14-18g/dl]	11,9
platlets	[150-400 *1000/µL]	134
INR	[0,9-1,25]	1,5
ALT	[0-45 L]	37
γ-GT	[0-5l]	1300
AP	[50-136 U/L]	349
bilirubin	[0,2-1,5 mg/dL]	9,2
γ-fetoprotein	[<10 ng/mL]	4,1 e 1

On abdominal ultrasound, a tumour of 8 cm in diameter in the right lobe of the liver as well as several hepatic metastasis and numerous enlarged lymph nodes along the abdominal aorta were seen. Analysis of bone marrow and peripheral blood smear (Figure 1) did not give any indication to the existence of a myeloproliferative syndrome or any other hematological disease.The result of liver biopsy confirmed the existence of undifferentiated hepatocellular carcinoma. Immediately after liver biopsy, the patient was started on chemotherapy following the CHOP scheme (cyclophosphamide, doxorubicin, vincristin, prednisolone) but to no avail. Four days following admission, the patient died. *Summary*. Leucocytosis in the context of paraneoplastic syndromes is rare. Some case reports also refer to leucocytosis as high as 50.000 leucocytes/µL in bronchial, pancreatic, corpus carcinoma and in malignant melanoma. Leucocytosis of more than 90.000/µL is an extremely rare finding. A tumour-induced G-CSF stimulation is the cause of paraneoplastic leucocytosis. In case of a marked leucocytosis without the existence of hematological disorder it is essential to consider the possibility of paraneoplastic syndrome.

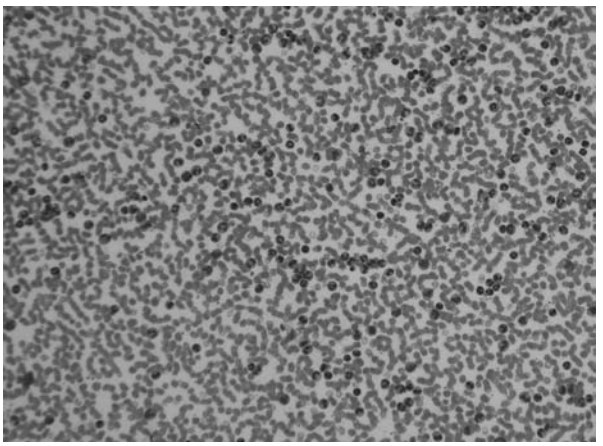

Figure 1. Peripheral blood smear (Pappenheim stain).

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IDIOPATHIC THROMBOCYTOPENIC PURPURA (ITP) - ANALYSIS OF ITS PREVALENCE, DIAGNOSTICS AND THERAPY IN THE SOUTH MORAVIAN REGION (CZECH REPUBLIC)

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Introduction. The reported annual incidence of ITP in adults in the world is approximately 5.8-6.6 cases of 100 000 inhabitants; the annual incidence in children is about 4-5 cases of 100 000 inhabitants. The exact incidence of ITP in the Czech Republic was not known yet. Diagnostics of patients with ITP are not fully standardized yet and therapy is a subject of expert recommendations. *Design and Methods*. To find out what the exact incidence and prevalence of ITP is and how the patients with ITP are treated at various outpatient clinics, we have proposed a questionnaire study of adult ITP in the South Moravian Region. In our questionnaire study we have focused on to find out data of ITP prevalence, ITP diagnostics and therapeutic preference of physicians. We sent the questionnaires to all of outpatient clinics in the South Moravian Region, where 1127718 inhabitants live according to the last population census. Results. All hematologists filled the questionnaires out. At the date of analysis, 564 adults with ITP were registered (406 patients with chronic ITP; 69 patients with the chronic resistant type of ITP; and 18 patients with Evans syndrome). According to our analysis, the annual incidence of ITP is 6 cases of 100 000 inhabitants, and its prevalence is 50.3 cases of 100 000 inhabitants. We have not recorded any death due to ITP (hemorrhage) in our patients. Our data demonstrated that the hematologists prefer diagnostics of ITP per exclusionem (88%). 47% of hematologists perform diagnostic bone marrow puncture. Radiolabeled scintigraphy before splenectomy is preferred by 53% of physicians. The key treatment modalities are corticosteroids and splenectomy. During pregnancy, intravenous immunoglobulins are used. The therapy of the chronic ITP is based on the use of cyclosporin A (53%) and the use of cyclofosfamide (29%). The use of danazol, rituximab and mycophenolate mofetil was limited to one main central department. *Summary*. The gained epidemiologic data of ITP confirm the worldwide data. The diagnostic and therapeutic procedures of the hematologists in our region reflect a considerable heterogeneity of diagnostics and the treatment, first of all, in the patients with the chronic type of ITP.

1320

ALTERATIONS OF PLATELET FUNCTION IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background. Patients with chronic myeloid leukemia (CML) present alterations of platelet function due both to BCR-ABL mutation and to medication. *Aims*. The aim of this study is to highlight alteration in platelet function in these patients relative to usual medication. *Design and Methods*: This prospective study included 24 patients diagnosed with CML during 3 years in the Hematology Department of the Universitary Emergency Hospital Bucharest. Patients were analyzed separately according to the administered medication. Platelet function was investigated by platelet aggregation on Chrono-log aggregometer and flowcytometry on BD FACS Calibur. Samples were done from PRP and PPP obtained by centrifugation. As stimuli, we used ADP, collagen, epinephrine and ristocetin. A group of 24 healthy volunteers was used for comparative results. Statistics was performed using ANOVA 2. *Results*. We obtained a much weaker response to epinephrine and collagen of platelets from patients with chronic myeloid leukemia versus controls (p<0001). This response matched both amplitude and slopes of aggregation curve. The slope and amplitude of ristocetin curves in patients are comparable with healthy volunteers, but the expression of CD42a and CD42b is lower (p<0.001). The lag phase of ADP and collagen curves is altered in patients with CML recorded a length and a greater amplitude without statistical significance.

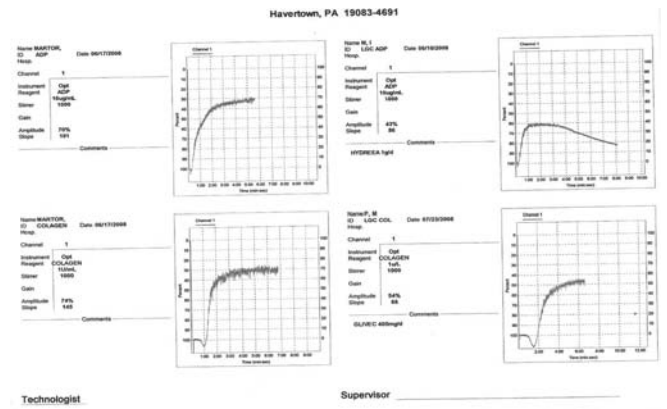

Figure 1.

Deaggregation was observed in 9/24 cases analyzed and it was not found on any control. When both tests were performed, we found an association between the weak expression of CD41 and deaggregation (2/5cases), but without statistically significance. The analysis on separate groups according to the received medication showed significant changes to the group of patients who received hydroxyurea. The amplitude and slope of aggregation curves, as well as the expressions of CD41 and CD61 were much smaller in the group of patients who received hydroxyurea (p<0001) followed by the group of patients who received imatinib and by the group receiving interferon. Also, patients who received hydroxyurea or purinethol have a greater length and amplitude of the lag phase for both ADP and collagen. Patients receiving hydroxyurea had more frequently platelet deaggregation - 5/8 cases compared to patients receiving Interferon (1/4) or imatinib (2/11). We also observed a weak expression on CD41 in 2 of the patients receiving hydroxyurea (from the group where flowcytometry was accessible). Explanation could be c-GMP generation by activating soluble guanil-cyclaze, because hydroxyurea is known to influence the induction of NO generation. Dasatinib proved to have an Aspirin-like influence that could explain the frequent hemorrhages of these patients. *Conclusions*. Poor response of patients with CML to different stimuli may have many explications, both BCR-ABL mutation itself and through various membrane or pool storage defects. We were able to identify the influence of different medications taken by CML patients on the aggregation pattern.

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QUALITY AND IN VIVO RECOVERY OF WASHED PLATELET CONCENTRATES (PRELIMINARY RESULTS)

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Background. Transfusion of plasma containing blood components such as proteins, immunoglobulin A or cytokines have been associated with anaphylactic life-threatening or severe non-hemolytic transfusion related reactions. It appears to be clinically rational to apply washing platelet concentrates (PCs) for sensitized thrombocytopenic patients who had experienced severe transfusion related allergic reactions or IgA deficiency, as well as for infants with neonatal alloimmune thrombocytopenia who require maternal PLTs. However, evidence on *in vitro* and *in vivo* quality of washed platelets (PLTs) is limited. *Aim*. The purpose of this study was to evaluate the characteristics: mean platelet volume (MPV), PLT count and aggregation of PCs before and after washing procedure and the *in vivo* post transfusion platelet recovery (PPR). The possible transfusion related reactions among the patients were also registered. *Design and Methods* We obtained 5 consecutive PCs from normal volunteer donors by automated platelet apheresis (Fresenius Com.tec). PCs were separated in 2 units with 3x1011 PLT /280-300 mL for each. One unit was washed manually with 150 mL of 15% ACD solution and the other part was stored unwashed. Platelet sedimentation was reached by centrifugation for 8 min at 3500g. After removal of supernatant plasma and a storage time of 30 min, the platelet pallet was re-suspended in 300 mL N/S 0.9% and was transfused 3-6h after the procedure. The MPV, the PLT number and aggregation (ADP, collagen, epinephrine) were evaluated in all units. Washed and unwashed PCs were transfused to the same patient in 2 consecutive days. PLT counts were obtained one hour after transfusion for PPR calculation. Transfusion related reactions were also recorded to all the involved patients. *Results*. A 9.2% and 0.3% reduction was observed, in PLT count and in MPV respectively after washing procedure. Washing PCs showed a decreased in ADP, collagen and epinephrine induced ability of aggregation compared with the unwashed PCs. The PPR of the patients who received washed PLTs was 7.5% lower compared to the patients who received unwashed PCs. Allergic reactions were not reported when washed PCs were transfused, even in the cases with positive history of allergic episodes. *Summary.* Washing stresses PLTs and as a result seems to impair aggregation in ADP, collagen and epinephrine. However, PLT counts after transfusion of washing PCs seem to be comparable to those unwashed and the lack of adverse events may support the utility and feasibility of this procedure within blood donation departments. Clinical and large scale studies need to define the functional hemostatic capacity of the washed PCs.

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SUCCESSFUL TREATMENT WITH RITUXIMAB OF THROMBOTIC THROMBOCYTOPENIC PURPURA IN A PATIENT WITH IGA DEFICIENCY

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Background. Thrombotic thrombocytopenic purpura (TTP) is a rare disease characterized by microangiopatic haemolytic anemia, thrombocytopenia, altered neurological symptoms, renal impairment and fever. The use of plasma exchange (PEX), plus corticosteroids or other immunosuppressive agents, has dramatically decreased the mortality rate. Complete remissions have been described in some patients with refractory TTP treated with rituximab. In patients with IgA deficiency, plasma infusion may be followed by severe allergic reactions. Aim: We describe the first case of TTP with Ig A deficiency successfully treated with rituximab. *Case report*. A 48 year old woman with anemia and thrombocytopenia was referred to our centre in June 2007. At admission, she presented asthenia and some petechiae in abdomen and legs without neurological signs and fever. Hematological findings were: hemoglobin (Hb) 6.3 g/dL, platelets (Plt) 89x10^9/L and withe blood cells (WBC) 4.7 x10^9/L. The peripheral blood smears showed a large number of schis-

tocytes. Total bilirubin (TB) level was 1.14 mg/dL and lactate dehydrogenase (LDH) was 2917 U/l.; ADAMTS13 activity was 32% (reference value 46-160); antibodies against ADAMTS13 were not detectable. The diagnosis of TTP was made and the patient underwent treatment with PEX. During the first PEX, the patient presented chills, fever and dyspnoea. Prednisone (1mg/Kg body weight/day) was started; moreover the remaining 11 PEX were associated with steroid premedication without other side effects. The patient achieved a complete remission of anemia and thrombocytopenia, but in June 2008 the TTP relapsed. Main hematological findings were: Hb 8 g/dL; Plt 97x10^9/L; LDH 1299 U/L). Treatment with prednisone and PEX was started, but anaphylactic shock (severe hypotension, dyspnoea, laryngospasm, chills, fever) occurred after the first plasma infusion. The dosage of IgA showed a serum level <0.05 mg/dl. In absence of plasma from Ig A-deficient donors, treatment with high-dose intravenous v.) Immunoglobulin was made without improvement. Treatment with rituximab (375 mg/mv./weekly/ x 4) was started. After five days from the first dose of rituximab, the platelets were 110x10^9/L and LDH was 420 U/l. After four doses of Rituximab, the patient was discharged and she is still in complete remission without treatment. *Conclusions*. The Ig A deficiency is generally associated to severe reaction following plasma infusion. In our TTP patient, the first treatment caused hypersensitivity to plasma and prevented carrying out PEX in occasion of the relapse. The treatment with rituximab induced a durable complete remission of her TTP in our patient. To our knowledge, this is the first case report of TTP with IgA deficiency, successfully treated with rituximab.

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INTRAVENOUS METHYLPREDNISOLONE PULSE THERAPY IN ACUTE ITP CHILDREN WITH OVERT HEMORRHAGE

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Background and Aims. Immune thromobocytopenia purpura (ITP) is an acquired hemorrhage disorder characterized by immune-mediated platelet destruction. However, single-institution, long-term data with initial intravenous methylprednisolone pulse therapy are limited. The objective of this study is to review the clinical response of ITP with overt hemorrhage received intravenous methylprednisolone pulse therapy and risk factors for chronic ITP at a single medical center. *Design and Methods*. 52 newly diagnosed, previous untreated acute ITP children with overt hemorrhage and platelet count less than 20K admitted and treated with intravenous methylprednisolone pulse therapy from Jan.1997 to Dec. 2007. The dosage of methylprednisolone is 30 mg/kg/day (max. 1gm) for 3 days. A complete response (CR) to pulse therapy was defined as platelet count ≥150K. A partial response (PR) was defined as an increase platelet count ≥50K. No response (NR) was defined as no response to pulse therapy with persisted platelet count less than 50K. *Results*. Among the 52 children, the median age was 45 months (range 7-186 months), 31 male and 21 female. The median initial platelet count was 6K (range 1-19K). A preceding infection was found in 32 and vaccination in 4. The intravenous methylprednisolone pulse therapy response rate (CR+PR) was 86.5% (45/52). No response rate was 13.5% (7/52). 16/52 (30.7%) cases became chronic ITP. 14/16 cases were initial pulse therapy responders. No significant adverse effects noted during intravenous methylprednisolone pulse therapy. By uni-variate analysis, we found the age over 10 years old was the risk factor for chronic ITP. (p<0.05). *Conclusions*. Initial intravenous methylprednisolone pulse therapy in our experience is an effective, safe and less expensive treatment for childhood acute ITP, with high response rate initially to prevent the overt hemorrhage during severe thrombocytopenic period. Age over 10 years old of acute ITP patients still had higher risk for subsequent chronic disease.

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TREATMENT OF IMMUNE THROMBOCYTOPENIC PURPURA ; 15 YEARS EXPERIENCE OF A SINGLE MEDICAL CENTER

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Background. Idiopatic thrombocytopenic purpura (ITP) also reffered as immune or autoimmune thrombocytopenic purpura is an aquired disease characterised by low platelet count, normal bone marrow, usually with an increased number of megakariocytes and the absence of any other disease. The majority of patients respond on short term, at an initial corticosteroid therapy, which is used as first line therapy. Sometimes after steroid therapy disease relapse and in this case there can be used other lines of therapy including splenectomy. *Aims*. We tried to evaluate the therapeutic results in a group of patients with ITP which have been treated in a single medical center. *Method*. From I 1990 to I 2005, 207 patients were hospitalised and treated in the Hematology Department of Timisoara, the Western part of Romania, 132 female and 75 males. The median age of patient at diagnosis was 34, 12 years (range 16-68 years). The mean time from diagnostic was 19, 31 months. The mean platelet count before treatment was 23,520/µl with limits between 1000 and 110.000/µL. Petechial and ecchymosis were the most commun components presenting symptoms at 51% patients, 34% have presented gastro-intestinal bleedings, 10% had bleedings in the central nervous system and 5% had other bleedings. In our department, the corticotherapy was the first line of treatment and it was applied to 68% of patients. Patients with platelet counts <20x10^9/L and/or major bleeding symptoms were treated with high dose methilprednisone (HDMP) intravenous immunoglobulin (IVIG) and/or combination therapy of HDMP and IVIG (21%). The rest of the patients (11%) which didn't respond appropriately to the steroidic therapy received combined treatment of steroid and IVIG, vinca alkaloids. Approximatelly 41% of patients received transfusions of thrombocytes between 1 and 7 days, in gastrointestinal major bleeding cases, cerebral and severe thrombocytopenia. Complete remission (CR) was defined as the maintenance of platelet count 150x10^9/L and partial response (PR) was defined as symptomatic improvement with an increase in platelet count to more than 50x10^9/L. Platelet counts below 50x10^9/L were defined as non-response. Patients were put under watch for 3,5 years (6 months to 14,2 years). Results: 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 approximatelly 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 splenectomised presented CR and 11% of patients presented PR. The long therm follow-up in CR and PR proves a good stabil and durable response in time for more than 7 years. *Conclusions*. The natural coure of the disease is variable. 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 sigur and durable response after the splenectomy. Modern treatments like monoclonal antibodies can bring a bigger benefit.

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GALANTAMINE HAS NO EFFECT ON PLATELET FUNCTION IN THE ELDERLY

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Background. Galantamine, a centrally-acting cholinesterase inhibitor, has been used in the treatment of mild-to-moderate dementia of Alzheimer Disease. Increased mortality, mainly due to cardiovascular events such as myocardial infarction and stroke, was observed in placebo-controlled trials of galantamine. It is not clear whether galantamine might produce functional influence on platelet response. The aim of the present study is to investigate the effect of galantamine on *in vitro* aggregation of human platelets, since increased *in vitro* platelet aggregation appears to be predictive of cardiovascular events. *Design and Methods*. 15 healthy, non-smoker elderly subjects, without any co-morbidity and medication, were selected. Average plasma concentration of galantamine at steady state is 42 ng/mL. So, galantamine solutions were prepared in three different concentrations as 20, 40, and 80 ng/mL similar to galantamine concentrations in the plasma that observed after clinical therapeutic oral applications. Venous blood samples were drawn from the ante-cubital vein and anticoagulated with sodium citrate solution. The hematocrit was determined and the blood sample was divided into four equal parts. According to the amount of plasma (derived from the hematocrit), the calculated volume of galantamine solution and the diluent without galantamine as control were added to blood samples which yielded 0, 20, 40, and 80 ng/mL galantamine concentrations in the plasma. We isolated the platelet-rich plasma (PRP) from supernatant. Turbidometric aggregation performed according to the protocol. Platelet aggregation responses were evaluated with 5 µM adenosine diphosphate (ADP) and 2 µg/mL collagen. Platelet aggregation curves were calculated automatically by the device and maximal aggregation (%) was obtained from the aggregation curve. *Results*. Percentages of maximum aggregation (Mean±SEM) were 51.33±4.61, 54.20±5.30, 55.07±5.07 and

57.07±4.16 for ADP and 61.87±3.40, 60.40±4.90, 65.27±3.36 and 63.40±2.36 for Collagen (Control, 20, 40 and 80 ng/mL galantamine, respectively). Galantamine showed no effect on platelet aggregation induced by ADP and collagen in human PRP in a dose-dependent manner. *Conclusion*- We have thought that platelet aggregation is not associated with the mechanisms of the increased mortality mainly due to cardiovascular events observed in placebo-controlled trials of galantamine. Further *in vivo* studies with larger populations are needed to evaluate platelet aggregation and other risk factors in the galantamine usage.

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LDH IS PREDICTOR OF DISEASE DURATION IN THE FIRST ONSET OF THROMBOTIC THROMBOCYTOPENIC PURPURA

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Background. Acquired thrombotic thrombocytopenic pupura (TTP) is an acute disorder characterized by microangiopathic hemolytic anemia, consumptive thrombocytopenia and widespread microvascular thrombosis with diffuse ischemic damage, most of all caused by an immunomediated deficiency of the von Willebrand factor-cleaving protease, ADAMTS-13. It can be fatal in 90% of patients when untreated and have potentially short and long term devastating consequences. A severe deficiency of ADAMTS-13 is sufficient but not necessary for clinical manifestations and outcome of TTP. The introduction of plasma exchange (PE) as first-line treatment improved survival to the acute event. However, TTP may worsen after an initial response during plasma therapy, and the number of PE sessions required to achieve remission is variable. Nowadays no clinical and laboratory parameters are still well established as markers predicting clinical response. *Aim*. To determine whether patient characteristics, clinical presentation, and laboratory features could correlate with response after the first episode of acute TTP.

Table 1. Main features of patients at first episode of TTP.

Number of patients	21
Age; mean ± SD	36 ± 11
Gender; F/M	16 / 5
Idiopathic TTP	14 (67%)
Secondary TTP	7 (33%)
Presenting features	
- Fever	8 (38%)
- CNS impairment	14 (67%)
- Renal impairment	4 (19%)
Platelet count, x10^9/L; mean ± SD	25 ± 26
Hb, g/L; mean ± SD	84 ± 22
LDH, U/L; mean ± SD	1291 ± 906
PE sessions; median (mean ± SD)	8 (11 ± 6)
PE response	15 (71%)
Post-PE platelet count, x10^9/L; mean ± SD	210 ± 138
Duration of acute event, days; median (range)	14 (5-56)
Deceased	2 (10%)
Relapsed	7 (33%)

CNS, central nervous system; Hb, hemoglobin; LDH, lactate dehydrogenase; PE, plasma exchange

Patients and Design and Methods. We retrospectively analyzed clinical and laboratory records of patients presenting with their first episode of acute TTP. Inclusion criteria were the presence of at least three of the following: 1) thrombocytopenia (<150x10^9/L) without other explaination; 2) negative direct Coombs' test hemolytic anemia with schistocytes on peripheral-blood smear; 3) increased serum levels of lactate dehydrogenase (LDH>400 U/L); 4) presence of signs or symptoms consistent with CNS or other organ ischemia. Other thrombotic microangiopathies should be excluded. Between 2000 and 2008 we enrolled 21 adult patients, 16 females and 5 males, mean age being 36±11 years. TTP was idiopathic in 14 patients (67%), whereas 7 patients (33%) had a secondary TTP, due to an underlying autoimmune disease. In 14 patients ADAMTS-13 activity and IgG anti-ADAMTS-13 were assessed at presentation using commercially available FRETS-VWF73 and ELISA assays respectively. Severe protease activity deficiency was considered as equal or less than 10%. A high IgG titer was defined as levels of 50 U/mL or more. Response to PE was defined as normalization of platelet count (>150x10^9/L) and LDH (<400 U/L) for at least 2 days after PE discontinuation. *Results.* Clinical and laboratory patient characteristics are shown in Table 1. ADAMTS-13 activity was severely reduced in 86% whereas autoantibodies were present with high titre in 57% of patients. The median number of PE sessions performed was 8 (mean 11±6, range 3-39). 15 patients (71%) achieved a remission after PE sessions; 4 patient failed to respond to PE treatment and needed adjunctive immunosuppressive therapy other than steroids. The event was fatal in two patients. The median time of episode was 14 days (mean 20±16, range 5-56). Among clinical and laboratory characteristics, there was a direct correlation between LDH values at presentation and the term of acute event (r=0.594, p=0.007). However, LDH levels were not significantly different in PE responders and refractory patients. *Conclusions*. Higher LDH level at diagnosis does not seem to be predictive of poor PE response but it may be a useful parameter for identification of patients with a more serious disease, needing a more intensive apheretic treatment to achieve a clinical remission.

1327

PLATELET COUNT AND SERUM THROMBOPOIETIN LEVEL AS PREDICTORS FOR MORBIDITY AND/OR MORTALITY IN THROMBOCYTOPENIC NEONATES

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Background. Platelet count is not a predictor of serum thrombopoietin level in thrombocytopenic and non-thrombocytopenic neonates. Serum thrombopoietin in thrombocytopenic infants is largely related to the cause of thrombocytopenia and the underlying disease. Many perinatal factors can affect thrombopoietin level like sepsis, birth asphyxia, respiratory distress, maternal hypertension, and even type of delivery. *Aim*. To evaluate the role of platelet count and serum thrombopoietin levels in prediction of the morbidity and mortality in newborns with thrombocytopenia. *Design and Methods*. A prospective study on 119 thrombocytopenic neonates; 54 full term and 65 preterm (Mean platelet count 40.13±33.1, gestational age range 28-42 wks) had been conducted. Congenital malformations known to be associated with thrombocytopenia or platelets dysfunction, infants who received blood, platelets or plasma transfusion or underwent exchange transfusion were excluded. Sera from venous blood were stored at -70 oC for thrombopoietin assay which was done using a qualitative ELISA technique (Quantikine RD systems, Inc. Minneapolis, USA). The test was repeated on the change of clinical status (recovery or deterioration). Results: A well-defined trend for lowering of thrombopietin level was noted on clinical recovery of bleeding and reversal of platelet count to normal (p<0.001 for matched pairs whether preterm or full term). Survival is significantly related to the basal platelet count in full term (Mean rank was 16.32 and 9.94 in alive and died full terms respectively with p=0.04), but insignificant among thrombocytopenic preterms. Platelet count is statistically negative correlated to thrombopoietin level in neonates in general and especially in preterm (r=-59, -0.69 respectively, with p<0.001), yet no statistically significant correlation between platelet count and duration of recovery was noted in preterms. Platelet count was found to be a better predictor for duration of recovery of thrombocytopenic neonates when compared to other factors specially thrombopoietin level. *Conclusions*. Thrombocytopenic neonates had very high levels of thrombopoietin. Despite the high thrombopoietin level in neonates died with severe thrombocytopenia, yet, mortality is related to the cause and outcome of thrombocytopenia rather than the serum thrombopoietin level. It is recommended to diagnose and treat the underlying cause of thrombocytopenia rather than to generalize the therapy based on thrombopoietin level.

1328

THE EFFECT OF ZINC SUPPLEMENTATION ON THE WHITE BLOOD CELL AND PLATELET COUNT LEVELS DURING VIRUS C HEPATITIS THERAPY

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Background. Zinc is essential for the structure of over than 300 tissue enzymes. Also, zinc has been suggested to have an antiviral activity; however some claimed that the value of zinc intake is controversial in some cases. Zinc is relatively safe and well tolerated when taken orally. It plays an important role in alteration of oxidant-mediated tissue injury, and phagocyte cells formation It Prevents damage of cells participating in innate immunity besides it enhances the activity of natural killer cell function. Free oxygen radicals can produce tissue damage .zinc as antioxidant Is important for neutralizing free radicals, including enzymes like

super oxide dismutase, catalase and glutathione peroxides. Patients with chronic hepatitis C have significant glutathione deficiency. During treatment of virus C by the combination of interferon and ribavirin, follow up with WBC count and platelets count are routinely done and cessation or changes of the therapy if reduction of these parameters occur. Aims:leucopenia and thrombocytopenia is a problem during the treatment with interferon and ribavirin, this work try to find the value of zinc supplementation in the prevention of some side effects in order to avoid the cessation of the course of therapy if this effect *Design and Methods.* 44 patients with chronic C hepatitis diagnosed by positive PCR and liver biopsy, they are divided into two of matched age and sex groups. White blood cell count (WBC) and platelet values were estimated before and after they received twice weekly standard doses of interferon and daily oral doses of ribavirin therapy, half of them added 175 mg zinc gluconate orally (equal 25 mg zinc) three times daily (group I) and to the other half (Group II) placebo was added. This is for 24 weeks, three patients from group I and five patients from group II are excluded as they did not continue due to the occurrence of side effects or failure to response. Results The result of this work shows signficant lower values of both WBC ($p<0.05$). And platelets count ($p<0.05$). In the group II than group I .The mean value of the WBC is 4.2* 1000 per m m3 and 5.8 *1000 per mm^3 respectively and the mean value of platelets is 121*1000 per mm 3 and 188* 1000 per mm 3 respectively. *Conclusions.* This preliminary study shows that zinc supplementation may be added to the therapy of interferon and ribavirin in chronic c hepatitis in an attempt to minimize the side effects. We advise further work on this subject with the use of more white and platelet function study in addition to their count and performing sternal puncture, also more patients will be needed.

1329

SHARING INFORMATION TO BUILD KNOWLEDGE: ESTABLISHMENT OF NEW NATIONAL REGISTRIES FOR THROMBOTIC THROMBOCYTOPENIC PURPURA AND NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

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Background. Thrombotic Thrombocytopenic Purpura (TTP) and Neonatal Alloimmune Thrombocytopenia (NAIT) are uncommon, but associated with significant morbidity and mortality. Resource implications of management, including provision of specialised blood products, are substantial and increasing. In NAIT, appropriate screening, early intervention, and management are areas of uncertainty. In TTP, the application of diagnostic tests, management of relapsed or refractory disease, and the role of emerging therapies all remain undefined. *Design and Methods.* Clinical registries provide a means to aggregate the full range of community experience with a condition and provide data to inform management in diseases where conduct of clinical trials is difficult. Monash University Department of Epidemiology and Preventive Medicine have extensive registry experience and an established methodology, which was employed in these projects in conjunction with the clinical expertise and networks of the Australian Red Cross Blood Service as part of the Transfusion Outcomes Research Collaborative (TORC). Results New registries have been established for each of these diseases to determine incidence and natural history, explore factors influencing clinical outcomes, inform patient management and inspire further research. For each registry, patients are identified and registered by the treating clinician. Data are recorded via specifically designed web-based forms, capturing details of presentation, management and outcomes. An independent Steering Committee comprising stakeholders and clinical experts monitors each registry. Sharing information with participating clinicians and hospitals is also a high priority. Participating hospitals represent every state and territory in Australia. For TTP, over 25 hospitals are participating, and over 15 hospitals for NAIT, including all tertiary referral centres for these diseases. Approximately 100 cases annually of TTP and 30 of NAIT are anticipated. *Conclusions*. Even major centres encounter relatively few cases of TTP or NAIT. Consequently it is difficult to establish optimal management, and there is significant variation in clinical practice. Rarity also hampers clinical trials. These national registries for TTP and NAIT will improve clinical practice and inform future clinical trials.

1330

GESTATIONAL THROMBOCYTOPENIA, IS IT NOT SIMPLY PHYSIOLOGICAL HYPERSPLENISM?

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Background. Thrombocytopenia is an ominous sign during pregnancy. Although one of the main reason for it is gestational thrombocytopenia, a benign condition occurring in 8% of all pregnancies and accounting for more than 70% of cases of thrombocytopenia in pregnancy, there is no pathonomonic sign for it and the diagnosis is by exclusion of other, sometimes emergent, causes of thrombocytopenia, such as HELLP syndrome, DIC, Pre Eclampsia, TTP, ITP etc. *Aims*. To establish a simple test for the diagnosis of gestational thrombocytopenia. *Design and Methods*. Nineteen pregnant women with an eventual diagnosis of gestational thrombocytopenia were investigated including standard physical examination, blood film, coagulation testing, liver function tests and abdominal ultrasonography. *Results*. Of the 19 women, 16 had splenomegaly on both physical examination and ultrasound. In the remaining 3 spleen size was at the upper limit of normal on ultrasonography. The reason for this was physiological gestational splenomegaly, which resolved immediately after the pregnancy. *Conclusion*. We suggest that in pregnant women with asymptomatic thrombocytopenia a finding of physiological splenomegaly strongly suggests the diagnosis of gestational thrombocytopenia. Larger studies are needed to confirm this hypothesis that gestational thrombocytopenia is simply due to hypersplenism secondary to the pregnancy.

1331

EVALUATION OF THE EFFICACY AND SAFETY OF A HUMAN RECOMBINANT ACTIVATED FACTOR VII (HRFVIIA) PREPARATION IN A PEDIATRIC PATIENT WITH GLANZMANN THROMBASTHENIA

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Background. The absence of agonist-induced platelet aggregation and the lack of fibrinogene receptor (GPIIb/IIIa) on the platelet surface proved the diagnosis of Glanzmann thrombasthenia in a male pediatric patient of Romany descent with severe hemorrhagic manifestations. DNA sequencing revealed a novel homozygous deletion of cytosine (1619delC) in the gpIIb gene. Neither GPIIb nor its truncated form could be detected in the platelets of the patient by Western blotting while a small amount of GPIIIa was demonstrated. Aims. We wanted to evaluate the efficacy and safety of a human recombinant activated Factor VII preparation (hrFVIIa, Novoseven®, Novo Nordisk, Bagsvaerd, Denmark) preparation, administered for the treatment of severe bleeding episodes of this patient in the clinical setting. *Design and Methods*. We have studied the patient data files and sheets retrospectively between 2004-2007, and joining the International Glanzmann Thrombasthenia Registy, and prospectively from 2008. *Results*. The patient presented here had bleeding episodes that had recurred every 2 to 3 months, initially as mild nosebleeds; these were replaced subsequently by more severe gingival bleeding episodes. As these progressive bleeding episodes were unresponsive to standard haemostatic therapy, the patient has been put on rhFVIIa therapy since 2004. The 20 bleeding episodes (between March 18, 2004 and December 30, 2008) reviewed here comprised epistaxis in 15 and gingival bleeding in 5 instances, for which the patient received hrFVIIa treatment on 313 occasions altogether. Between 2004 and 2007, 90 μg/kgbw doses, administered at 3-hour intervals for 5 to 6 consecutive days (a mean cumulative dose of 7500 μg/bleeding episode) were required to stop bleeding. In 2008, the intensity as well as the duration of bleeding episodes increased - while therapeutic efficacy declined. The patient required an augmented doses of 120 μg/kgbw dose for 14 and 20 days, respectively (a cumulative dose of 20,818 μg and 15,210 μg, respectively). No severe adverse effects were noticed in any case of hrFVIIa administration. *Conclusions*. In summary, the tested hrFVIIa preparation has proven effective in controlling the hemorrhagic episodes without significant side effects of this patient with Glanzmann thrombasthenia. Nevertheless, the long and increasing duration and intensity of individual bleeding episodes - as observed during the clinical course of this patient - may suggest that alternative treatment modalities should

be considered in the treatment of Glanzmann thrombasthenia in general and in the case of the presented patient in particular.

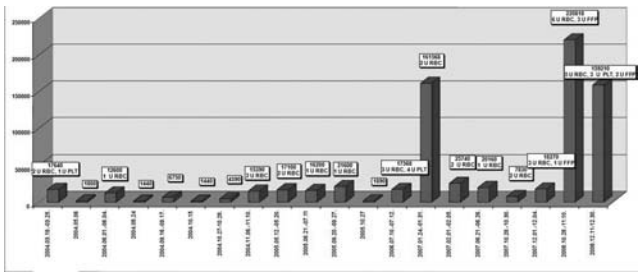

Figure 1. Cumulative dose (μg).

1332

MEAN PLATELET VOLUME IN ST ELEVATION AND NON-ST ELEVATION MYOCARDIAL INFARCTION

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Background. Platelet activation is thought to play a key pathogenetic mechanism in acute coronary syndromes. It was shown that platelet size correlates with their reactivity. *Aim*. We investigated the mean platelet volume (MPV) and platelet count in patients with both ST elevation myocardial infarction (STEMI) and non-ST elevation myocardial infarction (NSTEMI). *Design and Methods*. Fifty-two patients with NSTEMI, 56 patients with STEMI were enrolled in the study. Age- and gender-matched 50 healthy subjects constitute the control group. Venous blood samples for whole blood analysis were drawn on admission and analyzed by an autoanalyser. *Results*. There was statistically no difference between the STEMI and NSTEMI groups in investigated parameters. However, MPV was significantly higher in STEMI patients than NSTEMI patients and controls. Moreover, platelet counts for both STEMI and NSTEMI groups were significantly lower than controls. Platelet counts in the STEMI and NSTEMI patients did not show any statistical difference. *Conclusions*. Our study showed higher MPV values in STEMI as compared to NSTEMI patients and controls. MPV but not platelet count differs between STEMI and NSTEMI patients. The underlying mechanism should be investigated in further researches, which may shed light on developing new therapeutic strategies for this particular acute coronary syndrome.

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NIEMANN-PICK TYPE B DISEASE IN A MIDDLE AGE MAN

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Niemann-Pick Type B is a rare autosomal recessive lysossomal storage disease caused by deficient acid sphingomyelinase activity and the consequent accumulation of Sphingomyelin. The diagnosis is usually made in infancy or childhood following the investigation of a splenomegaly. These patients also have mild, but progressive, interstitial lung disease, growth retardation and pancytopenia caused by secondary hypersplenism. Most of them have little or no neurological involvement and often survive into adulthood. The presence of the characteristic foam cell in the bone marrow supports the diagnosis that has to be confirmed by measuring the acid sphingomyelinase activity in peripheral leucocytes, cultured fibroblasts and/or lymphoblasts. The disease is pan ethnic and is associated to allelic mutations in the ASM gene. Enzyme replacement therapy clinical trial has recently begun in adult patients. The authors report a Niemann-Pick type B disease presented in a male at the age of 44 years. He complained of abdominal discomfort which ameliorated with a cholesterol-lowering drug. Case report: A 44 year old Caucasian male sought medical attention for abdominal discomfort and mild dyspnoea for medium exercise. Referred no other complains, has normal stature and had been a regular blood donor for the past 8 years. He presented an enlarged spleen (20x18x14cm by ultrasound), mild thrombocytopenia (100.000/mL) and dyslipidemia. Bone marrow slides, staining by May-Grünwald-Giemsa, showed many blue-staining foamy macrophages, "sea blue" histiocytes like. The diagnosis was confirmed by a low sphingomyelinase activity in cultured fibroblasts (2.06 nmol/h/mg of protein; normal: 18-300). HDL cholesterol was low (0.57 mmol/L N 0.9-2.3) and chitotriosidase was high (342 nmol/mL N10-85). Hb 17.6 g/dL, HTC 52%, leucocytes 7.5x10^9/L, neutrophyles 4.2x10^9/L, platelets 98x10^9/L. Respiratory function tests showed low CO diffusion and rest hypoxemia. He was started on Ezetimibe10mg/Sinvastatin 20 mg and few months later he referred no dyspnoea, neither abdominal discomfort. At the age of 49 years he remains asymptomatic, the spleen size is stable, hematological and biochemistry parameters are similar and there are no functional signs of pulmonary disease progression. *Discussion*. The Niemann-Pick Type B clinical case described here is a very mild form of the disease, diagnosed in the fourth decade of life, with a very slow disease progression. During the last 5 years cholesterol-lowering drug Ezetimibe /Sinvastatin reduced patient abdominal discomfort; however, it is not clear whether it has any influence on disease progression.

1334

THE EFFECT OF 3RD GENERATION COLLOIDS ON PRIMARY HAEMOSTASIS IN PREGNANT WOMEN

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Background. Although the effect of colloids in haemostasis have been demonstrated in many studies, limited data exist with regards to potential changes in primary haemostasis after fluid preloading with colloids in pregnant women. *Aims*. The aim of this study was to evaluate the effects on primary platelet- mediated haemostasis in response to preloading with 6% Hydroxyethyl starch (HES) colloid solution and Pinger's Lactate (LR) in healthy pregnant women, prior to spinal anaesthesia for Cesarean delivery (CD). *Design and Methods*. We enrolled 20 healthy pregnant women with uncomplicated pregnancies at term (37-41 weeks of gestation) presenting for CD under spinal anaesthesia. Women with significant medical or obstetric co-morbidity and patients receiving drugs affecting haemostasis, were excluded from the study. Demographic and baseline obstetric characteristics were similar among groups.

Table.

		Sum of Squares	df	Mean Square	F	Sig.
PLT	Between Groups	7488,450	1	7488,450	2,419	,137
	Within Groups	55720,500	18	3095,583		
	Total	63208,950	19			
col/adp membrane*	Between Groups	1960,200	1	1960,200	7,655	,013[1]
	Within Groups	4609,000	18	256,056		
	Total	6569,200	19			
col/epi membrane *	Between Groups	8000,000	1	8000,000	4,843	,041[1]
	Within Groups	29734,000	18	1651,889		
	Total	37734,000	19			
HB	Between Groups	,098	1	,098	,046	,833
	Within Groups	38,520	18	2,140		
	Total	38,618	19			
Hct	Between Groups	1,152	1	1,152	,078	,783
	Within Groups	265,010	18	14,723		
	Total	266,162	19			
VWFRCo	Between Groups	385,442	1	385,442	,189	,669
	Within Groups	36751,818	18	2041,768		
	Total	37137,260	19			

1. Significant Difference

Patients were randomized to receive fluid preloading with either 1500 mL RL (group A) or 500 mL 6% HES (group B). Prior to preloading an initial blood sample was obtained (T0 samples). The fluid preloading was administrated over 30 min. A second sample (T1 sample) was subsequently taken from the contralateral arm. We measured in samples T0 and T1 the following parameters: Hb , Hct , PLT, platelet function using Platelet function Analyzer (PFA-100, Dade Behring), vWF activity (vWFR-Co). Platelet function was assisted with collagen/epinephrine and collagen/ADP as agonists. Results were expressed as closure time. Platelet count was measured using XT-1800 blood analyzer (Sysmex) and vWFR-Co using BCS XP coagulation analyzer (Dade Behring). *Results*. Statistical analysis was performed with the use of SPSS 16. Data were analyzed with ANOVA test. We observed no significant differences in all param-

eters between groups A and B in T0 samples. We found statistically significant differences (p<0.05) only in col/epi and col/ADP after preloading with fluids (T1 samples). Despite difference, closure times remained in normal ranges. *Conclusions*. Our results suggest that HES have a mild effect on platelet function, probably without clinical significance, but further studies with larger number of patients are needed to confirm this.

1335

THE EFFECTIVENESS OF RITUXIMAB IN CLINICAL AND LABORATORY IMPROVEMENT OF TTP: REPORT OF THREE CASES

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Background. Thrombotic Thrombocytopenic Purpura (TTP) is a rare disorder characterized by thrombocytopenia, microangiopathic haemolytic anemia and microvascular thrombosis. Unless a prompt diagnosis is made, TTP shows a poor prognosis with a mortality rate reaching more than 90% in some case series. Idiopathic forms are usually associated with the presence of antibodies against ADAMTS-13, a circulating protease that cleaves von Willebrand factor multimers under high shear stress conditions.

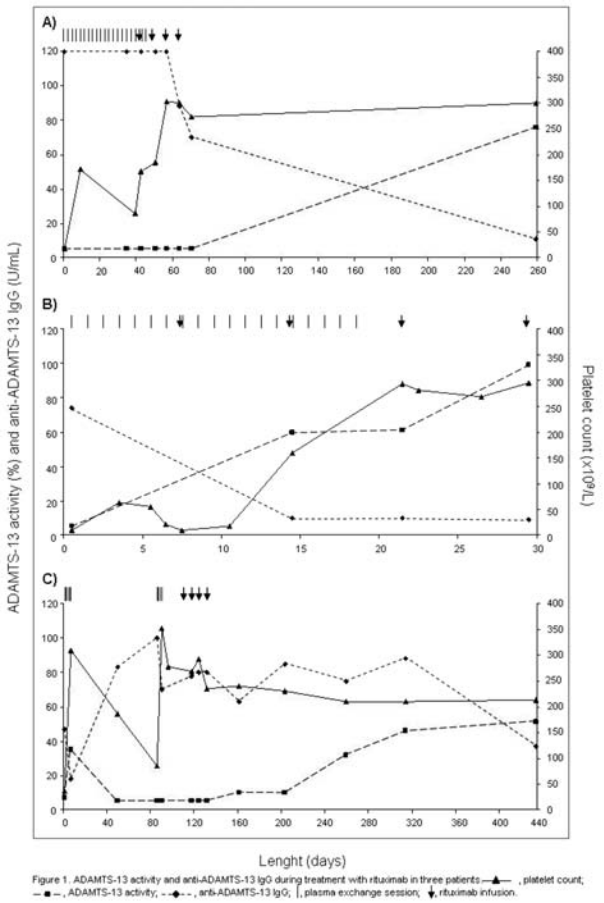

Figure 1. ADAMTS-13 activity and anti-ADAMTS-13 IgG during treatment with rituximab in three patients —▲— , platelet count; —●—, ADAMTS-13 activity; - -●- - , anti-ADAMTS-13 IgG; |, plasma exchange session; ↓, rituximab infusion.

Figure 1.

Prognosis of TTP has been improved by plasma exchange (PE) therapy, but mortality of disease has remained at 20%. Moreover in 20 to 50% of patients who achieve remission experience a chronic relapsing form, needing different approaches. In the last few years rituximab, an anti-CD20 monoclonal antibody, came up as both rescue therapy in the acute PE-refractory episodes and prophylactic treatment in patients who maintain a high titer of anti-ADAMTS-13 antibodies even through clinical remission. *Aim*. We describe the clinical and laboratory course of three patients affected by idiopathic TTP treated with 4 weekly infusions of rituximab 375 mg/m^2. Two patients were treated during their first PE-refractory episode and the third one was prophylactically treated during remission following his first relapse. ADAMTS-13 activity and IgG autoantibodies were assessed at onset and during rituximab treatment using commercially available FRETS-VWF73 and ELISA assays respectively *Results*. Patients 1 (Figure 1A) and 2 (figure 1B) were treated during acute event. They both had severe reduction in ADAMTS-13 activity (<10%) and high titre IgG anti-ADAMTS-13 (>120 and 74 U/mL respectively). The episodes lasted 47 and 24 days and the number of PE sessions performed was 20 and 19 respectively. Clinical and haematological remission (platelets count > 150x10^9/L) was achieved only after first rituximab administration in both cases. ADAMTS-13 activity normalization and anti-ADAMTS-13 antibody disappearance were demonstrated after 7 months and one week since the first infusion in patient 1 and 2 respectively. Patient 3 (figure 1C) experienced two episodes of acute TTP both responsive to PE. During remission following his second acute event he was prophylactically treated with rituximab because of persistent drastically reduced ADAMTS-13 activity (< 5%) with high titre of anti-ADAMTS-13 antibodies (70 U/mL). We observed a sustained clinical and haematological remission lasting for 15 months with normalization of ADAMTS-13 activity 11 months after first dose infusion (51%), but persisting low titre of autoantibody (37 U/mL). *Conclusions*. Rituximab is a useful immunosuppressive treatment both in acute PE-refractory and chronic relapsing TTP related to anti-ADAMTS-13 antibodies; however, despite an immediate clinical response in patients with an acute episode of TTP, we observed a variability in timing of ADAMTS-13 activity normalization and autoantibody disappearance, whose causes deserve further investigations.

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THE INFUENCE OF ANTIPLATELET DRUG THERAPY TO LIPID AND GLUCOSYLATED HAEMOGLOBIN LEVELS OF DIABETIC PATIENTS (MULTI-CENTERED STUDY)

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Background-Aims. To study a possible effect of anti-platelet drugs to HbA1C levels and the lidis of diabetic patients. *Design and Methods*. The study included 194 diabetic patients, 106 men and 88 women, wtih mean age, respectively, 60.9±11.3, 56.2±11.8 years, who where in combined anti-diabetic (metphormin), and anti-lipid (atorvastatin)therapy. From them, 85 where additionaly taking anti-platelet drug therapy (aspirin or clopidogrel), on either primary prevention level, (9 patients), or, more likely, secondary prevention level (76 patients), due to coronary disease, brain stroke, peripheral angiopathy etc.. All the patients where examined and evaluated for Total Cholesterol, LDL Cholesterol and -bA1C. *Results*. Mean avarege and standard deviation where like this (as you can see at the Table).

Table.

Test (n=194)	Under Anti-Platelet Therapy (n=85)	No Anti-Platelet Therapy (n=109)
HbA1C (%)	7,3 (±1,9)	7,9 (±2)
Total Cholesterol (mg/dl)	217 (±31,9)	239 (±30,6)
LDL Cholesterol (mg/dl)	98,2 (±20,4)	113,5 (±16,9)
Triglycerides (mg/dl)	139,6 (±28,2)	145,2 (±35,1)

Conclusions. Diabetic patients under anti-platelet drug therapy present much satisfactory levels as compared to the rest. HbA1C levels, Total Cholesterol and LDL cholesterol between the two groups, present statisticaly significant difference (p<0.01). 2) On the contrary, triglyceride levels seems to be unaffected from combined or not use of anti-platelet drugs (p>0.01). 3) Therefore the need for anti-platelet drug therapy for diabetic patients is a neccesity, not only for secondary prevention strategy, but for primary one as well, and in greater extent since the latter is oddly poor.

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EDTA-DEPENDENT PSEUDOTHROMBOCYTOPENIA AFTER CHANGING INSULIN THERAPY IN A CASE WITH INSULIN-DEPENDENT DIABETES MELLITUS

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Background. EDTA-dependent pseudothrombocytopenia (PTP) could be observed in healthy subjects, but it could also accompany certain diseases. Aims. In this report, development of PTP after changing insulin therapy in a case with insulin-dependent diabetes mellitus (IDDM) is presented. *Case Report*. A 36-year old male patient was evaluated for thrombocytopenia. It was learned from his history that IDDM was diagnosed eight years ago and Humulin M 70/30 (70% NPH isophan suspension mixed with 30% regular insulin produced with recombinant DNA technology) was started with the doses of 18 unit in the morning and 10 unit in the evening. Approximately half and a year ago, Humulin M 70/30 was stopped and NovoRapid FlexPen (Insulin aspart-fast acting human insulin analogous produced with recombinant DNA technology) 8 unit in the morning, 12 unit in the midday and 10 unit in the evening and Lantus Optipen (insulin glargine-long acting insulin analogous) 12 unit at night were started. The patient was not taking any other medication. There was no remarkable finding on physical examination. The values of complete blood count in which thrombocytopenia was detected were as follows: WBC 8.3x10^9/L, RBC 4.87x10^{12}/L, hemoglobin 15.4 g/dl, hematocrite 43.4%, mean corpuscular volume 89.0 fl, mean corpuscular hemoglobin 31.6 pg and platelets 56x10^9/L. Complete blood count freshly from EDTA-anticoagulated blood was as follows: WBC 5.2 x10^9/L, RBC 4.97 x10^{12}/L, hemoglobin 15.3 g/dl, hematocrite 44.2%, mean corpuscular volume 89.0 fl, mean corpuscular hemoglobin 30.7 pg and platelets 226x10^9/L. Huge platelet clusters were observed in peripheral blood film prepared from EDTA-anticoagulated blood after one hour delay. After diagnosing PTP as the cause of thrombocytopenia, we checked his previous medical reports, and we have noticed that his platelet count was in normal range (e.g. 243x10^9/L, 148x10^9/L, 278 x10^9/L, 225x10^9/L and lastly 188 x10^9/l five months before changing of insulin therapy). WBC and platelets were counted as 7.2x10^9/L and 41x10^9/L, respectively after two months of insulin changing; at the same laboratory blood was recounted freshly and at this occasion WBC and platelet count was found as 4.5x10^9/L and 221x10^9/L, respectively and this situation was interpreted as due to coagulation in first sample. The patient and the doctor managing insulin therapy were informed about the cause of thrombocytopenia was PTP and the possible reason for this situation might be due to change in insulin therapy according to his medical reports. *Conclusions*. This is the first case report of PTP related to insulin-dependent diabetes mellitus and insulin or insulin analogous therapy.

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CLASSIC SPLENECTOMY VERSUS LAPAROSCOPIC SPLENECTOMY FOR IMMUNE THROMBOCYTOPENIC PURPURA IN PATIENTS WITH SEVERE REFRACTORY THROMBOCYTOPENIA

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Splenectomy is an effective therapeutic modality for the treatment of immune thrombocytopenic purpura (ITP) with a rate of 70-90% of hematologic response at patients with refractory ITP. The objective of the surgery is to stop the bleeding by eliminating the organ responsible for the clearance of antibody-coated platelets. *Aim of study*. Evaluating the eficacy of classic splenectomy versus laparoscopic splenectomy for immune thrombocytopenic purpura in patients with severe refractory thrombocytopenia. *Design and Methods*. we studied 44 patients with ITP hospitalized in Clinic of Hematology from Craiova (Romania) between 2003-2008. All patients were diagnosed as having ITP, other causes of thrombocytopenia were ruled out by bone marrow aspiration. All patients were initial treated with corticosteroids (prednisone or high dose dexamethasone). Indications for splenectomy were thrombocytopenia with bleeding manifestations and/or lack of response to conservative treatment and low platelet count while on massive steroid. Results: 9 patients from 44 had indications for splenectomy. 3 of them received classic splenectomy and 6 laparoscopic splenectomy. 8 patients had a complet hematologic response after splenectomy. After a period of 2- 4 years 4 of 6 patients with laparoscopic splenectomy relapsed because of accessory spleens. *Conclusions*. classic splenectomy is preferable to laparoscopic splenectomy for ITP at patients with severe refractory thrombocytopenia, because the existence of the risk of relapse through accessory spleens.

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STUDY OF THE USE OF ANTIPLATELET MEDICATION BY DIABETIC PATIENTS IN PERIPHERAL HOSPITALS (POLYCENTRIC STUDY)

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Background and Aims. To register the percentage of diabetic patients who receive antiplatelet medication in a peripheral hospital, considering that the main cause of the morbidity but also the mortality in these patients, is related to vascular complications (either as microangiopathy, or as macroangiopathy), and consequently, this medication aims to the primary as well as the secondary prevention of these complications. On the other hand, the further goal is to underline the necessity of this medication's broader usage. *Material and Methods*. In total, the study involved 347 cases of diabetic patients (136 men and 211 women), with the average age of 63,9±11,9 years and mean time from the disease's appearance the 9,2±4,3 years. Results: As it was found out, 61 patients received in total antiplatelet medication, that is percentage 17,58%. This group included 34 men (percentage 25%) and 27 women (percentage 12,8%). It should be underlined that after further control it was found out that 48 of these patients (percentage 78,7%) suffered already from established coronary disease and received the antiplatelet medication because of the existing disease and also many times after the appearance of relative episodes. *Conclusions*. Considering the previous mentioned, worryingly low percentages of antiplatelet medication in diabetic patients in these county regions, it becomes evident that the prevention in this sector is particularly inefficient and is almost never applied on time. 2) It is, therefore, necessary to ensure the sufficient knowledge and the further activation of all the doctors and particularly the general practitioners at the level of primary health care, since they are in direct and frequent contact to the patients of these difficult of access borderlands, so that these patients get adequately informed about the importance of the preventive receive of antiplatelet medication and its usage is finally generalized.

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PLATELET FUNCTION IN BILHARZIAL HEPATIC FIBROSIS BEFORE AND AFTER SPLENCTONY

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Schistosomiasis is an endemic disease in Egypt Bilharzial hepatic fibrosis is one of its serious complication, leading to portal hypertentison and oesophageal varices. Many factors have been incriminated in the pathogenesis of bleeding in schistosomal patients, including rupture of oesophageal varices. Thrombocytopenia, thrombosthenia, defective coagulation mechamism as welll as increased fibrinolytic activity. In whom hepatosplenic schistosomiasis coexistence with chronic active hepatitis, unresolved hepatitis and/or cirrhosis an additive factor for pathogenesis of bleeding have incriminated. *Aim of work*. We have planned this work in order to assess platelet aggregation as atest for platelet function in hepatosplenic schistosomiasis and to evaluate the role of splenectomy and spleno renal shunt in improvement of platelet aggregation. *Design and methods*. We revised all clinical, hematlogical, haemostatic and platelet aggregation of 72 case of bilharzial hepatosplenomegally, portal hypertension and variceal bleeding presented at gastrointestinal surgery centre in Mansoura university out of these cases, splenectomy was done to 51 cases 32 (with bilharzial hepatic fibrosis, chronic active hepatitis (C.A.H), 7 with bilharzial hepatic fibrosis+unresolved hepatitis and 2 with mixed cirrhosis). spleno renal shunt (SRS) was done to 10 cases (6 cases with bilharzial hepatic fibrosis, 3 cases with bilharzial hepatic fibrosis+unresolved hepatitis and one case with mixed cirrhosis) and others were mangad by sclerotherapy. Follow up of the splenectomized and post SRS cases after 1d, 3d, 5d, 7d, 14d, 1m, 3m, 6m postoperative for clinical, neurological, medical assessment, blood counts, coagulation profiles and platelet aggregation (yardumain; etal 1989). Result Preoperative analysis of our data revealed that:-Platelet count, bleeding time, prothrombin time, activated partial thromboplastin time fibrinogen concentration, antithrombin 111 concentration are significant decreased in all groups however despite of the lower level of antithrombin 111, thrombosis is rare complication because of low prothrombin level, normal amount of antithrombin111 surface-bound com-

plex and normal or elevated β 2 macroglobulin which could partially compensate for antithrombin111 deficiency.Platelet aggregation with collagen, ADP, ristocetin, thrombin and arachidonicacid are significant decrease, in all groups and more evident in whom bilharzial hepatic fibrosis coexists with C.A.H and unresolved hepatitis due to the presence of platelet antibodies which is an additive factor for much impairment of platelet aggregation. post operative analysis of our data revealed that: splenectomay improved hypersplenic cytopenia and resulting in icreased in platelet count reaching its maximum after 2 weeks. Splenectomy improved hemostatic parameters including prothrombin activity, activated partial thromboplastin time, fibrinogen concentration and increased in anti thrombin 111 these changes together with thrombocytosis indicated hypercoagulable state having take place but the high anti thrombin 111 level counteract the observed hypercoagulable state. Splenectomy improve platelet aggregation to ADP, collagen, ristocetion, thrombin and arachidonic acid in patient with bilharzial hepatic fibrosis reaching its maximum after 2 weeks and persist till 6 months. In whom bilharzial hepatic fibrosis coexists with C.A.H and unresolved hepatitis, platelet aggregation to ADP, collagen and ristocetin are increased post splenectomy reaching its maximum after 3 months and persists till 6 months. However, in those with mixed cirrhosis platelet aggregation are increased postsplenectomy but not to extent as those with bilharzial hepatic fibrosis. Spleno renal shunt(SRS) improved hypersplenic cytopenia but not extent as splenectomy, because, spl enectomy not only improve congestion but also remove the organ responsible for blood cell sequestration. Spleno renal shunt(SRS) improve haemostatic parameter because of relief of congestion. Spleno renal shunt (SRS) improve platelet aggregation to ADP, collagen and ristocetin in all groups. However in those with unresolved hepatitis the increase is no more as those postsplenectomy because in shunt procedure, the spleen which may be responsible fore antibody formation not remove. *Conclusions*. Patients with more lowered antithrombin 111 preoperatively & more increased in platelet aggregation postoperatively are more liable to thrombosis post operatively. Patients with high F.D.ps preoperative & no more improvement in platelet aggregation postoperatively are more liable to bleeding postoperativly.

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EVALUATION OF PLATELET FUNCTION DURING PREGNANCY USING THE PLATELET FUNCTION ANALYZER (PFA-100)

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Background. The PFA-100 is a point-of-care platelet function analyzer which measures the speed of formation of a platelet plug *in vitro*, expressed as closure time (CT) in seconds. This device could potentially be used to assess primary haemostasis. *Aim*. This study aimed to evaluate platelet function during uncomplicated pregnancy using the PFA-100 and compare PFA values between pregnant and non-pregnant women . *Design and Methods*. The study population was divided in two groups: Group I (n=20) healthy non pregnant women (control group) , Group II (n=20) healthy pregnant women. Platelet function was evaluated in whole blood with the PFA-100 (Platelet function Analyzer PFA -100, Dade Behring), using collagen/epinephrine (col/epi) and collagen/ADP (col/ADP) as platelet agonists. Results were expressed as closure time (sec). We also measured platelet count and Hct using XT-1800 blood analyzer.

Table.

	Group A			Group B		
	Mean	Sd.	Min-max	Mean	Sd.	Min-max
col/epi	115, 5	16, 74	88 - 141	107, 85	33, 26	71 - 221
col/ADP	80, 75	10, 82	62 - 100	78, 05	13, 89	56 - 110
Hct	42, 94	3, 16	39,50 - 50,4	37, 64	3, 93	30,60 - 48,30
PLTs	253, 55	65, 82	155 - 368	230, 60	67, 99	131 - 402

Results. Age and platelet count were similar across the two groups (p>0.05), while Hct values were different at a statistical significant level as expected due to pregnancy anemia. Results are reported as mean, std deviation and min-max. Statistical analysis was performed with the use of SPSS 16 and data were analyzed with T-test. A p<0.05 was considered significant. *Conclusions*. In normal pregnant women, platelet reactivity is known to be increased. The mean CTs of our normal pregnant population were lower than that of non pregnant population but still in normal ranges and without statistical difference. This is suggestive of mildly enhanced primary haemostatic function. This study provides a normal range of PFA CT for our healthy pregnant populations and confirms the platelets activation of normal pregnancy. Further studies with larger number of patients could possibly reveal a statistically significant difference.

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RITUXIMAB IN REFRACTORY IDIOPATHIC THROMBOCYTOPENIC PURPURA TREATMEN IN CHILDREN

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Background. Immunosuppressive therapy is a method of choice for refractory idiopathic thrombocytopenic purpura (ITP) treatment in children. In last years the monoclonal CD-20 antibodies are more used in treatment. *Design and Methods*. Rituximab was used in treatment of three children with refractory idiopathic thrombocytopenic purpura. Informed concern forms had been obtained before starting the treatment. All patients did not respond to the basic steroid therapy (platelets count was less than $30x10^9$/L). All of them had a presence of hemorrhagic syndrome's features such as nasal bleedings and skin hemorrhagic syndrome. Rituximab was used once a week, 375 mg/m^2, during four weeks. Efficacy evaluation was based on both platelet count normalization and disappearance of hemorrhagic syndrome. After four injections of Rituximab, two patients reached a complete hematological response (platelets count increased up to 184-218$x10^9$/L) and one child had a partial hematologic response, and his plateles count increased up to $59x10^9$/L. A retrospective analysis has indicated that response has still been present, i.e. longer than for five months. There are no side effects detected. Thus, administration of Rituximab in treatment of refractory ITP allows us to achieve a significant effect. The latter would have been impossible should steroids have been used. In addition, the administration both leads to avoiding of long time usage of high dose steroids and their side effects, and significantly improves quality of life.

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INVESTIGATION OF THE FREQUENCY AND THE NECESSITY OF ANTICOAGULANT MEDICATION IN ELDERLY PEOPLE (POLYCENTRIC STUDY)

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Background and Aims. The study of the use of anticoagulants in elderly people, and the estimate of the potential benefit in relation to the possibility of hemorrhage. *Design and Methods*. Patients with risk factors for thrombo-embolic episodes, such as an old thrombo-embolic episode and atrial fibrillation, participated in the study. The receive or not of anticoagulant medication was investigated, the cases of patients with an INR level out of the therapeutic limits were registered, and all the inpatients but also the newly hospitalized patients with hemorrhagic tendency because of the anticoagulant medication, were included in the study. *Results.* In total 109 patients over 60 years old were found, who had at least one risk factor for thrombo-embolic episode. Only 43 of those (percentage 39,4%) received anticoagulant medication, while about one in three under medication with dicumarol derivatives had the INR level within the therapeutic limits. The majority (about 2/3) of the rest had higher values than the desirable and fewer (about 1/3) lower values. *Conclusions*. It is, therefore, evident that, although the anticoagulant medication is particularly valuable for the prevention of thrombo-embolic episodes in people with certain risk factors, in many cases it's not used. On the other hand, it is shown that, especially in the elderly people who apparently run a greater risk of hemorrhage, the anticoagulant medication should be used after caution and careful thought, since the possible benefit should be always weighed in relation to the possibility of

hemorrhage. Finally, it should be underlined that the percentage of cases where the effect of the dicumarol derivatives stands out of the therapeutic limits is quite high, fact that imposes a frequent and careful control of the INR.

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SERUM ADIPONECTIN AND RESISTIN LEVELS AND THE OCCURRENCE OF MYELODYSPLASTIC SYNDROME

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Background and Aims. Recent evidence suggests that obesity may be implicated in the etiology of myelogenous leukemia and myelodysplastic syndrome (MDS). We thus attempted to explore whether altered secretion of the adipokines: adiponectin and resistin, a hormonal system 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 total and high molecular weight (HMW) adiponectin and resistin, in the etiopathogenesis of MDS after adjusting for a potential confounding effect of body mass index (BMI), height, weight and leptin. We also explored associations between adiponectin and resistin and established MDS prognostic factors. *Design and Methods*. Blood samples were collected from 101 cases with incident, histologically confirmed primary MDS, and 101 hospital controls, admitted for non-neoplastic and non-infectious conditions, matched on gender, age and year/month of diagnosis (±1 month) between 2004 and 2007. Serum total adiponectin and resistin concentrations were determined by radioimmunoassay (LINCO Research Institute, St Charles, MO). Moreover, serum HMW adiponectin was measured using ELISA (ALPCO Diagnostics, Salem, NH). Statistical analysis of the data was performed with SAS 9.1 for Windows XP. *Results*. Cases presented significantly higher height and weight than control subjects ($p<0.001$), while differences of BMI were only of borderline significance ($p=0.12$). Cases had significantly lower serum levels of adiponectin, HMW adiponectin and resistin than controls ($p<0.001$, $p=0.003$ and $p=0.019$ respectively). HMW adiponectin levels were significantly different in MDS type ($p=0.028$) being higher in chronic myelomonocytic leukemia (CMML) but both HMW adiponectin and resistin were not significantly different amid IPSS stratification schemes both before and after adjusting for age, gender and BMI. Although, total and HMW adiponectin were both significantly inversely associated with MDS when modeled either in quartiles or continuously, HMW didn't offer any substantial additional predictive value over total adiponectin (OR=0.91 v. 0.93 for a 1 μg/mL change, respectively). Also, adjusting for age, gender, BMI, adiponectin and leptin, serum resistin levels were significantly inversely associated with MDS occurrence (OR= 0.20, 95% CI: 0.08-0.52). *Conclusions*. Total and HMW adiponectin may present a protective role in MDS, whereas resistin levels may be decreased via a compensatory response to the upregulation of other inflammatory parameters etiologically linked to myelodysplasia. These observations need to be replicated and warrant further investigation.

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TREATMENT OF PATIENTS WITH HIGH-RISK MYELODYSPLASTIC SYNDROMES RECEIVING AZACITIDINE WHO ARE ENROLLED IN AVIDA, A LONGITUDINAL PATIENT REGISTRY

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Background. A phase III study demonstrated that azacitidine, a hypomethylating agent, provides clinical benefit including prolonged survival in high-risk MDS patients (*Fenaux et al, Blood. 2007;110:Abstract 817*). Use of azacitidine in high-risk patients in the community-based setting has not been well characterized. *Aims*. Investigate the characteristics, treatment patterns, and transfusion status of patients with high-risk MDS who are enrolled in AVIDA (a longitudinal, multicenter patient registry designed to prospectively collect data from community-based hematology clinics on the natural history and management of MDS patients who are treated with azacitidine). *Design and Methods*. Patients with an International Prognostic Scoring System (IPSS) risk classification score of intermediate-2/high at baseline were included in the analysis. Transfusion independence was defined as no transfusions for at least 56 days in patients with historical (6 months prior to AVIDA) transfusion requirements and at least 56 days treatment duration. The first day of the 56-day period with no transfusions was noted as the time at which patients first achieved transfusion independence. *Results*. As of January 5, 2009, 79 patients (55 males, 24 females; median age 74 years [range, 45-88]) with an IPSS score of intermediate-2/high have been enrolled in AVIDA; 60 (76%) with intermediate-2 and 19 (24%) with high score. Median time from first MDS diagnosis until azacitidine treatment was 1 month (range, 0-103), suggesting minimal delay in treatment. At baseline, 64 (81%) patients had 5% or higher bone marrow blasts and 38 (48%) had a "poor-risk" karyotype. Cytopenias were reported in 2 or 3 lineages for 61 (77%) patients. A total of 281 cycles of azacitidine have been administered either by subcutaneous (48%) or intravenous (52%) route. These intermediate-2/high-risk patients have received a median of 3 cycles (range, 1-14); 49/79 (62%) patients have received at least 2 cycles. Red blood cell (RBC) transfusion status data are available for 23 patients who received RBC transfusions in the 6 months prior to treatment and at least 56 days of azacitidine. Of these, 10 (44%) achieved RBC transfusion independence; 9/10 (90%) first achieved RBC transfusion independence during the first 2 cycles. Platelet transfusion status data are available for 5 patients who received platelet transfusions in the 6 months prior to treatment and at least 56 days of azacitidine. All 5 (100%) have achieved platelet transfusion independence during the first 2 cycles. In these high-risk patients, azacitidine was generally well tolerated; most common adverse events were fatigue (23%), constipation (19%), nausea (17%), and thrombocytopenia (17%). *Conclusions*. These data demonstrate that patients with an IPSS score of intermediate-2/high are being treated in the community-based setting and can achieve transfusion independence while receiving azacitidine at a rate comparable to that reported a clinical trial (Aza-001). AVIDA provides a unique opportunity to characterize the MDS patient population that is receiving chemotherapy in the community-based setting. Effect of azacitidine on the outcome and overall quality of life in this patient subpopulation will become clearer as more patients with high-risk MDS are treated.

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ERYTHROID FLOW CYTOMETRY IMMUNOPHENOTYPE IN MYELODISPLASTIC SYNDROMES. PRELIMINARY RESULTS IN PERIPHERAL BLOOD

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Background. Cytomorphology has been the main approach to the diagnosis of Myelodisplastic Syndromes (MDS) for many decades. Recent studies show that multiparameter flow cytometric characterization of specific bone marrow population according to their lineage and maturation stage is of great clinical utility for the diagnosis of MDS. Actually, bone marrow needle aspiration is necessary for the diagnosis and classification of MDS and there is no peripheral blood screening that easily diagnoses this common hematologic malignancy. Erythroid dysplasia is found in almost all patients with MDS and is the only morphological abnormality in those with refractory anemia, that is, the pure erythroid disorders of the WHO classification. Although flow cytometry immunophenotyping is a reliable method for quantitative and qualitative evaluation of hematopoietic cells and can provide better reproducibility than morphology, evaluation of erythroid dysplasia represents a challenge in the immunophenotypic analysis of MDS, mainly because of the limited availability of specific antibodies. Recent the availability of monoclonal antibodies to the erythrocyte antigens have made such a study possible. *Aim*. The purpose of this study was to develop a flow cytometric approach (immunophenotype and forward and side scatter characteristics - FSC/SSC) to the evaluation of erythroid dysplasia in peripheral blood cells of patients with MDS, to easily diagnose early stages of this malignancy. *Design and Methods*. We evaluated peripheral blood samples from 16 *de novo* adult low grade MDS patients and 16 adult healthy blood donors. Informed consent was obtained in all cases. Whole peripheral blood samples were stained using a direct or indirect immunofluorescence technique without lyse in which the following monoclonal antibodies where used in triple staining against mem-

brane proteins like human blood groups, glycophorins, Transferrin receptor and hemolisis inhibitor proteins : CD36, CD55, CD59, CD71, CD174, CD236R, CD238, CD239, CD240DCE, CD240R and Glycophorin A. For reticulocyte identification DRAQ 5 dye was used. Acquisition was performed in a FACScalibur flow cytometer with CellQuest software and low flow, with FSC and SSC parameters at logarithmic scale. Data analysis was performed with Infinicyt software. Statisitc tests T-student and U-Mann-Whitney were used with SPSS software. *Results*. Comparison of FSC/SSC characteristics of normal healthy donors samples and MDS patients samples allowed the identification of aberrant phenotypes in MDS patients. The FSC and SSC parameters permitted the characterization of five different red blood cells subpopulations that presented abnormal distribution in MDS samples. Immunophenotypic aberrancies were detected in reticulocytes and also in mentioned red blood cells subpopulations. Low expression of CD236R, CD240DCE and Glycophorin A were the aberrant phenotypes with statistic significance. *Conclusions*. These results suggest that peripheral red blood cells flow cytometry studies can be useful in the earliest diagnosis of low grade MDS patients by the identification of aberrant immunophenotypes and abnormal FSC/SSC characteristics.

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PROGNOSTIC SIGNIFICANCE OF EOSINOPHILS AND BASOPHILS IN MDS: EVALUATION OF A CORE DATA SET OF 1008 MDS PATIENTS

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Background. Myelodysplastic syndromes (MDS) are a group of myeloid neoplasms characterized by abnormal differentiation and maturation of myeloid cells, reduced bone marrow (BM) function, and a genetic instability with enhanced risk to transform to secondary acute myeloid leukemia (AML). Among other factors, lineage involvement and maturation arrest are considered to be of prognostic significance in MDS patients. However, whereas the prognostic value of neutropenia, thrombocytopenia, and monocytosis have been documented, little is known about the impact of eosinophils and basophils. Patients and *Design and Methods*. We examined the prognostic significance of eosinophils and basophils in 1,008 patients with de novo MDS. Patients were enrolled from 3 centers of the Austrian-German MDS workinggroup and were analyzed retrospectively. Blood eosinophils and basophils were quantified by light microscopy, and their impact on survival and leukemia-free survival calculated by Cox regression. *Results*. Eosinophilia (>350/µL) and basophilia (>250/µL) were found to indicate a significantly reduced survival (p<0.05) without showing a significant impact on leukemia-free survival. The absence of eosinophils and basophils was also found to indicate poor survival. In multivariate analyses, eosinophilia and basophilia were found to be LDH-independent prognostic variables with IPSS-specific impact. Whereas an elevated LDH was found to be a major prognostic determinant in IPSS LOW, INT-1, and HIGH subgroups, eosinophilia and/or basophilia was found to be a superior prognostic indicator in INT-2 patients. *Conclusions*. Evaluation of eosinophils and basophils in MDS is helpful and may complement the spectrum of variables to optimize prognostication in MDS.

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RETROSPECTIVE EVALUATION OF IRON OVERLOAD PREVALENCE IN MYELODYSPLASTIC SYNDROMES AT THE DIAGNOSIS

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Background. In the practical management of myelodysplatic syndromes (MDS), iron balance is carefully evaluated overall in transfused patients to select those eligible for iron chelation. Serum-Ferritin (s-F) is the only reliable laboratory parameter to assess IOL in such patients. *Aim of the study*. There are few reports about the prevalence of iron overload (IOL) in not transfused patients. We wanted to evaluate this prevalence using different approaches *Design and Methods* We retrospectively examined data concerning iron balance of 81 MDS patients at the diagnosis, never transfused. We identified IOL not only by s-F (available in 74 subjects) but also by Transferrin Saturation Index (TSI) (available in 62). In 55 patients both parameters were present. TSI was calculated as percentage of the ratio between serum iron and total iron binding capacity (TBIC); s-F was routinely assayed. We chose this approach because in other clinical setting like Hereditary Hemochromatosis (HHc), TSI is indicated as an early marker of IOL and it has a diagnostic value also before the rise of s-F. Moreover, s-F can rise for liver damage and inflammation, loosing its specificity as IOL indicator. *Results*. Table 1 shows the results obtained. In our experience, the prevalence of cases with severe IOL (ferritin >1000 ng/mL) is similar to that reported by other Authors (*Gatterman. Hematol Oncol Clin North Am 2005*).

Table 1.

Iron balance evaluated according TSI n=62	TSI>50%	21/62	33,8%
Iron balance evaluated According s-F n=74	>300 ng/ml	29/74	39,2%
	>500 ng/ml	18/74	24,3%
	>1000 ng/ml	4/74	5,4%
Iron balance evaluated According TSI and s-F n=55	TSI>50% n=18 18/55(32,7%)	and s-F>300 ng/ml 13/18 Total cases 13/55	72% 23,6%
	TSI>50% N=18	and s-F>500 ng/ml 10/18 Total cases 10/55	55% 18,2%
	TSI>50% n=18	and s-F>1000 ng/ml 4/18 Total cases 4/55	22% 7,2
Iron deficient patients	TSI<20% n=9	and s-F<20 ng/ml 3/9 Total cases 3/55	30% 5,4%
Inflammatory iron balance	TSI<20% n=9	and s-F>50 ng/ml 6/9 Total cases 6/55	66,6% 10,9%
Normal iron balance	n=19	19/55	34,5%

Nevertheless, the prevalence of a quite relevant IOL (s-F>500 ng/mL and<1000) is not unimportant (14/74 patients; 18.9%). On the other hand, if IOL is evaluated according to the criteria recommended for HHc (TSI>50% alone or together with s-F>300 ng/ml), its prevalence is much higher (33.8%; 23.6% respectively). In the group with both TSI and s-F available, the prevalence of IOL, identified according to TSI>50%, is significantly higher than that identified according s-F>1000 (32.7% versus 7.2% Fisher's exact test: p=0.0015) In addition, the contemporary evaluation of TSI and s-F allows recognizing MDS with inflammatory pattern of iron metabolism. In our group of MDS patients 10,9% showed this condition. *Conclusions.* We retain that iron balance, in not transfused MDS, should be investigated both by ferritin and TSI. This approach seems to be more sensitive in individuating IOL, and it allows recognising other alteration of iron balance. Further studies should clarify if the less severe IOL states are able to generate oxidative stress in the complex clinical context of MDS. This is a very important issue because many evidences support the link between oxidative stress and the DNA damage as a fundamental contribution to neoplastic evolution of MDS (*Rassool Cancer Res 2007*).

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TREATMENT OF CMML BY AZACYTIDINE (AZA): A PRELIMINARY REPORT ON 23 PATIENTS (PTS)

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Background. Treatment of CMML remains difficult, with no drug having shown a clear clinical benefit. AZA has demonstrated a survival benefit in higher risk MDS, in a study that included a small number of CMML (*Lancet Oncol, 2009, online*). *Design and Methods*. The French health agency (AFSSAPS) designed a still ongoing pt named program (ATU) of AZA in higher risk MDS and poor risk AML. 24 pts with CMML or AML arising from CMML included in this program, before April 08 and having completed 1 cycle of AZA (75 mg/m^2/d during 7 days per 28 d cycle) are analysed here. Response was evaluated according to IWG 2006 criteria for MDS, and IWG-AML 2003 criteria for AML. *Results*. Median age was 66y (range 52-85), M/F: 16/7. At inclusion, 6 patients had CMML-1, 10 CMML-2 and 7 AML secondary to CMML according to WHO classification. Karyotype was normal (n=9), isolated -7/7q- (n=2), +8 (n=2), del20q (n=1), other single (n=8) complex (n=1) and a failure (n=1). Median interval from diagnosis to treatment was 1.5 years (range 0 months-5.6 years). Previous treatment consisted of low dose chemotherapy (n=1), Intensive chemotherapy (n=7), Allogeneic SCT (n=2), ATO (n=3) or ESA (n=9). The median number of cycles of AZA administered was 4 (range 1-19). 2 pts received only 1 cycle due to early death (n=1) and progression (n=1). 4 additional pts received less than 4 cycles due to hematological toxicity (n=3) or physician's decision (n=1). 7 pts (30%) responded including 3 CR, 3 marrow CR and 1 HI-E. Sex, Age, WBC count, monocyte count, Hb level, platelet count, WHO diagnosis, previous high dose chemotherapy and cytogenetics did not differ significantly between responders and non responders. 7/17 (41%) pts who received more than 4 cycles responded. Among the 16 MDS pts who received more than 1 cycle, 6 (38%) responded (3 CR, 2 marrow CR and 1 HI-E). Only one AML responded (marrow CR lasting 13 mos). 3 of the 7 responders relapsed after 13, 14 and 19 mos, and 4 remained responders after 10+, to 17+ mos. Median overall survival was 29 months in responders and 6.7 months in non responders. *Conclusions*. Those preliminary results confirm the activity of AZA in advanced CMML.

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α-DEFENSINS SECRETED BY IMMATURE DYSPLASTIC GRANULOCYTES INHIBIT THE DIFFERENTIATION OF MONOCYTES IN CHRONIC MYELOMONOCYTIC LEUKEMIA

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Background. Chronic myelomonocytic leukemia (CMML), which is the most frequent myelodysplastic/myeloproliferative disorder, occurs in elderly patients. One of the main diagnosis criteria of this clonal hematopoietic disease is a persistent peripheral blood monocytosis. We observed recently that, in most patients with CMML, part of the cells that accumulate in the peripheral blood and are usually classified as monocytes are actually dysplastic granulocytes with a CD14$^-$/CD24$^+$ phenotype. *Aims*. Compare the proteome profile of CD14$^-$/CD24$^+$ cells with the one of CD14$^+$/CD24$^-$ monocytes from CMML patients or healthy donors. *Design and Methods*. Clinical and laboratory findings were evaluated for several CMML patients. *Results*. The proteome profile of CD14$^-$/CD24$^+$ cells is dramatically distinct from the one of pathological and normal CD14$^+$/CD24$^-$ monocytes. More specifically, CD14$^-$/CD24$^+$ CMML cells synthesize and secrete large amounts of α-defensin 1-3 (HNP1-3). Recombinant HNP3 inhibits the M-CSF driven differentiation of human peripheral blood monocytes into macrophages, e.g. prevents characteristic morphological changes, the expression of CD71 and CD163 surface markers. Using transwell and suramin inhibition of ATP receptor or antibody-mediated depletion experiments, we demonstrate that HNP1-3 secreted by CD14$^-$/CD24$^+$ cells inhibit M-CSF-induced differentiation of CD14$^+$/CD24$^-$ cells from healthy donors or CMML patients. *Conclusions*. Altogether, these results suggest that a population of immature granulocytes, namely CD14$^-$/CD24$^+$ cells, contributes to the CMML phenotype through inhibiting the differentiation capabilities of monocytes.

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NEUT-X AND NEUT-Y PARAMETERS ARE USEFUL TO DETECT NEUTROPHIL DISPLASIA IN MYELODYSPLASTIC SYNDROMES

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Background. Diagnosis of myelodysplastic syndromes (MDS) relies on the morphological features seen on the blood or bone marrow. In peripheral blood we can see nuclear anomalies as hypolobulation, clumping, nuclear sticks and hypersegmentation and citoplasmic anomalies as hypogranulation. *Aims*. The main objective of this study is to see if hematology analyzers can detect neutrophil displasia. *Design and Methods*. Hemograms procesed in Sysmex XE-2100 hematology analyzers generate some research data like NEUT-X and NEUT-Y parameters. Blood samples anticoagulated with EDTA-K3 were analyzed before 6 h after extraction. Neutrophil granularity were optically graded as normal, hypogranular or both types of neutrophils in the same sample. Statistical analysis was performed using SPSS. *Results*. Between Aug 08 and Jan 09 we evaluated a control group of 50 cases, 50 deliveries, 50 anemias, 50 idiopathic leukopenias, 50 cases with hypergranulated neutrophils and 50 MDS. In the control group the mean and standard deviation (SD) were 1346±28.2 and 420±19.3 respectively for NEUT-X and NEUT-Y. In anemia group values were 1341±30.9 and 420±24.6 (p>0.05) and in the leukopenia group: 1346±30.2 and 427±34.0 (p>0.05). Deliveries group and hypergranulated neutrophils cases had significantly higher values: 1371±30.0 and 453±17.8 , 1394±38.3 and 556±111.4 (p<0.05). The mean and SD for MDS group were 1286±72,8 and 385±50,9 significantly lower than all other groups (p<0.05). In the group of MDS we included 4 refractory anemia, 11 refractory anemia with ringed sideroblasts, 1 5q- syndrome, 8 refractory cytopenia with multilineage dysplasia, 8 refractory cytopenia with excess blasts, and 18 chronic myelomonocytic leukemia (this type is included in myelodysplastic/myeloproliferative syndromes in the OMS classification). There were not significant differences in NEUT-X and NEUT-Y values between morphological types of MDS. In the subgroup of MDS with optic hypogranulation the mean and SD for NEUT-X and NEUT-Y were 1255±65.1 and 368±43.6; values were 1338±49.4 and 414±41.8 for MDS without optic hypogranulation (p< 0.05). NEUT-X and NEUT-Y less than 1285 and 375 respectively had a specificity of 98% and a sensitivity of 48% and 44% for MDS detection. The same cutoff will detect 68% and 58% respectively of MDS with optic hypogranulation. *Summary.* NEUT-X and NEUT-Y parameters are useful to detect neutrophil displasia in MDS.

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CLINICAL AND BIOLOGICAL EFFECTS OF 5-AZACITIDINE FIVE DAYS/MONTHLY SCHEDULE IN SYMPTOMATIC LOW-RISK (IPSS: 0-1) MYELODISPLASTIC PATIENTS

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Background. Nucleoside 5-Azacitidine (5-Aza) administered at a dose of 75 mg/m^2/day subcutaneously for 7 days, every 28 days, induces high hematologic response rates and reduces progression to acute myeloid leukaemia (AML) in the high risk MDS patients. *Aim*. The use of 5-Aza in the earlier phases of MDS could reduce the proliferative advantage of MDS clone and favours the regrowth of normal hematopoiesis. In the setting of low-risk MDS patients lower doses of 5-Aza could be enough to induce hematologic responses. We attempted to use an alternative schedule, 75 mg/m^2 subcutaneous daily for 5 consecutive days every 28 days, for a total of 8 courses, to evaluate its efficacy and tolerability in low risk MDS patients. Pharmacogenomic studies (the gene expression profile and single nucleotide polymorphism analysis), and the determination of cytokine's pattern, before and after 5-Aza treatment, were planned to identify new biological markers to predict the response. The first step will be to performe biological studies in 5 responsive and 5

resistent patients; based on these results the analysis could be extended to more patients. *Design and Methods*. Between May and December 2008 we enrolled 15 patients in the multicentric clinical trial. According to WHO criteria 6 patients had refractory anemia (RA), 5 patients refractory cytopenia with multilineage dysplasia (RCMD) and 4 patient refractory anemia with excess blasts-1 (RAEB-1); all patients were classified as Low Risk (IPSS score 0-1). Age at diagnosis ranged between 56 and 82 years. All patients failed EPO therapy and were in chronic red blood cell (RBC) supportive care with a median transfusions requirement of 4 units/monthly. *Results*. The response treatment criteria was according to IWG 2006. 5 out of 15 patients completed the 8 courses of therapy; 4 patients obtained an hematologic improvement (HI) with an erythroid response whereas 1 patient maintained a stable disease. The others 10 patients are ongoing. The drug was very well tolerated. Hematologic toxicity consisted in neutropenia (WHO grade III) and thrombocytopenia (WHO grade II) in one patient but it was transitory and no delay of treatment was necessary. *Conclusions*. Our preliminary results show that the 5-Aza five days/monthly schedule is very well tolerated and it appears to have an efficacy similar to the seven days/monthly schedule, at least in low-risk MDS setting. Considering that the optimal schedule and duration for demethylating agents has not yet been established, further MDS patients recruitment is warranted to confirm the efficacy of this alternative 5-Aza low dose regimen. Biological studies will be performed after the clinical study and they will be correlated to the treatment response. The results could be useful to elucidate the genetic bases for individual susceptibility to the beneficial (or adverse) effect of 5-AZA, in order to optimise the therapy.

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SECONDARY RADIATION-INDUCED MYELODYSPLASTIC SYNDROME: APOPTOSIS AND TELOMERE LENGTH

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Background. Myelodysplastic syndromes (MDS) in radiation exposed are characterized by a combination of ineffective hematopoiesis with late radiation post-effects. Increase of apoptosis and changes of telomere length were demonstrated in irradiation experiments and exposed humans. Aim of the study was to reveal possible modifying role of the late radiation induced changes on apoptosis and telomere length in myelodysplasia. *Design and methods*. Peripheral blood samples were studied in 17 exposed (mean dose 0,42 Sv) and 6 non-exposed MDS-RA patients and 10 non-exposed controls. Apoptosis was studied in Annexin-V test, telomere length - by PNA flow-FISH assay. *Results*. MDS-RA samples have demonstrated high levels of granulocyte and lymphocyte apoptosis and significantly lower relative telomere length (RTL) index in comparison with healthy controls. Lower RTL indices and no difference in apoptosis were shown between MDS-RA subgroups due to the fact of previous irradiation. Mean RTL indices in the exposed RA subgroup were lower than in comparison group. Correlations with age were demonstrated with early apoptosis but not RTL. AML cases in comparison with MDS showed marked decrease of cell numbers at all stages of apoptosis but no difference of group RTL from control due to individual variation. *Summary*. This study shows a possibility of relationship between apoptosis induction and the telomere region changes in MDS-RA at the remote period after radiation exposure.

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A RATIO OF LYMPHOCYTES AND NEUTROPHILS GRANULARITY AS A NEW APPROACH FOR SCREENING MYELODYSPLASTIC SYNDROM BY FLOW CYTOMETRY

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Background. The myelodysplastic syndrome (MDS) is a group of clonal bone marrow disorders characterized by peripheral cytopenia, inefficient hematopoiesis, and unilineage or multilineage dysplasia. It is known that the level of granularity of neutrophils is the most important morphologic feature of MDS. The genetic features of MDS often include clonal, nonrandom chromosomal deletions (e.g., 7q-, 5q-, 20q-, 6q-, 11q- and 13q-) that appear to inactivate tumor suppressor genes required for the normal development of myeloid cells. The aim of this study is a creation of simple and modern screening test for first step diagnostic of MDS. *Design and methods*. 20 patients with median age 68 (58-83) years diagnosed with MDS and 20 samples of healthy individuals were enrolled into this study. The protocol of study included peripheral blood smears microscopy, evaluation of blood cell populations by hematological analyzer (GenS, Beckman Coulter, USA), and flow cytometry (FC500, BC). It was used a single tube protocol of multiparametric flow cytometry using a panel of monoclonal antibodies: D14-FITC, CD16-PE, CD33-PC5 and CD45-PC7 (BC). Neutrophils and lymphocytes were identified using the special gating strategy and the combinations of FS/SS, CD45/SS, CD45/CD16 and CD33/CD14. Molecular genetic tests were performed for identification specific genetics abnormalities for MDS. Results. There were determined the mean scatters (MS) of neutrophils and lymphocytes of peripheral blood. The ratio between neutrophils and lymphocytes MS was significantly lower in MDS patients than in the group of healthy individuals (p=0.016), AUC=0.824, sensitivity 81.8%, specificity of 81.2%. Based on cytogenetic results, patients with MDS can be subdivided into cases with (i) normal karyotypes (75% cases), (ii) del(5q-) (15% cases), (iii) del(6)(q23) (5% cases), and (inv) del(7)(q22q31) (5% cases). *Conclusions*. The information about hypogranularity of the neutrophils is a well known feature of MDS. We have suggested single tube flow cytometry protocol to determine the level of granularity of different leukocytes populations. The lymphocyte MS is constant for healthy individuals and for MDS patients. The ratio between neutrophils and lymphocytes MS allows to differentiate peripheral blood of healthy individuals and MDS patients regardless of the severity of disease. These data are numerical, quantitative and objective. The ratio between neutrophils and lymphocytes MS might be useful tools to detect or flag the neutrophil hypogranularity and helps in the differential diagnosis of MDS.

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EXPRESSION OF CYCLIN A1 MRNA IN PATIENTS WITH MYELODYSPLASTIC SYNDROME AND ITS CLINICAL SIGNIFICANCE

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Background. Myelodysplastic syndromes are bone marrow stem cell disorders resulting in disorderly and ineffective hematopoiesis manifested by irreversible quantitative and qualitative defects in hematopoietic cells. In a majority of cases, the course of disease is chronic with gradually worsening cytopenias due to progressive bone marrow failure. Approximately one-third of patients with MDS progress to AML within months to a few years. Aims: The purpose of study was to evaluate the expression of cyclin A1 mRNA in patients with myelodysplastic syndrome (MDS) and their clinical value. *Design and Methods*. The expression of cyclin A1, cdk2 and p21 cip1 mRNA in the bone marrow from 56 patients with MDS and 10 normal control were measured with Quantitative real-time reverse transcriptase polymerase chain reaction (RQ-PCR) technique. *Results*. The positive rate and the expressing level of cyclin A1 in MDS patients (69.64%; 0.964±1.879) were significantly lower than those in nromal control (0%; 0.012±0.014) (p<0.01). Among de novo MDS patients, the expressing mRNA level of cyclin A1 was higher in the MDS-RAEB group (1.895±1.769) than that in MDS-RA group (0.629±1.583) (p<0.01). The expressing mRNA level of cyclin A1 in post-treatment group was significantly lower than that in prior treatment group (p<0.01). *Summary*. It is concluded that the mRNA expression of cyclin A1 in MDS patients is higher than normal control, the abnormal expression of cyclin A1 might be a prognostic marker in MDS patients.

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WITHDRAWN

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RISK FACTORS AND OUTCOME OF SEVERE INFECTIOUS OR HEMORRHAGIC EVENTS DURING THE COURSE OF MYELODYSPLASTIC SYNDROMES

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Background. Neutropenia and thrombocytopenia are common cytopenias in myelodysplastic syndromes (MDS). *Aim*. To evaluate the impact of severe infectious or hemorrhagic events on morbidity and mortality during the course of MDS is unknown. *Design and Methods*. A retrospec-

tive study was conducted to describe infectious or hemorrhagic events requiring hospitalization in MDS. The impact of risk factors on the outcome was also evaluated: comorbidities, prior treatment, disease status, degree of neutropenia or thrombopenia, treatment. Results-Thirty infectious events (IE) occurred in 22 MDS (3 RA, 2 RARS, 10 RAEB-1, 3 RAEB-2, 4 AML according to WHO's classification). Mean IPSS was 1.43 and high risk MDS (IPSS >1) represented 53% with a mean age of 72.4yr (min.54-max.90). Four patients required intensive unit care transfers due to acute respiratory distress syndrome (3 cases) or hypovolemic choc (1 case) and all died. Overall mortality rate was 13%. Site of infection was commonly pulmonary (10 cases), cutaneous (5 cases) and digestive tract (4 cases). Microorganisms category was proved in 6 cases (5 bacteria, 1 virus) and supposed to be bacterial in 19 cases or fungal in 4 cases. Mean absolute neutrophils count (ANC) was 2.4 G/L, 17 patients have neutropenia (ANC <1.5G/L) with 11 ANC <0.5G/L. Mean period of hospitalization was 12.7 days (min.1-max. 45) and increased with neutropenia degree, 15.8 and 8.5 days for ANC <1.5 or >1.5G/L respectively. Most IE received more than 1 treatment, mean 1.9 drugs. Favorable outcome was observed in 80% of events. Twenty four hemorrhagic events (HE) occurred in 17 MDS (2 RA, 1 RCMD, 5 RAEB-1, 4 RAEB-2, 5 AML). Mean IPSS was 1.5 and high risk MDS (IPSS >1) represented 55%, mean age was 72.1yr (min.57-max.86). Localizations of bleeding were commonly cerebral (7 cases), cutaneous or mucous (13 cases). Mean platelets count was 21G/L. Mortality rate was 29% and reached 71% for cerebral bleeding. No risk factor (platelet count, prior therapy) was correlated with the occurrence of HE except disease progression. Fatal outcome was associated with an increased Charlson comorbidity index (respectively 4.85 versus 3.35). In the same period MDS hospitalizations for IE, HE, red blood cells transfusions or treatment procedures represented 6.8, 15.0, 57.1, 21.1% respectively. *Conclusions*. IE and HE were common events wich represented 20% of MDS hospitalizations and have high mortality rate of 13 and 29% respectively. Poor outcome is observed in cerebral bleeding and comorbidities worsened prognosis. At the opposite IE outcome was favorable in 80% of cases and degree of neutropenia was correlated with increased hospitalization period.

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PROGNOSTIC SIGNIFICANCE OF β2 MICROGLOBULIN PREDICTING SURVIVAL IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Background. β2 microglobulin (β2M) is a 12-kDa light chain protein that binds to the β chain of class I MHC (MHC1) molecules. Serum β2M levels are known to reflect renal function and membrane turnover, which is associated with tumor mass and growth rate. Elevated serum levels of β2M are reported to predict poor survival in several hematologic malignancies. *Aims*. To evaluate the significance of β2M as a prognostic marker in patients with myelodysplastic syndromes (MDS) in our Center. *Design and Methods*. We examined 199 patients with MDS who were admitted to our Center from 2000 through 2007. B2M levels were measured in 154 patients. Of these 154 patients, 12 patients with a diagnosis of chronic myelomonocytic leukemia and 5 patients with RAEB-T were excluded from subsequent analysis. The remaining 137 patients provided the database for this analysis. B2M and karyotype were recorded along with other hematologic, biochemical and clinical covariates. Diagnosis was established using World Health Organization (WHO) and IPSS criteria. Serum β2M was quantified by a radioimmunoassay. Results. Nine patients (6%) had refractory anemia (RA), 35 (25%) refractroy anemia with excess of blasts-1(RAEB-1), 20 (14%) refractory anemia-2 (RAEB-2), 15 (11%) refractory anemia with ringed sideroblasts (RARS), 45 (33%) refractory cytopenia with multilineage dysplasia (RCMD) and 15 (11%) refractory cytopenia with multilineage dysplasia with ringed sideroblasts (RCMD-RS). The median β2M level was 2,75 mg/L (range: 1,02-15,73 mg/L). It did not differ significantly among the WHO groups (RA, 3.88 mg/L; RARS, 3.4 mg/L; RCMD, 2.83 mg/L; RCMD-RS, 2.66 mg/L; RAEB-1, 2.47 mg/L; RAEB-2, 2.72 mg/L; 5q-, 2.34 mg/L) as well as among IPSS risk groups (low, 2.96 mg/L; Int-1, 2.52 mg/L; Int-2, 2.95 mg/L; high, 2.81 mg/L). Patients with β2M levels > 2 mg/L had a worse survival compared to those with levels < 2 mg/L (p= 0.046). Univariate analysis identified cytogenetics, hemoglobulin level, platelet count, bone marrow blasts, intensive treatment and β2M levels as significantly associated with survival. Patients who received intensive treatment had significantly shorter survival than other patients (38 months vs 57 months), probably due to selection of RAEB-1 and RAEB-2 for intensive treatment. High β2M was associated with shorter survival both in patients with good/intermediate and poor karyotype (p=0.002). Multivariate analysis confirmed that β2M provided additional prognostic information to that provided by IPSS. *Conclusions*. High β2M is associated with a worse survival in MDS patients. ts measurement is strongly recommended, as it can be used, additionally to IPSS, as a prognostic tool for a better stratification of MDS patients.

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MYCOPHENOLATE-PREDNISONE PLUS ERYTHROPOIETIN IN THE TREATMENT OF MYELODYSPLASTIC SYNDROMES

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Background. Response rate to Erythropoietin (Epo) in patients with myelodysplastic syndromes with high level of serum erythropoietin and transfusions is very low. Moreover, most of MDS patients with an initial response to Epo loss this response during the follow-up. Aims: To evaluate the efficacy and safety of mycophenolate-prednisone before recombinant Epo β (ML20559 study) in the rescue of MDS patients that loss the response to Erythropoietin or with very low probability of response to Erythropoietin (Scandinavian Score). *Design and Methods*. In a multicenter study, 10 patients with MDS and loss the response to Erythropoietin or with very low probability of response to Erythropoietin (Scandinavian Score) because of basal serum Epo >300 u/L and transfusion requirements, were treated with oral mycophenolate 2000 mg/d plus 0.5 mg/kg/d of prednisone during 12 weeks, prednisone was tapered to 10 mg/d. Mycophenolate and prednisone were maintained along the treatment. After 12 weeks, recombinant erythropoietin β (NeorecormonR) 30000 u/week was added to non-responders from 12 to 18 weeks. In non-responders, from 18 to 24 weeks Neorecormon was increased to 60000 u/week. For response IWG criteria were used. Results. After 2 weeks of treatment one patient withdrew the treatment, the remaining 9 cases completed it. In 5 cases an erythroid response (HI-E) was observed including 2 HI-E minor responses (1 case decreased transfusion requirements > 50% with Epo 30000 u/w, and 1 patient increased Hb from 8.5 to 10.1 with Epo 60000 u/w) and 3 HI-E major responses (in one transfused patient the Hb after mycophenolate-prednisone was 8.9 g/dl without transfusion and increased to 9.7 with Epo. In a second case with an initial Hb of 8.1 g/dL, the level increased to 9.9 with mycophenolate-prednisone, minor response, and to 10.9 with Epo 60000 u/w, major response. In the third case, a sideroblastic anemia under transfusions, Hb using Epo 30000 u/w was 8.3 g/dl without transfusions and increased to 9.6 g/dl with Epo 60000 u/w). The treatment was well tolerated. However, 4 cases developed diarrhea and 1 case hyperglycemia, those adverse events were managed with medical treatments and all patients completed the treatment. *Conclusions*. In this preliminary study including MDS patients that loss the response to Epo or very low predictive score of response to Epo, mycophenolate-prednisone followed by the addition of Epo β could be a promising therapy. More extensive studies should be carried out to explore this combination therapy and to clarify its role in MDS treatment.

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EFFECT OF DEFERASIROX ON OXIDATIVE STRESS AND VASCULAR DYSFUNCTION IN MDS PATIENTS WITH IRON OVERLOAD

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Background. Secondary iron overload due to frequent blood transfusions has been related to the pathogenesis of ischemic cardiovascular disease.

Table.

Molecular marker	Before deferasirox treatment MIF±SD	After deferasirox treatment MIF±SD	p
VCAM-1	194.5 ± 5.8	169.8 ± 4.6	0.005
ICAM-1	201.0 ± 8.0	145.3 ± 5.2	<0.001
E-Selectin	89.8 ± 2.2	70.3 ± 2.2	<0.001
P-Selectin	43.0 ± 1.8	21.3 ± 1.7	<0.001
β1-Integrin	34.8 ± 1.0	21.3 ± 1.7	<0.001
EPC	0.002 ± 0.001	0.036 ± 0.020	0.015
EC	0.020 ± 0.010	0.095 ± 0.040	0.011
Total leukocyte hydrogen peroxide	163.1 ± 17.9	93.0 ± 20.2	0.002
Total leukocyte intracellular glutathione	120.5 ± 33.9	228.0 ± 109.4	0.109
Total leukocyte mitochondrial membrane potential	14.5 ± 7.7	23.68 ± 8.9	0.169

Abbreviations: EC: Endothelial cells; EPC: Endothelial progenitor cells; E-selectin: Endothelial selectin; ICAM: Intercellular adhesion molecule; MFI: Mean fluorescence intensity; P-selectin: Platelet selectin; VCAM: Vascular cell adhesion molecule.

Iron burden can lead to endothelial dysfunction and vascular injury by promoting the generation of reactive oxygen species (ROS), which, in turn, impairs vasodilatation and promotes platelet and leukocyte adhesion, thereby contributing to plaque disruption and thrombosis (*Circulation 2003;107[20]:2601-6*). Iron chelators, including the novel chelator deferasirox, have demonstrated to effectively suppress generation oxidative stress markers (*Arterioscler Thromb Vasc Biol. 2005 Nov;25(11):2282-8*). *Aims*. To evaluate the effect of deferasirox on endothelial progenitor cells (EPCs) and other molecular markers of atherosclerotic progression in patients with myelodysplastic syndrome (MDS) and secondary iron overload. *Design and Methods*. Chelation treatment consisted of deferasirox 20 mg/kg/day, which had to be administered uninterruptedly for at least one month. Oxidative stress biomarkers were studied by flow cytometry in white blood cells (WBCs) as total leukocytes, neutrophils, lymphocytes and monocytes. Mitochondrial membrane potential, ROS, intracellular glutathione levels and the expression of the following adhesion molecules VCAM-1, ICAM-1, E-selectin, P-selectin and β1-integrin in monocytes and neutrophils were analyzed using a dual-laser FACSCalibur and Cell Quest software (Becton Dickinson and Company). *Results*. These preliminary results correspond to the first four patients with low-risk MDS and iron overload enrolled. The inclusion period of this study is still open. No adverse events related to deferasirox administration were observed. Mitochondrial membrane potential, intracellular glutathione and endothelial progenitor cells were increased after treatment with deferasirox in all cell types, whereas generation of ROS and adhesion molecules significantly decreased after deferasirox treatment in most cell types. *Conclusions.* Treatment of iron overload with deferasirox may help to reduce vascular injury and atherosclerotic progression through a mechanism involving reduction of EPC levels, oxidative stress, and adhesion molecules. Final results will be presented at the meeting.

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CLINICO HEMATOLOGICAL CHARACTERISTICS AND TREATMENT OF 150 NEW MDS PATIENTS. SINGLE CENTER EXPERIENCE DURING 7 YEARS (2000-2007)

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Background. Myelodisplastic syndroms (MDS) are clonal hematopoietic stem-cell disorders characterized by ineffective dysplastic hematopoiesis involving one or more cell-lineages and characterized by peripheral blood cytopenias and high risk of progression to acute myeloid leukemia (AML). *Aims*. We gathered data on patient characteristics and treatment of 150 MDS patients seen in our hematology center during a period of 7 years (2000-2007). *Design and Methods*. We studied all new MDS cases diagnosed and treated since January 2000 in our Clinic. All patients were studied on admission with full blood count, bone marrow aspirate smears (BMA) and some were studied with cytogenetics and bone marrow biopsy specimens (BMB). We evaluated morphological features for all MDS patients including the availability of May Grunwald-Giemsa well stained BMA smears and BMB specimens. BMA were examined for diserythropoiesis (DE), disgranulopoesis (DG) and dismegakariopoesis (DM) as defined by WHO criteria. For all MDS patients, we analyssed the percentage of blasts and ringed sideroblasts. *Results*. From the 150 patients, 89.33% (134 patients) had primary MDS and 10.6(16 pat.) were diagnosed as treatment related MDS. The M:F ration was 1.42, median age 69 years (range 20-83 years). The median presenting Hb was 8,3 g/dL (range 3,5 -11,3), platelet count $73x10^9$/L (range 2.0-610.0) and WCC $3,1x10^9$/L (1,1-3,9). The distribution among MDS types was RA 21,33%, RA-RS 11,33%, RCMD 12%, RCDM-RS -2%, RAEB-13,33%, RAEB 2-16%, MDS-U-16% and 5q-syndrome- 8%. A karyotype analysis was available in 69,33% of patients (104 patients) and 66.66% were with a normal karyotype. According to the International Prognostic Scoring System (IPSS), 34.67% of the patients belonged to the low-risk, 30% to the Intermediate-1, 20% to the Intermediate -2 and 15.33% to the high-risk group. There were 88 males and 62 females. From the 150 patients that we studied 81,33% were treated in our Hematology Department; 24,6% of those were treated in daycare hospital and 75,6% were admitted on the hospital. The reasons for hospitalization were high-risk group patients, disease progression, disease complications like: infections, hemorrhages and bad general conditions. From all the treated patients, in 41 cases, patients were admitted for intensive chemotherapy and any kind of treatment that requires inpatient care. None of the patients received a Stem-Cell Transplantation. 84.66% of patients received at least one unit of packed red-cells and 60% received at least one unit of platelets. The median number of hospitalizations per patients was 2 (1-11). With a mean follow-up of 30,5 months (range 0,31-82,6) 54% (81 pts)died, 12% (18 pts) transformed to AML after a median of 13,21 months. 60 patients died of MSD related complications- 68.33% sepsis,15% bleeding, 10% AML, 6.67% (4pts) deaths, were not related to MSD. The median OS was 27,8 months. The median OS in RA/RAPS, RCMD/ RCMD-RS, RAEB-1, RAEB-2 were 79,11; 75,10, 10,52 and 5,34 months respectively. *Conclusions*. With regard to MDS subtype distribution, patients seen in our hematological center did not differ much from the MDS population as a whole. Our patient needed hospitalization for inpatient care either for management of MDS related complications or intensive treatment of the underlying bone marrow disease.

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CLINICAL EFFECTS OF DECITABINE IN MYELODYSPLASTIC SYNDROME (MDS)

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Background. MDS is characterized by heterogeneous clinical presentation, involving multilineage dysplasia, ineffective hematopoiesis and transformation to acute leukemia. MDS pathogenesis is characterized by hypermethylation of DNA and abnormal histone acetylation, affecting functions of oncogenesis and tumor suppressor genes. By reducing hypermethylation of tumor suppressor genes ("desilencing"), decitabine may balance gene expression and normalize cell function. Study objective: Assessment of clinical effects of decitabine in patients with MDS. *Design and Methods*. We prospectively observed 16 patients with MDS (7 men and 9 women; mean age 62 years) treated with decitabine 15 mg/m^2 i.v. q8h for 3 days within each 6-week cycle. According to FAB classification, 14 patients were diagnosed with RAEB and 2 patients with RAEBT. Abnormal karyotype was found in 6 patients, including hypodiploidy in 2 patients, del 5q in 2 patients, and complex abnormalities in 2 patients. Results: Of the 16 patients, 9 have completed 3-4 cycles of decitabine therapy and 7 have completed 1-2 cycles; 14 are still on therapy and 2 died after 1-2 cycles from disease progression. Mean Hb level was 74.8±8 g/l at baseline and 96.9±8 g/l after 3-4 cycles of therapy (p=0.05). Among patients who completed 3-4 cycles of therapy, the mean number of red blood cell transfusions per 6 weeks decreased from 4 to 2, mean platelet count increased from 70.6±13x10^9 to 136.6±3.5x10^9 (p<0.001), and blast cell counts in bone marrow decreased from 9.9±2.1% to 3.9±0.8% (<0.01). There was no hematological response in patients who completed 1-2 cycles of therapy. Assessment of response according to modified IWG criteria (*Cheson BD. Blood 2006;108:419*) in patients completing 3-4 cycles of decitabine treatment gave the following responses: 1 complete response (CR), 6 complete marrow responses (mCR), and 2 stable disease (SD). Median time to first response was 3 cycles and median time to best observed response was 4 cycles. Mean duration of response was 6.5 months. The non-hematological adverse events were reported following 1-2 cycles of treatment: upper respiratory tract infections (n=8), mucous mycotic lesions (n=4), and herpes infection (n=1). Following 3-4 cycles of therapy, upper respiratory tract infections were reported in just 2 patients. No other serious complications were reported. *Conclusions*. This study demonstrated that decitabine is characterized by clinical effect, and longer treatment duration led to better response rates. Decitabine was well tolerated and no apparent signs of cumulative toxicities were detected throughout treatment. Thrombocytopenia and leucopenia were transient and did not require adjustment of dose and treatment-free intervals for decitabine. Continuation of decitabine treatment for at least 3-4 cycles in MDS patients is well tolerated and is appropriate to obtain the best response, while continued therapy beyond reaching best response is instrumental to reach prolonged survival.

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5-DAYS SCHEDULE OF DECITABINE MAY BE EFFECTIVE AS IN HIGH RISK MDS EITHER IN RELAPSED AML

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Hypomethylating agents have provided a new option in the treatment of the patients with myelodysplastic syndromes. Coming up from seventies as antileukemic drugs azacitidine (AZA) and decitabine (DAC) after schedule and dose modification were proved to be very active in myelodysplastic syndromes. The overall response rate in intermediate and high risk MDS was reported to be as high as 73%-81% with 34%-36% of CR for decitabine in 5-days schedule in monocenter trial (H.Kantarjian, Blood 2007, 109, 52-57; Cancer 2007;109:265-273) and 60% with 7% CR for AZA in a CALGB randomized trial (*Silverman JCO, 2002; 20: 2429-40*). It was also demonstrated that AZA gave a significant OS advantage in randomized comparison with conventional care regimens (*P. Fenaux, 2007 ASH abstr 817*). The big point of the reported studies is low general toxicity of the drugs. This promising data became a basis for initiating by Russian leukemia Study group a multicenter trial testing the efficacy of the 5-days schedule of DAC in high risk MDS and relapsed AML presenting as MDS. *Design and Methods.* From Aug 2007 till February 2009 18 patients from 6 hematological centers were enrolled in the study: median age 49y (26-77 yy), RAEB-9pts (IPSS Int-2 - 3pt, high risk-4, 1-no cytogenetics, 1- secondary MDS), RCMD-3 pts (IPSS Int-1 - 1pt, Int 2 -2 pt), RARS (IPSS Low - 1 pt), AML - 4 pts (de novo -2, relapsed after MUD -1 pt, relapsed after MDS transformation -1 pt) CMML 1 pt. 7 patients were pretreated with different therapies. DAC was used in a 5 days schedule: 20 mg/m^2 once daily 1-hour i.v. infusion for 5 days, three courses were applied till response evaluation. The treatment was stopped at AML progression or absence of any improvements after 3 courses. Median number of courses is 3 (1-9), median follow-up from treatment start - 9 mo (1-17 mo). *Results*. Among 9 RAEB and 3 RCMD pts we observed 2 stable CR (after 2-3 courses), 1- mCR, 1 HI (after 3 courses), 5 progression to AML after 1-4 courses (Me-2), 1 pts did not respond. So, 4 of 10 advanced MDS pts (40%) achieved hematological remission and a mediana response duration of 10 months. DAC was ineffective in low risk RARS patient with prolonged disease history (2 years). CR was registered in one CMML patient after 3 courses with 6 months response duration, after progressed to AML and died. In 2 out 4 AML patients marrow CR was obtained, two patients progressed to overt AML and died. In one pt CR is stable (+10 mo), the other one relapsed at 9 months of mCR and died. It worth to note that this mCR was the third in this pt and DAC was applied after MUD allogeneic transplantation. Cytogenetic response was documented in 3 of 9. General toxicity of DAC was acceptable, though the majority of the patients experienced after DAC prolonged neutropenia with infections and thrombocytopenia transfusion dependant. All patients were hospitalized at least once. *Conclusions*. Though the studied group is small and rather heterogeneous we nevertheless may conclude that decitabine provided 7of 16 (44%) CR+HI in very high risk MDS and AML patients. Effect was usually achieved after the 3rd course. Decitabine may be an option for relapsed after stem sell transplantation AML and in AML secondary to MDS, though AML should not be highly proliferative. The drug should be used in leukemia departments by doctors aware of myelosupression complications.

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LOW FREQUENCY OF RAS GENE AND ABSENCE OF FLT3-ITD GENE MUTATIONS IN INDIAN PATIENTS WITH MYELODYSPLASTIC SYNDROMES: AIIMS STUDY

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Background. Chromosomal abnormalities and molecular detection has potential importance for diagnosis and prognosis of MDS, although the mechanisms underlying the development of MDS and their progressive evolution to AML are still largely unknown. Since, no studies have been reported from India on the prevalence of N-RAS, K- RAS point mutation in codon 12 and FLT3-ITD mutations in patients with MDS, we undertook this study. *Objectives*. To find out the frequency of RAS and FLT3-ITD mutations in Indian MDS patients Method DNA and RNA were extracted from bone marrow/peripheral blood. Using RT-PCR the patients were screened for length mutations in FLT3 gene. PCR-RFLP and nested PCR-RFLP were used for the detection of point mutation in codon 12 of N-RAS and K-RAS. *Results*. A total of 53 patients (median age 39 yrs, range 9-78yrs; M: F 2:1; median TLC-3.9x10^9/L, range 0.8-116x10^9/L. Median platelet count- 87x10^9/L, range 1-349x10^9/L, Median hemoglobin -6.8 g/dl , range 2.7- 16.1 g/dL, were studied. One out of 53 patients (2%) was found positive for N-RAS and four patients were positive for K-RAS (8%) mutation. FLT3-ITD mutation was studied in 60 patients; all the patients were found negative. The mean observation of all the patients was 30 months and the median overall survival was 28 months. Nine patients died during follow up. The presence of N-RAS codon 12 mutation was associated with the poor survival. FLT3-ITD mutation was not observed in any of our cases, which is in contrast to 3% reported from the West. *Conclusions*. Thus, it appears that the RAS and FLT3 mutations are uncommon in MDS patients in India.

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DETECTION AND MANAGEMENT OF IRON OVERLOAD IN MDS: A EUROPEAN SURVEY

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More than 90% of all patients with myelodysplastic syndromes (MDS) will present with anaemia at the time of diagnosis, and 60% will experience severe anaemia at some stage during the course of their disease. Patients requiring regular red cell transfusion are at risk of developing iron overload. There is evidence to show that iron overload is often poorly detected and managed in the MDS setting and the reasons for this are not well understood. MIDIS - the MDS Iron-overload Detection Insight Survey - aims to gain insight into important barriers to optimal detection and management of iron overload in MDS. This European survey is being carried out by the MDS Foundation and the European School of Haematology in partnership with Novartis Oncology. A questionnaire was developed and reviewed by MDS experts to ensure that the questions were relevant, accurate and appropriately phrased. The English questionnaire was translated into French, German, Dutch, Spanish, Swedish and Italian. Physicians involved in the management of MDS were invited to complete either paper or on-line versions of the questionnaire. 125 responses were received from 24 countries by March 2009. Data are currently being analysed to identify key barriers and determine relationships between variables. Final results will be available in April 2009 and will be presented at the meeting. The results of the survey will be used to guide initiatives aimed at promoting optimal detection and management of iron overload in patients with MDS.

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VITAMIN C SHOW ANTITUMOR ACTIVITY AGAINST MYELODYSPLASTIC SYNDROME CELLS

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Vitamin C has few pharmacological actions and cancer is one of the most common diseases for which a beneficial role of vitamin C has been claimed. However, the use of vitamin C as an alternative for cancer treatment is controversial. Vitamin C is a potent water-soluble antioxidant, but, depending on concentration, may also have pro-oxidant and even mutagenic effects in the presence of transition metals. Myelodysplastic syndrome (MDS) is a heterogeneous stem cell bone marrow disorder characterized by the underproduction of one or more blood types cells, due to hematopoiesis dysfunction. This disease affect older adults and frequently progress to acute leukemia. MDS may arise de novo or may be secondary to treatment with chemotherapy or radiation therapy for another disease. For most MDS patients, the currently available treatments do not offer a chance for a cure and the only curative therapy for MDS is bone marrow transplantation. In this context, we aimed to evaluate the therapeutic potential of vitamin C in MDS, as in monotherapy and/or as adjuvant to conventional therapies. To attempt this purpose we use a well-established MDS cell line, the F36P cells, and incubated them in absence and presence of increasing concentrations of ascorbic acid (AA) or dihydroascorbic acid (DHA) and/or cytarabine (Ara-C), added at the beginning of the incubation time or daily, during 96 h. Cell growth and viability was evaluated using Trypan blue exclusion and the efficacy of the drugs determined by the IC50 using a dose response curves based on cell viability. Cell death analysis was performed by morphological studies and by flow cytometry, using annexin-V/propidium iodine incorporation. In order to evaluate the involvement of oxidative stress and mitochondria in the cytotoxicity induced by the vitamin C, we evaluated the production of ROS and GSH and mitochondrial membrane potential by flow cytometry. ROS generation were determined by staining cells with H2DCF-DA and DHE, the content of reduced GSH was measurement by staining with mercury orange and the evaluation of mitochondrial membrane potential was determined using JC1 dye. Our results show that both reduced (AA) and oxidized (DHA) form of vitamin C induced reduction in cell viability of MDS cells with IC50 values ranging from approximately 1 mM for DHA to 2,5 mM for AA, after 48h. These compounds induced cell dead in F36P cells by apoptosis in a time-, dose- and administration-dependent manner. Vitamin C-induced apoptosis was mediated by increased ROS production, especially by increasing superoxide production (DHA, 2,2-fold; AA, 1,8-fold). We also observed an increase in mitochondrial membrane potential depolarization (DHA, 4,2-fold; AA, 6,2-fold) (reduction of mitochondrial transmembrane potential). In summary, vitamin C may be a new therapeutic option for treatment of MDS as monotherapy or as adjuvant of conventional chemotherapy.

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ARA-C - ATRA - EPO COMBINATION TREATMENT IN ELDERLY PATIENTS WITH MYELODISPLASTIC SYNDROME

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Myelodysplastic syndrome (MDS) represents a heterogeneous group of clonal hematopoietic disorders characterized by hypercellular marrow with dysmyelopoiesis and peripheral blood cytopenias resulting from ineffective blood cell production. *Aim of study*: evaluating the response of treatment with all-trans retinoic acid (ATRA) combined with low-dose of cytosinearabinoside (ARA-C) and erythropoietin (EPO) in elderly patients with MDS. *Design and Methods*. we studied 21 patients with MDS hospitalized in Clinic of Hematology from Craiova (Romania) between 2007-2008 devised by age, sex, classified by FAB classification. The elderly patients with MDS received ARA-C 20 mg s.c twice daily in combination with ATRA 45mg/m^2/day for ten days.Anemia was treated with transfusions when haemoglobin value was less than 8g/dl, and with EPO 30.000 UI/week, s.c, when haemoglobin value was more than 8 g/dl. Response was evaluated monthly. *Results*. Median age of the patients was 73 years, with equal frequency at men and women. FAB classification showed: RA- 4 patients, RARS - 2 patients, RAEB - 7 patients, RAEB-t - 3 patients, unclassified - 5 patients. Response was maintained in 75% of responders after 12 weeks, and in 9.7% after 24 weeks of treatment. ARA-C - ATRA - EPO combination was well tolerated, the most frequent side effect was mucosites of ATRA. EPO - induced trombosis was present in one case. *Conclusion*s. ARA-C - ATRA - EPO combination is safe and determined significant response in elderly MDS patients who cannot banefit of more aggressive therapy.

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EFFICACY OF HYPOMETHYLATING AGENTS ON RHEUMATOLOGIC MANIFESTATIONS IN HIGH RISK MYELODYSPLASTIC SYNDROMES: FIRST REPORT OF A POTENTIAL INDICATION FOR THIS EMERGING THERAPY

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Background. Rheumatologic manifestations (RM) are frequently associated with myelodysplastic syndromes (MDS) and can occur before, at the same time or after the diagnosis of the MDS. Moreover, therapeutic approach is difficult and requires, to being effective, either rheumatologic or haematological or both medications. The use of immunosuppressive therapy (i.e. corticosteroids) during the course of the disease is complex due to increased risk of infections and other harmful complications. *Aim*. We report two observations of RM associated with MDS treated with hypomethylating agent with a spectacular effect on both haematological and rheumatologic features. *Design and methods*. Description of the two cases and comparison with RM characteristics observed in a 500 MDS cohort referred to our hospital during a four year period (2004-2008). *Results*. Case 1: a 74 year-old man with a polymyalgia rheumatica preceding MDS progression (RAEB-t) was treated with high dose corticosteroids. Decitabine was used, RM disappeared after 2 cycles and corticosteroid therapy was discontinued at cycle 5. One year later, complete remission of both diseases was confirmed. Case 2: an 82 year-old woman had seronegative polyarthritis, fever, altered performance status and RAEB-1 with complex karyotype and high level of RBC transfusions. She received Azacitidine. After the first course, apyrexia was obtained; after 4 cycles corticosteroid therapy was definitively stopped. With a period of follow up of six months, both situations remained in

complete remission. Prevalence of RM in our cohort was 4.5%. Main features are polymyalgia rheumatica, seronegative polyarthritis, psoriatic arthritis and remitting seronegative symmetrical synovitis with pitting edema. RM outcomes were various: either favourable with immunosuppressive therapy and independent of the MDS evolution, or unfavourable and identical to MDS outcome. *Conclusions*. Therapeutic approaches of RM associated to MDS have to be discussed after the emergence of new therapies of high risk MDS. Hypomethylating agents showed for the first time a positive impact on extra-haematological features in MDS. The same question could be asked about cutaneous manifestations of these diseases.

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RED BLOOD CELL TRANSFUSION INDEPENDENCE FOLLOWING THE INITIATION OF IRON CHELATION THERAPY IN MYELODYSPLASTIC SYNDROME: A CASE REPORT AND REVIEW OF THE LITERATURE

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Background. Iron chelation therapy (ICT) is often used to treat iron overload (IOL) in patients (pts) requiring transfusion of red blood cells (RBC) for chronic anemia. During treatment of a pt with myelodysplastic syndrome (MDS) and IOL with ICT, RBC transfusion requirement (TR) ceased and he became transfusion independent (TI). *Aims*. Here we report the clinical course of our pt and review the reported cases of TI or decreased RBC TR in pts with MDS receiving ICT. *Design and Methods*. The pt chart was reviewed. Reported cases were identified by PubMed search using the terms 'MDS' and 'ICT', and reviewing references cited in reports identified. Results: A 76 year (y) old man was referred in 2004 for management of MDS diagnosed in 1997, when investigations showed: white blood cell (WBC), $2.4x10^9$/L; neutrophils, $0.7x10^9$/L; hemoglobin (Hb), 133g/l; platelets, $108x10^9$/L. Bone marrow aspiration and biopsy showed refractory anemia (RA), karyotype +8 and -Y. IPSS was intermediate-1. Erythropoitin (epo) level was 148.3mIU/mL and stem cell assay showed no epo-independent growth. In 2004 the Hb dropped to 60g/l prompting initiation of RBC transfusion support. He required 3 RBC units every 4 weeks to maintain a Hb >90 g/L and complained of fatigue and functional limitation. Ferritin in 2004 was 1293 ug/L and 2197ug/L in 2006. He declined ICT with deferoxamine (DFO) but in 2006 agreed to receive deferasirox (DFX). Two months (mo) after starting ICT, the Hb increased spontaneously to 109 g/L and he has not required RBC transfusion since. Mean Hb over 24 months since starting ICT was 122g/l and ferritin decreased to 1356 ug/L in 2008. He reports excellent energy and an improved quality of life. There are 18 other cases reported of MDS showing improvement in Hb with ICT; 9 became TI. Characteristics of the 10 TI pts were: median age at MDS diagnosis 58 (range 18-74)y; male, n=5. MDS subtype: RA, n=5; RARS, n=2, RCMD, n=1; RAEB, n=2. IPSS (n=8): low, n=1; int-1, n=5, int-1/2, n=1; high, n=1. ICT was: DFO, n=7; DFX, n=3. Median time to RBC TI was 17.5 (1-24)mo and TI duration 13 (3-28)mo to date. *Conclusions*. 19 cases of MDS to date have been reported in whom improved Hb followed initiation of ICT; 9 had a decrease in RBC transfusion requirements, and RBC transfusion independence occurred in 9, our pt being the 10th. The remarkable course of our pt adds to evidence that ICT may be of clinical benefit for patients with MDS and IOL. The mechanism by which these effects occur remains an area for future investigation.

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AEE788 IS A VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITOR WITH A POTENT IN VITRO ACTIVITY IN ACUTE MYELOID LEUKEMIA

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Aberrant activation of tyrosine kinase receptors is frequently observed in Acute Myeloid Leukemia (AML). Activating mutations of the protein tyrosine kinase FLT3 can be found in approximately 30% of patients with acute myeloid leukemia, thereby representing the most frequent single genetic alteration in AML. AEE788, a novel dual receptor tyrosine kinase inhibitor of the EGF and the VEGF, is being studied in several solid tumors with remarkable success. It is not known, however, about the efficacy of this inhibitor in the treatment of AML. Therefore, we investigated the effect of AEE788 in the treatment of four human AML cell lines. Cell proliferation and apoptosis in NB4, THP-1, MOLM-13 and MV4-11 cells (the two last showing the FLT3/ITD mutation) incubated with 0.5-15 microM AEE788 were quantified though XTT colorimetric assay and combined annexin V/PI staining, respectively. Using ELISA, EMSA, inmunoprecipitation and Western blot analysis, we also studied the activation of VEGF/VEGFRs loop, FLT3 and their downstream effectors (Akt, ERK, STAT5 and NF-kB) after AEE788 treatment. Our data showed that AEE788 was a tyrosine kinase inhibitor of FLT3 and had a potent antiproliferative and proapoptotic activity in AML-derived cell lines that endogenously expressed an activated FLT3 receptor (THP-1, MOLM-13 and MV4-11). Consistently, in these cells AEE788 abrogated VEGF/VEGFRs activation and many survival signaling pathways, including Akt, ERK, STAT5 and NF-kB. Taken together, the potent activity of AEE788 might represent a promising new option of targeting FLT3 for the treatment of AML patients.

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AN IN VITRO COMPARISON OF AZACITIDINE AND DECITABINE ACTIVITIES IN ACUTE MYELOID LEUKEMIA

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Background. Vidaza (azacitidine; 5-azacytidine; AZA) is a nucleoside analog that is approved for the treatment of adult patients with intermediate-2 and high-risk myelodysplastic syndromes (MDS), and certain types of chronic myelomonocytic leukemia and acute myeloid leukemia (AML). AZA demonstrated the ability to significantly extend overall survival in a phase III clinical trial (*Fenaux, Lancet Oncology*); however, Dacogen (decitabine; 2'-deoxy-5-azacytidine; DAC), a structurally similar nucleoside analog, failed to show a survival benefit over conventional therapy in a similar trial. Given the different outcomes of these phase III clinical studies, we undertook an *in vitro* comparison of AZA and DAC mechanisms of action in AML cell models. *Design and Methods*. Five human AML cell lines were tested for sensitivity to AZA and DAC in 72hr cell viability assays (CellTiter-Glo). Two cell lines, KG-1a and THP-1, were selected for analysis of time- (24-72hr) and dose- (0.03- 3uM) dependent effects of AZA and DAC on cell cycle and molecular endpoints, including markers of DNA incorporation, DNA damage and apoptosis. Western analyses were performed for DNA methyltransferase 1 (DNMT1), phospho (Ser139) histone-H2A.X and cleaved poly (ADP-ribose) polymerase (PARP) proteins. DNA methylation was evaluated using LINE1 pyrosequencing and the Illumina GoldenGate platform. Flow cytometry was used to measure staining with NIM-DAPI and annexin V/7-AAD. AZA and DAC-mediated changes in gene expression were also evaluated, using Affymetrix GeneChip microarrays. Results: All AML cell lines were sensitive to both AZA and DAC in viability assays. High AZA concentrations (5-30uM) reduced cell viability to less than 15%, whereas equivalent concentrations of DAC failed to reduce cell viability below 50%. In KG-1a and THP-1 cell lines, both AZA and DAC decreased the level of DNMT1 protein and reduced DNA methylation in a dose- and time-dependent manner, with DAC mediating maximal changes at lower concentrations than AZA. Both drugs increased levels of the DNA damage marker phospho (Ser139) histone-H2A.X and DNA hypomethylation. Additionally, both drugs increased levels of the apoptosis marker, cleaved PARP, and increased staining for annexin V and 7-AAD. Differences between the drugs were observed in cell cycle analyses. At drug concentrations that induced DNA damage, AZA and DAC both increased cell death, as measured by the sub-G1 cell cycle population; however G2/M growth arrest was only observed with DAC. Finally, gene expression analyses revealed similarities and differences in the signaling pathways that were modulated by AZA and DAC. *Conclusions*. In summary, similarities and differences were observed in the molecular and phenotypic responses of AML cell lines to AZA and DAC *in vitro*. Detailed analyses in two human AML cell lines showed that both AZA and DAC caused DNMT1 depletion, DNA hypomethylation, and increases in markers of DNA damage and apoptosis; however, distinct effects on cell viability, cell cycle and gene expression were observed.

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DNA METHYLATION STATUS OF THE CORE AND UPSTREAM REGION PROMOTER AND GENE EXPRESSION LEVELS OF C/EBPA IN BRAZILIAN ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS AT DIAGNOSIS

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Background. The transcription factor CCAAT enhancer binding protein α encoded by the C/EBPA gene is crucial for the differentiation of granulocytes. Mutations in C/EBPA gene are reported in approximating 5-14% of acute myeloid leukemia patients. Furthermore, different negative regulation mechanisms of C/EBPA have been described in myeloid leukemia. These observations imply that the suppression of C/EBPA expression may contribute in the disruption of granulocytic differentiation in myeloid leukemogenesis. Recently, aberrant DNA methylation in the upstream promoter region of C/EBPA has been reported in lung and head and neck tumors and in myeloid leukemia leading to a down regulation of the gene expression. *Aims*. Based on these findings we analyzed expression, core promoter methylation status and bisulfite sequencing of upstream promoter region of C/EBPA gene in bone marrow (BM) samples of Brazilian patients with acute promyelocytic leukemia (APL) at diagnosis. *Design and Methods*. C/EBPA gene expression of BM samples of 18 APL patients and 7 healthy controls was accessed by Real Time PCR and normalized to GAPDH expression. Methylation core promoter status of BM samples of 40 APL patients and peripheral blood (PB) samples of 9 healthy controls was evaluated in genomic DNA bisulfite treated by Methylation Specific PCR (positions of methylated and unmethylated primers: -286 to -66 and -287 to -65 bp from transcription start site (TSS), respectively). To perform the sequencing of C/EBPA upstream promoter region in 11 APL patients (BM) and 3 healthy controls (PB), DNA was treated with sodium bisulfite and amplified by PCR (primers position: -1423 to -1121 bp from TSS). The PCR products were cloned into a vector, transformed bacteria were cultured overnight and the plasmid DNA was isolated and sequenced. For this study the percent methylation at individual CpG site was counted and an average methylation level was calculated for each patient (25 CpG sites were analyzed in each clone). *Results*. BM samples of APL patients (n=18) presented significantly lower levels of C/EBPA gene expression compared to healthy controls (n=7) (0.37±0.04 vs 1.13±0.23, respectively, p=0.0001). To understand the molecular mechanism of the low C/EBPA expression we investigated the DNA methylation status in core and upstream promoter region of C/EBPA using MSP and bisulfite sequencing, respectively. Regarding to core promoter, we detected no methylation in 40 APL samples, neither in 9 samples of healthy controls. On the other hand, the analysis in the upstream promoter region showed that 10 of 11 APL samples were methylated (median, 26%; range, 0-56%), with 5 patients showing methylation levels of >30%. DNA methylation levels in samples of healthy controls were low (median, 0%; range 0-4%). *Conclusions*. Our data support recent findings showing that APL patients present the upstream promoter region of C/EBPA hypermethylated, however, we detected a higher percentage of methylation in our samples compared to published data.

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PEDIATRIC ACUTE MEGAKARYOBLASTIC LEUKEMIA (M7) SHOWS MARKED MORPHOLOGICAL AND IMMUNOPHENOTYPIC HETEROGENEITY

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Background. M7 was first recognized, by the FAB group, as a separate subtype of AML in 1985. Since then variability in its relative frequency has been reported. A major cause of this variability could be attributed to morphological heterogeneity. Aims: In this work we aimed to investigate the true relative frequency of M7 within pediatric AML diagnosed at the NCI, Cairo University and to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7. *Design and Methods*. In the years 2003-2008, 510 cases were diagnosed as pediatric AML (age <18 years). The diagnosis was established according to standard methods including clinical picture, complete blood count, bone marrow aspirate/biopsy, cytochemistry and immunophenotyping. *Results.* M7 was encountered in 31/510 (6.1%) of the cases. Patients included 17 male and 14 female. They showed an age range of 2 days- 14years, median 14 months. Four of them were associated with Down's syndrome including the 2 days old one. Typical M7 morphology with large blasts showing cytoplasmic blebbing was obtained in 22 cases (71.0%); one of them showed many micromegakaryocytes with platelet budding and thrombocytosis of 750x10^9/L. This latter case expressed BCR/ABL fusion gene, P210 and proved to be acute crisis on top of CML. The other 9 cases (29.0%) showed morphology suggestive of AML in eight and of ALL in one case. Bone marrow blasts was < 20% in 5 cases and the diagnosis relied upon bone marrow trephine biopsy. Cytochemistry showed a characteristic pattern of acid phosphatase in all cases with strong cytoplasmic positivity distributed in the whole cell. Diagnosis was confirmed in all cases by expression of CD61 and/or CD41. Coexpression of other myeloid antigens namely CD13 and/or CD33 was encountered in 17 cases, CD7 in five cases, CD4 in four, CD56 in three, CD2 in two and CD 34 in 12 cases. Two cases were true biphenotypic TALL/M7, one of them expressing CD56 as well. *Conclusions.* M7 shows marked morphological heterogeneity with 29.0% lacking the typical features. These cases could be missed especially if they are expressing other myeloid markers or occasionally markers of other lineages. CD61 and CD41 should be tested in all cases of AML as well as in cases lacking other lineage markers. Cytochemistry especially the characteristic pattern of acid phosphatase could be highly suggestive of M7 and direct the attention to testing for CD61 and CD 41 expression.

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THE EFFECTS OF CERAMIDE, GLUCOSYLCERAMIDE AND SPHINGOSINE 1 PHOSPHATE IN RESVERATROL INDUCED CELL DEATH IN HL60 ACUTE PROMYELOCYTIC LEUKEMIA CELLS

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Background. Resveratrol (3,5,40-trihydroxy-trans-stilbene) is naturally occuring phytoalexin, which presents in grapes, and some other natural products. It is a potential anticancer agent which inhibits tumor initiation, promotion, and progression. This inhibitory effect of resveratrol causes cell cycle arrest and trigger apoptosis in various cancer cells. Ceramides are the central compounds of sphingolipids metabolism. They are known as second messengers regulating various cellular processes including cell growth, proliferation, differentiation and apoptosis. While ceramide acts as a strong apoptotic molecule, glucosylceramide and sphingosine-1-phosphate trigger cell growth and proliferation and inhibit apoptosis. Sphingosine kinase-1 (SK-1) is an enzyme catalyzing the phosphorylation of sphingosine to sphingosine-1-phosphate while glucosylceramide synthase (GCS) that converts ceramide to glucosylceramide. Thus, inhibition of GCS by N-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decanamide, hydrochloride (PDMP) or SK-1 by sphingosine kinase-1 inhibitor, or exogenous application of ceramide analog (C8:ceramide) result in increased accumulation of ceramides. *Aims*. It was shown by our and some other groups that resveratrol induces apoptosis in human HL60 acute myeloid leukemia cells. However, mechanistic information about resveratrol-induced apoptosis is not clearly defined. More importantly, the involvement of ceramide metabolising genes and their end products are not investigated in acute myeloid leukemia cells. In this study, we examined the roles of ceramide, glucosylceramide and sphingosine-1-phosphate in resveratrol-induced apoptosis in HL60 cells. *Design and Methods*. HL60 cells were grown in RPMI1640 medium containing 10% FBS and 1% penicilline-streptomycine. Cytotoxicity analyses of resveratrol, C8:ceramide, PDMP and SK-1 inhibitor were conducted by XTT cell proliferation assay. Then combinations of resveratrol with C8:ceramide, PDMP or SK-1 inhibitor were applied to HL60 cells and possible synergistic analyses were examined. Results: IC50 values of resveratrol and C8:ceramide or IC90 value SK-1 inhibitor and PDMP in HL60 cells were found to be 75 μM and 45 μM or 5 μM and 11 μM, respectively. There were 3-, 5-, 15-, 29-, 45-, and 55% decreases in cell proliferation of HL60 cells in response to 1-, 5-, 10-, 20-, 50-, and 100 μM resveratrol, respectively. Combination of resveratrol with 45 μM C8:ceramide decreased proliferation of HL60 cells to 75-, 76-, 79-, 80-, and 81%, respectively, as comparing to untreated controls. 11 μM of SK-1 inhibitor in combination with 1-, 10-, 15-, 20-, and 50 μM of resveratrol resulted in 19-, 39-, 45-, 54-, and 70% decreases in cell proliferation, respectively. Although PDMP induced cell death in HL60 cells in a dose dependent manner, we did not observe any synergy with combination of resveratrol. *Conclusions*. In this study, we aimed to show cytotoxic effects of resveratrol, exogenous ceramide analog,

C8:ceramide, GCS inhibitor, PDMP, and SK-1 inhibitor. The results of this study showed that increasing intracellular concentrations of ceramides or application of exogenous ceramides significantly increased cytotoxicity in HL60 cells. More importantly, it was shown for the first time by this study that there were synergistic effects of combination of resveratrol and SK-1 inhibitor or C8:ceramide but not PDMP at IC90 value in. Taking together all these results showed that ceramides may be involved in resveratrol-induced cell death.

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PROGNOSTIC IMPLICATION OF NRAS GENE MUTATIONS IN EGYPTIAN ADULT ACUTE MYELOID LEUKEMIA

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Background. The pathogenesis of acute myeloid leukemia (AML) involves the cooperation of mutations promoting proliferation/survival and those impairing differentiation. Point mutations of the N-RAS gene are the most frequent somatic mutations causing aberrant signal-transduction in acute myeloid leukemia (AML). *Aim*. To study the frequency and prognostic significance of NRAS gene mutations (NRASmut) in de novo Egyptian adult AML. *Design and Methods*. Bone marrow specimens from 150 patients with de novo acute myeloid leukemia and 10 controls were analyzed by genomic PCR-SSCP at codons 12, 13 (exon 1), and 61 (exon 2) for NRAS mutations. *Results.* NRASmut was found in 19/150 (12.7%) AML cases, represented more frequently in the FAB subtype M4eo (P = 0.028), and at codon 12, 13 (14of 19; 73.7%). Patients with NRASmut had a significant lower peripheral, marrow blasts (p=0.004, p=0.03) and non significant improved clinical outcome than patients without mutation. Complete remission rate was (63.2% vs 56.5%; p=0.46), resistant disease (15.8% vs 23.6%; p=0.51), 3 year overall survival (44% vs 42%; p=0.85) and disease free survival (42.1% vs 38.9%, p=0.74). Multivariate analysis showed that age was the strongest unfavorable factor for overall survival (relative risk [RR], 1.9; p=0.002), followed by cytogenetics (p=0.004). FAB types, NRAS mutation and leukocytosis were less important. *Conclusions*. NRAS gene mutation frequency and spectrum differ between biologically distinct subtypes of AML but do not significantly influence prognosis and clinical outcome.

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THE PROGNOSTIC SIGNIFICANCE OF ANTIGEN EXPRESSION BASED ON CYTOGENETICS IN 246 ADULT PATIENTS WITH ACUTE MYELOID LEUKAEMIA

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Background. A chromosomal abnormality is one of the most important independent prognostic factors in acute myeloid leukaemia (AML). Other parameter which may influence the prognosis is the expression of certain surface markers on the blast cell populations. The aims of the study were to determine the various antigen expressions in AML and also to determine the relationship between antigen expression and cytogenetics. *Design and Methods*. Antigen expression profiles of 13 cellular antigens of the blast cell populations from samples of peripheral or bone marrow in 246 patients were determined by a 4- colour flow cytometric immunophenotyping. The determination of the antigen positivity of the blast cell populations was based on the criteria of the cut-off point of 20%. Routine conventional cytogenetic analysis from bone marrow aspirate or peripheral blood was carried out in these patients. *Results*. The expression of the 13 cellular antigens in 246 patients with descending trend of CD33$^+$, CD13$^+$, CD117$^+$, HLA-DR$^+$, MPO$^+$, CD64$^+$, CD34$^+$, CD15$^+$, CD56$^+$, CD7$^+$, CD19$^+$, CD14$^+$ and CD2$^+$ occurred in 94%, 92%, 86%, 73%, 71%, 69%, 57%, 55%, 19%, 18%, 16%, 15% and 7%, respectively. There were significant associations between CD34 expression and karyotype (p<0.05), between expression of CD34 and CD7 (p<0.05) and between expression of CD34 and CD56 (p<0.05). However, the associations between expression of CD34 and CD19 (p=0.684) and with CD2 (p=0.608) were not significant. CD19$^+$, MPO$^+$ and CD2$^+$ were predominantly occurred in 43%, 100% and 22%, respectively in patients with favourable outcome group with CD7$^+$, 0.1% the least expressed. On the other hand, CD34$^+$ was predominantly occurred in 93% of patients with adverse prognosis. Other antigens expressions were relatively variable with regard to the prognosis. Sixty eight percent of patients with t(15;17) had the composite of CD34-/CD33$^+$/CD13$^+$/HLA-DR-, whereas 46% and 92% of patients with t(8;21) had the phenotypes of CD19$^+$/CD34$^+$ and CD19$^+$/CD15$^+$, respectively. *Conclusions*. The three most common antigens expressed in the patients were CD33, CD13 and CD117. The expression of CD34 and CD7 were found mostly in the adverse prognostic group of patients. Individual expression of MPO, CD19 and CD2 were the three most common expressed antigens in the favourable group. The majority of AML patients with t(15;17) expressed the composite of CD34$^-$/CD33$^+$/CD13$^+$/HLA-DR-, whereas the composite of CD19$^+$/CD15$^+$ was found in the majority of AML patients with t(8;21).

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ARRAY-CGH ANALYSIS FOR THE DETECTION OF CRYPTIC GENE DOSE CHANGES IN ACUTE MYELOID LEUKEMIA WITH NORMAL KARYOTYPE

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Background. A large proportion, 40-50%, of AML cases have either a normal karyotype or non-recurrent chromosomal alterations. Normal karyotype AML (N-AML) is a clinically and molecularly heterogeneous group with gene expression changes and frequently mutated genes, such as NPM1 and FLT3. Especially, cryptic gene dose changes could be identified by the introduction of array CGH (aCGH) technology and explain some of the heterogeneity of N-AML. *Aims*. To establish the frequency of cryptic gene dose changes and understand their potential significance in patients with N-AML. *Design and Methods*: Bone marrow samples were selected from of 8 patients with AML, diagnosed with normal karyotype by G-banding. They were negative for recurrent genetic abnormalities, AML1/ETO, BCR/ABL, MLL, CBFB and PML/RARA rearrangements, and confirmed by FISH or RT-PCR. Their percentages of blasts were average 75.5% (range 55~98%). The CGH analysis were performed with Agilent's 244K whole-genome oligonucleotide array according to the manufacturer's Oligonucleotide Array-Based CGH for Genomic DNA Analysis protocol version 2 (Agilent Technologies, CA, USA). Scanned images were quantified with Feature Extraction software version 9.0 and analyzed with DNA Analytics 4.0.76 software (Agilent Technologies). We used the signal-to-noise ratio and the normalized log2 ratio with male reference DNA (Promega, WI, USA). Results: Fourty intervals having the high frequency of gene dose changes, i.e. more than 80% (>4 among 8 patients with N-AML), were detected except sex chromosomes. These included 35 known genes and 4 unknown genes. Gains of gene were found at higher frequency than losses (36 and 3 respectively). Seventeen genes (48.6% among known genes) were previously reported as CNV region. Eight of the CNV genes (47.1%) contained partially CNV region. The most frequently amplified gene, EEF2, was founded in all patients, and the frequency of IRF2BP2, TOP2B and DDX5 were 87.5%. The CDK6, NUP98 and DNMT1 were also significantly amplified with high frequency and known as genes associated with tumor suppressor activity. *Conclusions*. Our study using aCGH revealed that gene dose changes are a common features in N-AML, although they don't have the recurrent genetic abnormalities by FISH or RT-PCR. Some genes with dose change were known as ones associated with tumor suppressors . These results should reveal the possibility that genomic patterns be used to segregate N-AML patients into groups with the potential role in the prediction of prognosis and the rational therapy. Further studies in as many patients as possible may help to confirm our findings and should be followed by functional analyses to explain the implications of our regions of interest in leukemia.

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PREAL-TIME QUANTITATIVE PCR DETECTION OF WILMS' TUMOR GENE EXPRESSION IN CHILDREN WITH ACUTE MYELOID LEUKEMIA AND ITS IMPACT ON PROGNOSIS

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Background. Wilms' tumor gene 1 (WT1) expression in a subpopulation of early CD34+ cells has suggested its involvement in hematopoiesis. WT1 expression at diagnosis has been associated with unfavorable prognosis in adult acute myeloid leukemia (AML). *Aims.* To assess the biological significance and the prognostic impact of WT1 mRNA expression, in pediatric patients with AML, using a real time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR). *Design*. Twenty-eight newly diagnosed paediatric AML patients were included. They

were subjected to history taking and clinical examination, Laboratory investigations included ; complete blood count, bone marrow examination and chromosomal analysis for cytogenetic stratification. Quantitative assessment of WT1 transcripts by Q-RT-PCR. Patients were compared to twenty healthy subjects served as controls. All patients were followed up over the period of the study to detect the clinical course and outcome stressing on the event of remission, relapse or any adverse events. *Resuts*. WT1 expression was significantly higher in AML patients compared to controls ($p<0.001$). Although a highly significant increase in mean WT1 levels were elicited in cases with high risk cytogenetics compared to those with low and intermediate risk ($p=0.007$), yet no significant difference was detected between different FAB subtypes ($p=0.22$). Higher WT1 expression at diagnosis was detected in patients resistant to first course chemotherapy compared to those who experienced complete remission ($p=0.0003$), in dead cases compared to alive cases ($p=0.002$) as well as in patients who experienced relapse versus remitted cases till the last follow up ($p=0.01$). The 5- year survival rate was 47% and 100% among those with WT >1 and WT1 <1, respectively (log rank test gave $p=0.05$). On multivariate analysis , cytogenetic risk had the most significant effect, followed by WT1 expression on survival rate.In Kaplan-Meier curve patients with WT1 >1 experienced relapse after a shorter time than cases with WT <1 ($p=0.04$). WT1 expression had the most significant effect on relapse. *Conclusions*. Higher WT1 expression in AML patients compared to controls and was an independent prognostic factor for relapse prediction. Higher WT1 expression was associated with unfavorable prognosis with shorter survival time and shorter relapse free time. WT1 could be included as part of the initial evaluation of AML to establish more defined risk groups.

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ACUTE MYELOGENOUS LEUKEMIA BLASTS CONVERT INTO ADHERENT MYOFIBROBLASTS

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Background. It is evidenced that a microenvironment has an important role for the transformation and proliferation of malignant clones from the epithelial cells, in which the cytokines produced from the normal stromal cells make the microenvironment for the chronic inflammation and create the place for the growth of malignant cells. The stroma-forming cells are reported to be derived from normal cells. Also, there has been reported that when fibrosis is observed in hematological malignancies, these fibroblast cells are originated from the normal clone. *Aims*. We show in this report that non-adherent leukemia blasts can convert into myofibroblasts to create a microenvironment for the proliferation of leukemia blasts in acute myelogenous leukemia (AML) cases. *Design and Methods*. When myeloblasts were cultured long term *in vitro*, their morphology changed into myofibroblasts, which had similar molecular characteristics to the original myeloblasts, and cytokines such as granulocyte colony-stimulating factor, interleukin-6 and vascular endothelial growth factor, were produced significantly. When cultured onto the expanded leukemia blast-derived myofibroblasts, the original leukemia blasts proliferated extensively. The generated myofibroblasts expressed CD133, which is one of the important markers for cancer stem cells. When non-adherent AML cells were transplanted into severe combined immunodeficiency (SCID) mouse, leukemia was observed after 2 months. Cells were prepared from SCID mouse, and adherent cells were analyzed, in which the leukemia-derived myofibroblasts were identified. *Conclusions.* These results indicate that leukemia blasts can create their own microenvironment for the proliferation.

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AN ANTI-APOPTOTIC PATTERN CORRELATES WITH MULTIDRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA PATIENTS: A FLOW CYTOMETRY AND MOLECULAR STUDY OF PRO-APOPTOTIC AND ANTI-APOPTOTIC MOLECULES AND THE MDR1 GENE

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Background. In recent years important advances have been achieved in the treatment of patients with acute myeloid leukemia (AML). However, resistance to the cytotoxic effects of chemotherapy has remained the main obstacle in obtaining a cure. The insights into the molecular pathways leading to drug resistance might provide particularly powerful prognostic markers for response to therapy and opportunities to identify potential targets for the development of new molecularly designed therapeutic strategies Aim: Based on these premises, the aim of the present study was to evaluate the expression of such as active Caspase-3 (aC-3), Bcl-2, cleaved Poly (ADP-ribose) polymerase (cPARP), and survivin (an IAP gene family member) in comparison to MDR1 gene expression in AML patients. *Design and Methods*. Bone marrow samples were analyzed in 17 AML patients with a mean age of 53,5±16,8 years, 6 females and 11 males, diagnosed on the basis of morphology, cytochemistry, immunophenotype and complementary cytogenetic and molecular genetic studies according to WHO criteria. The intracellular levels of aC-3, Bcl-2 and cPARP were measured by CBA (Cytometric Bead Array) and flow cytometry. The expression of Survivin and MDR1 genes was defined by RT-PCR. *Results*. The analysis detected heterogeneous patterns of the studied proteins in AML pts. Activated C-3 varied from 0-215,0 U/mL (mean 34,6±52,5 U/mL); the lowest intracellular levels of bcl-2 were detected at 275,3 U/mL reaching as high as 6000 U/mL in 4/17 patients (mean 3268,4±2055,2 U/mL); cPARP varied from 0 to 97,8 U/mL (mean 24,59±29.97 U/mL). No correlation was observed among the studied variables. Molecular analysis by RT-PCR revealed heterogeneous patterns of over-expression of survivin in 9/17 pts (52,9%), while MDR1 gene was over-expressed in 10/17 pts (58,8%). Interestingly, 7 of 9 survivin(+) pts (77,8%) were also MDR1(+), while MDR1 over expression was found in only 3/8 survivin(-) pts (37,5%) though p value was estimated as n.s.. Besides, pts with high levels of survivin mRNA showed significantly lower cPARP (11.8±14.3 U/mL vs 53.9±31.9 U/ml, $p=0,005$) and a tendency towards higher aC-3 (49.3±70.0 U/l vs 18.1±9.9 U/mL, $p>0.05$). In contrast, no association was found between th e MDR1 gene status and the intracellular levels of the examined proteins. In total, no significant relationship was found with relevant clinical and laboratory, except for patients with favourable recurrent cytogenetic/molecular abnormalities none of whom showed survivin over-expression compared to 9/13 (69,2%) of the remaining patients who were positive (Fisher's Exact test, $p=0.029$). Interestingly, patients with high survivin expression had low therapeutic response rate, as complete remission was achieved in only 16,7% of the cases, and poorer clinical outcome with a mean overall survival of 138 days, compared to 75% complete responders with a mean survival of 641 days in the survivin-negative group. *Conclusions*. The detection of survivin gene expression defined a subgroup of patients of anti-apoptotic profile, characterized by significantly lower levels of apoptosis as defined by the intracellular quantity of cPARP that showed unfavorable clinical behaviour, which might be also due to higher MDR1 gene expression levels. A possible explanation of this finding may be the overlap of common signal pathways that trigger their expression. Our results support the potential relevance of apoptosis-related markers in AML for further understanding the disease; however the heterogeneity and complexity of molecular interactions warrants further prospective studies.

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MMTV-HOMOLOGOUS SEQUENCES IN CHILDREN WITH ACUTE MYELOID LEUKEMIA

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Background. MMTV-homologous sequences were found earlier in tumor tissue and lymphoid cell DNA from sporadic breast cancer (BC) and Non-Hodgkin's lymphomas +BC patients. Two female BC patients in our cancer register had children suffered with acute myeloid leukemia (AML). Earlier retrovirus-like particles were found in peripheral blood cell of the patients with acute myeloblastic leukemia (*Seman et al., 1973*). We have analyzed children with AML treated in Moscow clinics to verify these observations. *Design and Methods*. Peripheral blood and bone marrow (BM) DNA samples were obtained from 14 patients with primary AML with t(8;21) in their karyotype and 6 ones with acute lymphoblastic leukemia (ALL). DNA samples extracted from 2-3 day cell culture or from the frozen BM cells (fixation with methanol : acetic acid, 3:1) were analyzed by PCR using specific primers for gp52-coding area of the env MMTV gene and Sag-coding area of 3'LTR MMTV. PCR products of 675 bp and 785 bp were cloned in pGEM-T vector and sequenced. RT PCR using RNA from the fresh BM cells and primers specific for the env MMTV gene was performed to evaluate MMTV-homologous sequence expression. *Results*. 6/14 (42.9%) BM DNA sam-

ples obtained from 4 girls and 2 boys of 5-15 years old, were env /LTR MMTV-positive, one boy has mother with BC and young healthy brother. While 6 ALL patients (3-11 years old) were MMTV-negative by PCR. Sequencing of the env MMTV and 3'LTR - related cloned PCR products has found 93-94% homology to the exogenous env MMTV gene (C3H strain, Mus musculus MMTV) and 92% homology to SAG protein gene of Mus musculus MMTV. The sequence transcripts were revealed by RT PCR in 4 RNA samples. ORF finder has shown 1 ORF of 567 bp long in gp 52-coding area of the env MMTV sequence and 1 ORF in Sag-coding sequence. BLAST analysis puts the sequences into tree clusters between endogenous MMTV RNA env gene/ right LTR and Mus musculus mammary gland cDNA branches. *Conclusions*. MMTV-homologous sequences were revealed in 6/14 children with AML. It indicates that MMTV-related retrovirus might involves both in human lymphomas and AML progression by infection of dendritic and/or pluripotent hemopoetic stem cells - blast progenitors.

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ACUTE PROMYELOCYTIC LEUKEMIA WITH T(15;17) (Q22;Q21) DEVELOPING INV(16)(P13Q22) SECONDARY AML

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Background. We describe a 50 year old female with acute promyelocytic leukemia (APML) with an abnormal karyotype of 47 chromosomes with a t(15;17) translocation and trisomy 8. Molecular analysis confirmed a PML-RARA (bcr3) gene fusion and the patient was entered into the MRC AML15 trial. She achieved a good induction response (4.7 log reduction, laboratory mean=2.54), and molecular remission post second course. The molecular remission for PML-RARA has continued, monitored typically every 3 months. Three years post diagnosis she developed pancytopenia and morphological assessment indicated the presence of blasts in the marrow. Arsenic trioxide treatment was commenced due to the suspicion of APML relapse. However, she was PML-RARA negative by cytogenetics and molecular analysis, therefore a diagnosis of therapy related AML was considered rather than relapsed APML. Cytogenetic analysis identified a pericentric inversion of chromosome 16, and a CBFB-MYH11 (type D) gene rearrangement was confirmed by molecular analysis. The patient achieved a dramatic response to FLAG with a complete molecular response (>4.7 log reduction) post course 1 and has since remained in molecular remission for both PML-RARA and CBFB-MYH11 transcripts. *Aims*. To carry out retrospective RQ-PCR analysis of the CBFB-MYH11 gene fusion and gain a unique insight into the kinetics of emergence of AML. *Design and Methods*. Serial blood and/or marrow RNA, banked as a consequence of the PML-RARA monitoring, was retrospectively analysed by RQ-PCR for CBFB-MYH11 transcripts. Results: CBFB-MYH11 transcripts were first identified 9 months post diagnosis of APML, 27 months pre secondary leukemia. Transcripts were detectable only at relatively low levels for 18 months before rapidly expanding. It is presumed that the pre-expansion plateau represented a clone with just a CBFB-MYH11 gene fusion, from which emerged a clone with a second mutation, that proliferated rapidly resulting in emergence of the secondary leukemia. *Summary*. The bi-phasic kinetics of emergence of the CBFB-MYH11 leukemia supports a two mutation model of AML. The appearance of CBFB-MYH11 transcripts after only 9 months, and the subsequent kinetics of the CBFB-MYH11 clone, suggests this clone may already have been present at diagnosis of APML, although it remains possible that it was induced early in the treatment of the original leukaemia. The kinetics of the clone once rapid expansion was observed suggests the second mutation, driving the more proliferative phase, may have occurred during subsequent courses of treatment for APML.

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CYTOKINE RECEPTORS EXPRESSION ON ACUTE MYELOID LEUKEMIA CELLS

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Background. Interleukin (IL)-3, IL-7, CCL5 and CXCL12 have been previously reported to induce growth, development, recruitment, and tissue localization of several hematopoietic malignancies, including acute myeloid leukemia (AML). *Aims*. The expression level of four cytokine receptors on AML cells was analyzed in relation to their FAB subtype, differentiation status, aberrant phenotype, and response to induction therapy; as knowledge of their receptor profile on AML cells may be valuable for the prognosis, pathobiology, subclasification and therapeutic decisions during the medical management of AML patients. *Design and Methods*. Mean Fluorescence Intensity Ratio (MFIR) of CD123, CD127, CXCR4 and CCR5 on CD34+ or CD117+ cells from EDTA/bone marrow samples of 21 newly diagnosed AML patients (M0=4, M1=2, M2=1, M4/5=14) and 3 healthy donors were detected by flow cytometry. Six-color combinations of markers were used to optimally gate the cell population of interest and to establish the differentiation status according to CD133 and CD34 level. Marker combinations used and the gating strategy are detailed in Figure 1. The cut-off value for positive MFIR was 2. *Results*. In 8 out of the 21 AML cases, leukemic cells expressed intermediate levels of CD127 (MFIR=2-5). Seven of them where AML M0-2; 5 expressed intermediate levels of CD133, high levels of CD34, and at least one phenotypic aberrancy. No CD127 expression was detected in the control group. Although CD127 has been previously reported to be restricted to lymphoid lineage, the role of IL-7 as a growth factor for certain hematological malignancies and breast cancer has been established and attributed to activation of STAT-5 and up-regulation of the anti-apoptotic molecule Bcl-2. 15/21 AML patients showed intermediate (n=6) and high (n=9, MFIR>5) CXCR4 expression. CXCR4 levels significantly correlated with the M4/M5 FAB subtype (p=0,04) but there was no correlation with the differentiation status or the presence of phenotypic aberrancies. One intermediate expression was determined within the control group. 13/21 AML patients expressed intermediate (n=10) and high (n=3) CD123 levels. Interestingly, two of the three high expressers were of AML M4/5 subtype and expressed high CD133 and CD34 levels and at least one phenotypic aberrancy. CD123 expression was found in 7 cases with one or more phenotypic aberrancy. We found significant correlation between CD123 expression and the illicit presence of lymphoid markers on AML cells (p=0,001). In line with previously published data, no CD123 expression was detected within the control group. CD123 expression was further correlated with poor response to induction therapy in our group (p=0,07). 11/21 AML patients expressed intermediate (n=7) and high (n=4) CCR5 levels. The expression level of CCR5 seemed to be inversely related to the differentiation status of leukemic clone. *Conclusions*. Cytokine receptor analysis in AML may provide important information of diagnostic relevance, such as the degree of differentiation and phenotype aberrancy of the malignant clone. Measuring their expression levels will also contribute to the detection of minimal residual disease and become prognostic candidates when larger groups of patients involved.

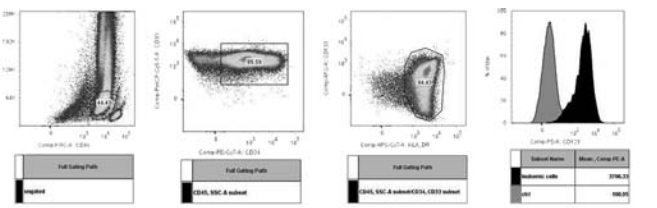

Fig. 1: Measurement of CD123 expression level on one AML representative case (M4 FAB subtype) by flow cytometry. The combination of markers used for gating was CD45-FITC/ CD123 PE/ CD33PC5.5/ CD34PC7/ CD133APC/ HLA DR APC/C7 for the sample tube and CD45-FITC/ IgG1- PE/ CD33PC5.5/ CD34PC7/ CD133APC/ HLA DR APCC7 for the control tube. MFIR was calculated by dividing MFI values of the samples and isotype control, respectively. Images were generated using the Flow Jo, Tree Star, Inc analysis software; data acquisition was performed with a three laser BD FACSCanto II instrument.

Figure 1.

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IMMUNOLOGICAL AND PROTEOMIC ANALYSIS OF FRESH AND CULTURED ACUTE MYELOID LEUKEMIA CELLS

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Background. Acute myeloid leukemia (AML) is a disease caused by dysregulation of proliferation, differentiation and death of immature myeloid leukemia stem cells. *Aims*. To assess the role of leukemic stem cells in the acute myeloid leukemia we attempted to isolate these cells and performed their immunological and proteomic analysis. *Design and Methods*. Leukemic cells were separated from bone marrow and peripheral blood samples of untreated first diagnosed patients with AML. Primary cultures of leukemic cells were established in the presence and absence of immunologic (antibody to CD34 molecule) or epigenetic

modulators of the gene expression - valproic acid and SAHA. Fresh and cultured cells were analysed for cell cycle, differentiation, apoptosis and senescence. Proteomic analysis was also performed by two dimensional electrophoresis and electrofocusation in polyacrylamide gels. The results were compared with myeloid leukemia cell lines MOLM-7, MOLM-9, JURL-MK1 and HL-60. *Results*. Leukemic cells of 25 consent patients with acute myeloid leukemia classified as M1 - M5 were separated by density gradient centrifugation and cultured for a period of 3-7 days in the presence of valproic acid, SAHA or antibody to the CD34 molecule. The proteomic analysis showed at least 1000 protein spots. We were however not able to identify the minor proteins responsible for dysregulation as products of oncogenes, transcription factors or tyrosine kinases in the resolution. Both compounds - valproic acid and SAHA inhibited the cell cycle, induced the programmed cell death and/or senescence both in primary cultures of patients cells and cell lines, however in different concentration ranges. Monoclonal antibody to the stem cell molecule CD34 inhibited the proliferation of CD34 positive myeloid cell lines but not CD34 negative HL-60 cells.*Conclusions*. Primary cultures of acute myeloid leukemia cells in the presence of fetal bovine serum result in the slow inhibition of proliferation, spontaneous differentiation and prolonged slow cells death or senescence. This process is associated with morphological, immunological and proteomic changes. All compounds used in the study inhibited cellular proliferation and induced cell death, however no direct correlation was found with disease stage or prognosis.

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TREATMENT OF LEUKEMIC CELLS WITH HSP90 INHIBITOR 17-AAG INDUCES G2/M ARREST AND APOPTOSIS

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Background. Acute myelogenous leukemia (AML) is among the deadliest of the lymphatic and bone marrow cancers. Patients diagnosed with AML have a five year survival rate of 21.3%. AML is characterized by an accumulation of undifferentiated and functionless myeloid precursors in the bone marrow and blood. Heat shock protein 90 (Hsp90) is a molecular chaperone that is highly conserved among all living organisms. It serves a critical function in many different signaling cascades by ensuring proper protein structure, activity, proteolytic turnover, and localization. Hsp90 constitutes 1-2% of total cellular protein. In spite of this, it is often upregulated in cancer. It has been proposed that Hsp90 serves in a buffering capacity to stabilize mutated proteins and may help the cancer cells survive in the harsh environment of the host. Because Hsp90 affects many different signaling mechanisms, intense research has been undertaken in treating cancer with Hsp90 inhibitors. *Aims*. 17-AAG, an analog of geldanamycin, is a potent Hsp90 inhibitor. It is currently undergoing clinical trials for the treatment of various blood malignancies. We sought to determine the effects of Hsp90 inhibition in AML cell lines. *Design and Methods*. The AML cell lines KG-1a and HL-60 were treated with varying concentrations of 17-AAG in cell viability assays. For all other assays, clinically relevant concentrations, 3 μM or less, were used. Cell viability assays were performed by counting live cells via dye exclusion. Proliferation and differentiation effects of treatment were measured by assessing the expression of cell surface markers CD71 and CD11b, respectively. Annexin V labeling was used to measure apoptosis with treatment. Cell cycle analysis was performed by cell fixation in ethanol, followed by propidium iodide staining. Marker experiments and cell cycle analysis were analyzed using flow cytometry. *Results.* After 48 hours of treatment, the cells remained static, which prompted further differentiation and proliferation studies. CD11b expression did not increase which indicates differentiation was not induced. CD71, a transferrin receptor, was markedly decreased with treatment showing that the cells were becoming less proliferative. Cell cycle analysis has revealed that the cells undergo G2/M arrest within 48 hours of treatment. Annexin V labeling increased on the cells over time demonstrating long term treatment was inducing apoptosis. *Summary*. These results suggest that inhibition of Hsp90 causes these leukemic cells to cease proliferating which is due to an arrest in G2/M phase of the cell cycle. Furthermore, extending treatment of the cells eventually leads to induction of apoptosis. Further studies are ongoing to determine the mechanism behind these observations.

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INTERNAL TANDEM DUPLICATION OF FLT3 GENE IN EGYPTIAN ADULT ACUTE MYELOID AND ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. FLT3 plays an important role in stem cell proliferation, differentiation, and survival. The most common mutation in the FLT3 gene is an internal tandem duplication (FLT3/ITD). Several studies have demonstrated that FLT3 mutations are a strong detrimental prognostic factor in AML and ALL. Aim: Our study was designed to evaluate the effect of FLT3/ITD status in AML and ALL on response to treatment and overall survival. *Design and Methods*. This study was carried out on 110 newly diagnosed adult acute leukemia cases including 61 AML and 49 ALL. Bone marrow or peripheral blood cells were collected from patients at diagnosis. All samples were analyzed for mutation in exon 11 of the FLT3 gene using genomic PCR method. ALL cases were treated according to our risk adapted chemotherapy protocol while mature B phenotype cases were excluded from the study. AML cases received induction with 3&7 regimens. Evaluation of response was carried out at the end of induction regimen. *Results*. ITD appears as an extra PCR band (mutant band) in addition to the 133-bp wild band was found in 21.3% (13 / 61) of AML cases. The highest frequency of FLT3/ITD was associated with M3 (40%) followed by M5 (37.5%) and M4 (33.3%) FAB subtypes, less frequent in M2 (13.6%), M1 (13.3%) and none in M0 and M7. In ALL cases FLT3/ITD was detected in (5/44) 10.2%. The highest frequency was associated with precursor B phenotype (11.7%) and less in T- ALL patients (7.1%). No association was detected between the status of FLT3/ITD on one side and age, gender, high leucocytic count, BM blasts, DNA index or CD34 expression on the other side in either AML and ALL. We couldn't find statistically significant difference in response to treatment between FLT3/ITD positive and negative AML and ALL cases. *Conclusions*.FLT3/ITD + in our AML patients was 21.3% as that reported in the literature, however the in adult ALL it was much higher than that reported in literature. Further study on a large scale is recommended to identify the prognostic impact of FLT3/ITD in adult ALL.

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MOLECULAR CHARACTERIZATION OF RARE TRANSLOCATION T(11;12)(P15;Q13) GENERATING NUP98-HOXC13 FUSION IN THE CASE OF *DE NOVO* FLT3-ITD POSITIVE ACUTE MYELOID LEUKEMIA

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Leukemia is frequently associated with recurrent chromosome translocations. One of the most promiscuous fusion partner gene involved in chromosomal translocations is NUP98 gene at 11p15 that encodes a component of nuclear pore complex. The most frequently observed fusion partners of NUP98 are the homeobox family of transcriptional factors (HOX), required for regulation of embryonic morphological development. This is the first case of de novo AML (Acute Myeloid Leukemia) patient with simultaneous presence of the t(11;12)(p15;q13) and FLT3-ITD (fms-like tyrosine kinase 3-internal tandem duplication) mutation. Molecular characterization of the t(11;12)(p15;q13) translocation was done using semi-nested RT-PCR analysis and direct sequencing. For detection of FLT3-ITD mutation we used PCR method. The position and size of ITD was determined using sequencing analysis. Molecular analysis showed that the exon 16 of NUP98 was fused in frame with exon 2 of HOXC13, giving raise to the functional fusion protein NUP98-HOXC13, that acts as aberrant transcriptional factor. Sequencing analysis of the FLT3-ITD revealed in-frame duplication of 82 bp in exon 14, involving juxta-membrane domain of FLT3, giving raise to the functional protein that provokes uncontrolled proliferation of the cells bearing this mutation. Our results represent another contribution to the theory of cooperating mutations necessary to cause acute leukemia (two hit hypothesis). Namely, NUP98-HOXC13 fusion and FLT3-ITD mutation act in collaboration in the process of leukemogenesis, in the way that FLT3-ITD provides proliferative advantage to myeloid progenitors whose differentiation is impaired by NUP98-HOXC13 fusion.

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PROGNOSTIC SIGNIFICANCE OF FLT3 MUTATIONS IN NORMAL KARYOTYPE ACUTE MYELOID LEUKEMIA PATIENTS IN INDIA

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Background. Acute myeloid leukemia patients without clonal chromosome aberrations at diagnosis constitute the largest cytogenetic group of AML, approximately 45%, and are classified in the intermediate prognostic category. It is important to identify prognostic markers that predict patients' outcome more precisely, thereby allowing development of molecular risk-adapted treatment strategies that may improve the clinical outcome. FLT3, a member of receptor tyrosine kinases 3 family is involved in differentiation and proliferation of hematopoietic stem cells. Flt3 mutations represent one of the most frequent molecular aberrations in acute myeloid leukaemia(20-35%). Flt3-ITD mutations in the juxtamembrane domain leads to ligand independent proliferation and thus cell survival, the other point mutation(5-10% frequency) present in kinase domain 2 of the receptor. Many AML patients with Flt3 mutations lack chromosomal aberrations (up to55%). Recent studies have shown that patients with FLT3 mutation show poor prognosis. Aim:- Aim of the study was to find out prognostic significance of FLT3 mutations in normal karyotype AML patients. *Design and Methods*. 104 normal karyotype AML patients were selected for the study, their diagnosis was based FAB classification. The distribution of AML patients in to subtypes was as follows:M0-7, M1-9, M2-33, M4-29, M5-18, and M6-7 and M7-2. Bone marrow samples were collected at presentation, after induction, after consolidation, at relapse and patients were followed up to 2 years. RT-PCR was performed for FLT3-ITD and FLT3-TKD(D835Y) mutations. Sequencing was done find out ITD length and domain mutation confirmation. The statistical analysis was performed by Chi-square and Mann-Whitney tests. *Results*. A total of 34 patients showed FLT3 mutations (28 with ITD and 6 with D835Y). The ITD length ranged from 20 bass to 220 bases. Patients with FLT3-ITD positive had a significantly higher median white cell count ($38.6x10^9$/L) than those with wild type ($5.4x10^9$/L (p=0.001). Complete remission was achieved by 81% of patients without a FLT3-ITD and by 44% of patients with the mutation. CR rates were further lower in patients whose ITD size was larger than 50 bases. More patients with FLT3 mutations experienced induction death (57%) than those without these mutations (19% (p=0.01). Six relapsed cases had FLT3-ITD mutation were positive for ITD and this mutation could have been responsible for lower EFS in cases of ITD positive cases compared to wild type FLT3 patients. No significant difference was observed in terms of overall survival between the two groups. No correlation was found in terms of WBC at presentation, CR rates, EFS and OS amongst wild type and mutated D835Y FLT3. *Conclusions*. Thus it can be assumed that the presence of FLT3/ITD in AML patients confers a worse prognosis in terms of CR rate, induction failure and EFS. Therefore prognostic markers like FLT3 mutation apart from cytogenetics may be useful in identifying high-risk patients for closer monitoring and possibly more prolonged and intensive therapy. Further FLT3 can also be used as a marker for minimal residual disease detection in cytogenetically normal patients.

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THE ROLE OF CDK1, CDK2 AND CYCLIN A1 IN ALL-TRANS-RETINOIC ACID MEDIATED DIFFERENTIATION OF U-937 LEUKEMIC CELLS

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All-trans-retinoic acid (ATRA) is a candidate for cancer therapy, in particular for the treatment of leukemia. The ATRA-induced cellular effect is complex, and probably involves multiple mechanisms. The aim of our present study is to investigate whether the key cell cycle regulatory proteins cyclin A1, CDK1 and CDK2 may contribute to ATRA-induced differentiation and cell cycle arrest in leukemic cells. In the present study, we treated U-937 cells with ATRA for 24, 48 and 72 hours. Terminal differentiation induced by ATRA was characterized by morphology changes and expression levels of the surface markers CD11b and CD11c measured by flow cytometry analysis. ATRA-induced differentiation correlated well with an accumulation of cells in G0/G1 phase. Real time PCR studies revealed that ATRA treatment significantly increases the mRNA level of the retinoic acid receptor β and decreases the levels of the retinoic acid receptor γ, the retinoic X receptor γ and cyclin A1. Further, ATRA-induced differentiation appears to be related to downregulation of protein expression of CDK1 and CDK2, the major kinase partners associated with cyclin A1. The reduction of protein expression was observed predominantly in the nuclear compartment. To further investigate the role of cyclin A1, CDK1 or CDK2 in ATRA-mediated cell cycle arrest and differentiation, we depleted these proteins in U-937 cells via siRNA-mediated knockdown. We are currently characterizing the effect of single knockdown or double knockdown of these proteins on differentiation, cell cycle progression and the cellular responses to ATRA-induced differentiation.

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PRIMARY HUMAN ACUTE MYELOGENOUS LEUKEMIA CELLS SHOW CONSTITUTIVE RELEASE OF SEVERAL MATRIX METALLOPROTEASES AND THEIR INHIBITORS

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Background. Angiogenesis seems to be important both for leukemogenesis and chemosensitivity in human acute myelogenous leukemia. Angiogenesis is regulated by the balance between pro- and antiangiogenic cytokines, but matrix metalloproteases and their natural inhibitors; tissue inhibitors of metalloproteases, may also be important. Aims:To investigate the constitutive release of matrix metalloproteases and tissue inhibitors of metallopoteases for a large group of consecutive acute myelogenous leukemia patients. *Design and Methods*, Primary human acute myelogenous leukemia cells were cultured *in vitro* either alone or together with microvascular endothelial cells and levels of matrix metalloproteases and tissue inhibitors of metalloproteases were determined in culture supernatants. *Results*. Primary human acute myelogenous leukemia cells showed constitutive release of matrix metalloproteases. For all patients detectable matrix metalloprotease 10 release was observed, and most patients showed detectable release of at least one addition matrix metalloprotease, usually matrix metalloprotease 9. Matrix metalloprotease release was increased by Protein kinase C agonists. Endothelial cells showed high release of matrix metalloproteases 10, and these levels were further increased by the presence of acute myelogenous leukemia cells during culture. *Conclusions*. Matrix metalloproteases may be important for leukemogenesis and chemosensitivity in human acute myelogenous leukemia.

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PROGNOSTIC IMPLICATIONS OF GENE ABNORMALITIES IN ACUTE MYELOID LEUKAEMIA WITH NORMAL KARYOTYPE (NK-AML): A SINGLE-CENTRE EXPERIENCE

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Research into genotypic leukaemia-specific alterations in the broad group of NK-AML has contributed to unravel this highly heterogeneous group of entities. Gene mutations, gene expression abnormalities and other molecular alterations affect critical functions in AML cells, and may exert profound effects on therapeutic response and outcome of disease. To ascertain the association between the presence or absence of a set of molecular markers and clinicopathological features and treatment outcome in NK-AML, diagnostic samples derived from 28 patients with NK-AML, consecutively diagnosed between December 2006 and January 2008, were analysed for FLT3-ITD, FLT3-TKD (D385), NPM1, CEBPA and MLL-PTD mutations. Median patient age at diagnosis was 66 years (15-79) and fifty-four percent were female. Thirty-nine percent had WBC>30x10^9/l at presentation. Twenty-five percent (7/28) were deemed as not meeting criteria to undergo intensive chemotherapy. Mutations prevalence was as follows: 50% NPM1+ (14/28 cases); 37% FLT3+ (10/27, all ITD, ranging from 1 to 10 duplications); 11% MLL-PTD+ (3/28); 11% CEBPA+ (3/27). Concurrency of genetic abnormalities was observed in 29% (8/28) of cases; one FLT3-ITD+/MLL-PTD+ case was observed; no cases of concurrency between NPM1+ and CEBPA+ and/or MLL-PTD+ were observed. Of NPM1+ cases, 54% (7/13) were also FLT3-ITD+ (p=0.089). NPM1+ cases were predominantly female (64%, p=0.256) and displayed higher peripheral blood WBC (57% >30x10^9/l, p=0.060). The overall incidence of NPM1+ was higher in FAB-M4 and M5, as compared with other subtypes (75% vs. 33%,

p=0.085); no association was detected between FAB subtype and FLT3 mutational status. Regarding immunophenotype, we observed a significant association between CD34+ and NPM1- (92%, 12/13, p=0.001) and that NPM1+ cases more frequently expressed monocytic lineage markers, such as CD11b (62%, 8/13, p=0.055). FLT3-ITD- cases displayed lower WBC counts (71% <=30x10^9/L, p=0.285). No other associations were apparent between clinico-analytical variables and mutational status. The observed induction death rate was 14% (3/21); CR rate was 83% (15/18) without significant influence of mutational status, although a higher frequency of CR achievement was observed in NPM1+ patients (89% vs. 78%, p=0.500). With a median follow-up of 8 months and a median DFS of 10 months, the observed cumulative OS and DFS were, respectively, 50% at 37 months and 59% at 36 months. By univariate analysis, higher but not significant OS and DFS were observed in NPM1+ (64% vs. 29% at 37 months, p=0.415 and 64% vs. 50% at 34 months, p=0.445, respectively); cumulative OS (p=0.891) and DFS (p=0.517) were, respectively, 53% at 35 months and 67% at 34 months for FLT3-ITD+ and 52% at 37 months and 55% at 36 months for FLT3-ITD- patients. In NPM1+ patients, concurrent FLT3-ITD+ did not appear to result in worse cumulative OS (71% vs. 66% at 35 months, p=0.245). MLL-PTD and CEBPA were excluded from follow-up analysis due to limited number of patients. In this single-centre sequential cohort of NK-AML patients treated with intensive chemotherapy, we observed a higher although not significant frequency of CR achievement and higher cumulative OS and DFS in NPM1+ cases. Documented CR, cumulative OS and DFS were similar for both FLT3-ITD- and FLT3-ITD+ patients.

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SELENIUM AND GLUTATHIONE PEROXIDASE STATUS IN ADULT EGYPTIAN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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This study was undertaken to evaluate selenium and glutathione peroxidase status in patients with newly diagnosed acute myeloid leukemia (AML) before and after induction therapy. Twenty five patients with newly diagnosed AML and fifteen healthy age and sex matched control subjects were included in this study. Serum selenium (Se) level by the graphite furnace atomic absorption spectrometric (GFAAS) technique and glutathione peroxidase (GPX) activity by an adaptation of Beutler method was performed for the patients before and after receiving the induction therapy. Serum selenium level was significantly lower in patients with AML versus control subjects (63.1µg/L ±8.8 vs. 77µg/l ±8.8 before therapy p<0.01 and 69 µg/l±6.8 vs. 77µg/L ±8.8 after therapy with a p<0.01).GPX activity was significantly lower in patients with AML versus control subjects (1.6 µ/gm protein ±0.4 vs.3.4 µ/gm protein ±0.7 pretreatment p<0.01and 1.9 µ/gm±0.6 vs.3.4 µ/gm protein ±0.7 post induction treatment with a p<0.01).serum selenium level and GPX activity significantly increased in AML patients after treatment. Patients who accomplished complete remission after induction harbored significantly higher selenium levels than resistant patients before and after treatment. There was no significant correlation between serum selenium level and GPX activity. Decreased selenium level and reduced GPX activity in AML patients support the association of carcinogenesis and subnormal selenium states.

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A SIMPLE PROGNOSTIC SCORING SYSTEM FOR NEWLY DIAGNOSED ACUTE LEUKAEMIA PATIENTS WITH NORMAL KARYOTYPE: A RETROSPECTIVE ANALYSIS ON 173 CASES

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Background. Cytogenetics is the most important prognostic factor for acute myeloid leukaemia (AML), enabling to categorize AML patients in three risk categories: favourable [t(15;17), t(8;21), inv(16)], intermediate [normal karyotype (NK), +8, abnormalities not included in other categories] and unfavourable [abnormalities of chromosome 3, -7/-7q, -5/-5q, abnormalities 11q23, complex karyotype]. The post-induction therapeutic strategy, including or not allogeneic-SCT, is not well established in patients with NK, who account for at least the 40 - 50% of cases and show an extremely variable long term survival, ranging from 20 to 70%. *Aims*. The aim of the study is to provide a simple prognostic scoring system, to better define the disease-risk in AML patients with NK and to optimally address the issue of post-induction therapy. *Design and Methods*. We retrospectively analyzed 173 AML patients with NK (NK-AML) consecutively treated from 1990 to 2005. Induction regimen was FLAI in 110 patients (64%) and ICE/DCE in 63 cases. After the first consolidation, patients were addressed to intensification therapy including allogeneic-SCT if aged less then 45 yrs, with a HLA compatible donor and at least one other high risk feature (WBC over 30x10^9/L, secondary AML, non response to the first induction therapy, multidrug-resistance Pgp over-expression and presence of Flt3-ITD). An allogeneic-SCT was performed in 59/173 cases (34%). By univariate analysis age at least 50 yrs, female sex, FAB subtype, Pgp positive phenotype, presence of Flt3-ITD, WBC count over 19x10^9/L significantly affected the CR rate. Moreover, age at least 50 yrs, FAB subtype, WBC count over 19 x 10^9/L and no response to the first induction cycle significantly affected disease free survival (DFS) and overall survival (OS). By multivariate analysis, age at least 50 yrs and female sex significantly affected the CR rate, whereas age at least 50 yrs, WBC count aver 19 x 10^9/L and no response to induction significantly affected the DFS and OS. Univariate and multivariate analysis were conducted using the logistic regression model for CR rates and the Cox proportional hazard model for survival. A numerical score was derived from the regression coefficients of each independent prognostic variable and was 1 for WBC count over 19 x 10^9/L and age at least 50 yrs and 2 for no response to first induction regime. The prognostic score for each patient was then calculated by totalling up the score of each independent variable. *Results*. The allotransplanted patients were censored at the time of SCT. For what concerns DFS, patients could be stratified in low (score=0), intermediate (score = 1) and high risk group (score=2), with a median DFS of 28, 10 and 5 months, respectively (p=0.0003). For what concerns OS, patients could be stratified in low (score=0), intermediate (score = 1 - 2) and high risk group (score = 3) with a median OS of 120, 17 and 5 monhts, respectively (p=0.0001). *Conclusions.* these preliminary retrospective data suggest that common available clinical variables may still represent a valid approach for determining a prognostic stratification for NK-AML patients.

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A PHASE II STUDY WITH CP-4055 AS SECOND SALVAGE THERAPY IN PATIENTS WITH AML

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Background. CP-4055 (elacytarabine [pINN]) is a novel cytotoxic nucleoside analogue. CP-4055 has similar mechanism of action to cytarabine, but unlike cytarabine, it is independent of nucleoside transporters for cellular uptake. *Aims*. To assess efficacy and safety of CP-4055 when given as second salvage therapy to patients (pts) with acute myeloid leukemia (AML). *Design and Methods*. CP-4055 2000 mg/m^2/d was administered over 24h (CIV) in a d1-5 q3w schedule to adult pts who had received at least 2 previous chemotherapy regimens and who had refractory/relapsed AML [CR after first salvage therapy lasting less than 6 months] and from whom informed consent was obtained. After every 20-pt cohort, an Independent Data Monitoring Committee (IDMC) evaluated the results for futility and safety for recommendation of study continuation. *Results*. The IDMC recommended continuation of enrolment based on the first 20-pt cohort evaluation. Forty pts [28 male and 12 female, median age 58 yrs (range 26-82)] have been treated at 8 centres. The majority of the pts had previous ara-C based therapy, 12 pts had not obtained CR1 or CR2. Only 1 pt did not receive CP-4055 d1-5

dosing. The most frequently reported related adverse events (AEs) >= grade 3 (CTCAE v3.0) were thrombocytopenia, leukopenia, febrile neutropenia, lymphopenia, fatigue and pyrexia. Four deaths occurred within 4 weeks after start of treatment. None of these were related to CP-4055. Clinical activity (IWG criteria for AML) was recorded for 6 patients, 4 CRs and 2 CRp, representing a response rate of 15%. Two of the responders had refractory disease (first induction after >= 2 regimens), three pts were in first relapse with one pt having duration of first CR < 6 months, two patients having duration of first CR > 12 months. One responder was in second relapse with duration of first CR < 6 months. The median time from start of treatment to remission was 32 days (range 28-126 days) and the median duration of remission was 61 days (range 6-130+ days). The median overall survival was 88 days (range 8-292+ days). *Conclusions*. CP-4055 administered at 2000 mg/m2/d CIV in a d1-5 q3w schedule to patients with late stage AML showed manageable toxicity. Encouraging clinical activity was recorded with a CR/CRp rate of 15% in this difficult-to-treat patient population.

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MITOXANTRONE, ETOPOSIDE, ARA-C AND LOW DOSE GEMTUZUMAB OZOGAMICIN (MY-MEC) AS SALVAGE THERAPY FOR RELAPSED-REFRACTORY AML PATIENTS

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Background and aims. Conventional salvage regimens for AML patients are often badly tolerated and induce few long lasting remissions. Gemtuzumab ozogamicin (GO) has shown promising activity in relapsed-refractory AML patients. We therefore explored the feasibility and the efficacy of a regimen combining mitoxantrone, etoposide and Ara-C (MEC) with low dose GO. *Design and Methods*. We present the first analysis on 30 patients with CD33+ refractory-relapsed AML receiving mitoxantrone 12 mg/sqm, etoposide 100 mg/sqm, Ara-C 1 g/sqm (days 1-4 in patients < 65 years and 1-3 in patients ≥65) and GO 5 mg at day 5 or 4. Responding patients were administered the same regimen as consolidation and allogeneic stem cell transplant when indicated. Patients: The median age of patients was 64 (range 33-74); M / F ratio was 22 / 8; FAB subtypes were M0 in 4 patients, M1 in 8, M2 in 10, M4 in 3, M5 in 3, M6 in 2. Thirteen patients were refractory to first line induction therapy, 17 were in first relapse (median first CR length 6 months, range 3-19). Previous induction regimens mainly included combinations of fludarabine with Ara-C and anthracyclines±GO. Twenty-one patients had *de novo* AML; in 9 patients AML was secondary to MDS (7) or myeloproliferative disorders (2). Cytogenetic analysis revealed a poor prognosis alteration in 5 patients (complex karyotype) and an intermediate alteration in the other 25. Haematological parameters before therapy were the following: WBC $5x10^9$/L (range 0,8-105); Hb 9,2 g/dl (8-13); Plt 66 x 10^9 / L (10-255). *Results*. The neutrophil (PMN > $0,5x10^9$/L) and platelet (>$25x10^9$/L) recovery required a median of 17 (range 12-25) and 20 days (range 14-36) from the end of therapy. Therapy was well tolerated, with 2 deaths occurring during induction (7%). Thirteen infectious complications were observed (9 sepsis, 2 pulmonary aspergillosis, 2 broncopneumonia). No VOD were reported and mild and transient signs of liver toxicity were observed in 3 patients only. Fourteen pts (47%) achieved CR, 3 (10%) showed a partial response, 11 (36%) did not respond; 2 (7%) patients died before response evaluation. Three patients underwent allogeneic stem cell transplant. Complete remission and survival lasted a median of 7 (range 3-26) and 8 months (range 1-27), respectively. Five out of 13 patients treated for refractory disease achieved CR (38%) whereas CRs have been 9 among 17 patients treated in first relapse (53%). Poor prognosis cytogenetics at diagnosis had a negative impact on CR rate (0% compared to 56% in patients with intermediate prognosis karyotype). CR rates have been 52% and 33%, in de-novo and secondary AML respectively. Eigth out of 14 patients in CR have relapsed. Overall 25 patients have died. *Conclusions*. In our hands MY-MEC proved to be a well tolerated and effective salvage treatment for refractory-relapsed patients with intermediate prognosis karyotype. However the short duration of CR requires the early application of allogeneic stem cell trasnsplant or innovative consolidation therapies.

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CLOFARABINE IN THE TREATMENT OF POOR RISK ACUTE MYELOID LEUKAEMIA - GALWAY UNIVERSITY HOSPITAL EXPERIENCE

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Background. Despite immense progress in the diagnosis and therapy of haematological malignancies, treatment of unfavourable risk Acute Myeloid Leukaemia (AML) remains a challenge. We present our 2 year experience (December 2006-December 2008) of clofarabine both as a single agent and in combination as treatment of unfavourable risk AML patients at our institution who were not suitable for standard therapy. *Aims*. We retrospectively analysed the efficacy and safety of clofarabine in the setting of unfavourable risk AML. *Design and Methods*. The medical records and data from laboratory system were analysed. *Results*. Over a period of 24 months 22 AML patients (11 M, 11 F) with poor risk features, deemed unsuitable for standard therapy, were treated with clofarabine, alone (8 patients) or in combination (14 patients) for up to 3 cycles of treatment. The median age was 67.5 years (24 to 76) with 16 patients > than 60. Cytogenetic data were available in all 22 patients (no favourable karyotype , 18% normal karyotype 45% - adverse karyotype 36%) had an abnormal karyotype, which was neither favourable nor adverse. Eighteen patients had active AML at the time of clofarabine therapy. In the majority of patients during induction and consolidation, clofarabine was administered as a 1-hour intravenous infusion at 20 mg/m2 daily dose for 5 consecutive days. In five younger patients (< 60 years) clofarabine was administered as a 1-hour intravenous infusion at 40 mg/m2 daily for 5 days. A second induction cycle was given to patients in the absence of progressive disease after the first induction cycle. Cycles were repeated every 4 to 8 weeks depending on response, marrow recovery and absence of ongoing toxicity. Selected patients also received daunorubicin and/or gemtuzumab ozogamycin as a part of initial induction chemotherapy. Up to 3 cycles of clofarabine based treatment were administered (median number of cycles in patients with active AML was 1.5). Four patients intolerant of standard induction received clofarabine as consolidation (20 mg/m2 daily for 5 consecutive days). A majority of patients received G-CSF. The overall response rate (ORR) for the 18 patients with active AML was 61%, 9 patients (50%) achieving a complete response (CR). The CR rate in adverse cytogenetics group was 50%, in normal cytogenetics group 50% and 62% in abnormal other than complex. 38% of patients did not respond to therapy (50% in adverse cytogenetics group, 50% in normal cytogenetics group and 25% in abnormal other than complex). Induction and consolidation were well tolerated with no unexpected toxicities. Predictably, all patients developed grade 4 neutropenia but the median duration was only 20 days (17-120). Induction mortality was acceptable at 17%. The median survival since diagnosis for the 18 patients treated for active AML is approximately 11 months. *Discussion*. In our experience clofarabine was very well tolerated as a single agent, both in induction and even more so as consolidation therapy. Clofarabine, both as a single agent and in combination therapy, is active in unfavourable risk AML with an acceptable safety profile. Our data are comparable with that published to date and demonstrates the feasibility of this treatment approach in routine practice. Clofarabine should be considered as a potential therapeutic option in unfavourable risk AML patients unsuitable for more intensive therapy or in the relapsed/refractory setting.

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IMMUNOHISTOCHEMISTRY (CD123, CD56 AND CD4) REVEALS MINIMAL BONE MARROW INVOLVEMENT IN CASES OF BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM

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Background. Blastic plasmacytoid dendritic cell neoplasm has been included as a new disease entity in 2008 WHO classification of myeloid neoplasms. It is a highly aggressive and rare form of hematologic neoplasm. Almost all patients show cutaneaous manifestation at initial presentation and approximately 60-90% of patients have bone marrow involvement. Typically, tumor cells of blastic plasmacytoid dendritic cell neoplasm show CD4, CD56 and CD123 positive phenotype. *Aims*. It is difficult to diagnose minimal bone marrow involvement of blastic plasmacytoid dendritic cell neoplasm by morphologic examination only. In this situation, immunohistochemical stain can be helpful to confirm min-

imal bone marrow involvement. This study was purposed to demonstrate the usefulness of immunohistochemistry to reveal minimal bone marrow involvement of blastic plasmacytoid dendritic cell neoplasm. *Design and Methods*. 8 patients who were diagnosed as blastic plasmacytoid dendritic cell neoplasm from June 2000 to September 2008 were investigated. Except 2 patients who didn't receive bone marrow study, immunohistochemistry of CD4, CD56 and CD123 were done on biopsy or clot section to confirm minimal bone marrow involvement. *Results*. On initial morphologic diagnosis, bone marrow involvement was found in only 1 patient of 6 who received bone marrow biopsy. But Immunochemistry revealed minimal bone marrow involvement of 3 patients (CD123:3/3, CD56:2/3, CD4:2/3) whose bone marrow had been morphologically normal. *Conclusions*. Minimal bone marrow involvement of blastic plasmacytoid dendritic cell neoplasm can be precisely detected by immunohistochemistry. So we recommend CD4, CD56 and CD123 immunohistochemistry for the patients with blastic plasmacytoid dendritic cell neoplasm even though initial bone marrow study shows normal morphology.

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COMPARISON OF SECONADRY ACUTE MYELOID LEUKEMIA WITH *DE NOVO* AML: CLINICAL FEATURES AND TREATMENT OUTCOME

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Background. Secondary acute myeloid leukemia (AML) includes AML in patients who were exposed to chemo- and/or radiotherapy previously (t-AML) or AML evolving from myelodysplasia or myeloproliferative disorder (m-AML). Aims: We intended to investigate the clinical features at diagnosis and the therapeutic outcomes of t-AML and m-AML, comparing with those of *de novo* AML (d-AML). *Design and Methods*. Between June 1989 and July 2008, 886 consecutive patients with newly diagnosed AML in Asan Medical Center, Seoul, Korea were included in our retrospective analysis. Patients were classified into the three groups of d-AML (n=816, 92.1%), m-AML (n=46, 5.2%) and t-AML (n=24, 2.7%). The data of clinico-pathologic findings and clinical outcomes were retrieved from the Asan Medical Center Leukemia Registry. *Results*. Median number of peripheral blast count at diagnosis was lower in patients with m-AML ($1.41x10^3$/uL) and t-AML ($2.09x10^3$/uL) than patients with d-AML ($6.15x10^3$/uL) (p=0.01). Proportion of blasts in bone marrow nucleated cells was also significantly different among the three groups (66.4%, 43.6%, and 55.0% for d-AML, m-AML and t-AML respectively; p<0.001). More than 80% of patients in each group received induction chemotherapy. The complete remission (CR) rate after remission induction chemotherapy in patients with m-AML was significant lower than those with d-AML and t-AML [11/38 (28.9%) vs 560/720 (77.9%) and 13/20 (65.0%), respectively, p<0.001]. The 5-year actuarial overall survival (5-OS) rate in t-AML and m-AML patients was lower than d-AML patients (9.8% and 12.8% vs. 32.1%, respectively; p<0.001). But we observed no statistically significant difference in disease free survival (DFS) and event free survival (EFS) among the three groups. More patients with t-AML tended to die of non-relapse causes than those with m-AML after the first CR achievement [4/13 (30.7%) vs 4/38 (10.5%)]. HCT was performed for 184 (22.5%) of 816 patients with d-AML, 12 (26.1%) of 46 patients with m-AML, and 3 (12.5%) of 26 patients with t-AML. All of the 4 patients whose HCT was the initial therapy for m-AML did not relapse but 1 died of non-relapse cause, whereas 3 (37.5%) of the 8 patients whose HCT followed remission induction chemotherapy for m-AML, relapsed and died. For t-AML, three patients undertaken HCT in the first complete remission state followed by remission induction chemotherapy but 2 (66.6%) of 3 patients relapsed after transplantation, and all subsequently died. *Conclusions*. Our results suggest that prompt HCT may be more preferable therapeutic option particularly for patients with m-AML. To improve the overall outcome of patients with t-AML, novel therapeutic strategies are needed.

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THE ADDITION OF BORTEZOMIB TO INDUCTION THERAPY OF REFRACTORY ADULTORY AML, PRELIMINARY RESULTS

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Background. Proteasome inhibition, the block of angiogenesis, modification of the NF-κ-B system seems to be a important target in refractory acute myeloid leukemia (AML). *in vitro* data clearly support, that bortezomib possesses antiproliferative and pro-apoptotic effects in different AML cell-lines, moreover bortezomib was able to improve anthracyclin and possibly ARA-C sensitivity in different AML cell-lines . More recently, a Phase I trial showed bortezomib monotherapy efficient (only in few percents) in childhood refractory acute leukemia. AIms We have tried bortezomib containing first or second line combinations in 35 (18 female, 17 male, mean age 59.76 years) patients with refractory or poor risk AML, in a retrospective fashion, analysing responses and safety.. *Design and Methods*. The combinations were as follows: HAM or Flag-Ida, combined with bortezomib 1,3 mg pro sqm, day O and seven). The following groups were considered as refractory or poor risk AML: 1.*De novo* AML, 2nd line: Lack of remission to first line standard treatment ("3+7"), n=4 (Velcade- Flag-Ida treatment) 2. *De novo* AML 1st line: bilineal or biphenotypic (flow-cytometry) n=3 (Velcade-Flag-Ida treatment) 3.De novo AML with complex karyotype or with flt-3 mutation, n=13, 1st line (Velcade-Flag-Ida n=10, Velcade-HAM protocol, n=3) 4.Secondary AML or AML with evidence of previous MDS, n=15, 1st line: (Velcade-Flag-Ida n=10, Velcade-HAM n=5) Results Complete remission (CR) 16/35, partial remission (PR) 10/35, no remission 8/35, progression during treatment: 1/35. CR had been achieved in all patients of group, and group 2 (biphenotypic, bilineal). The CR rate was quite appreciable in group 3, i.e. 9/13 (complex karyotype or normal karyotype with FLt-3 mutation) - the response rate was excellent with flt-3 mutated cases). In group 4. (MDS, secondary AML) the results were less impressive. There were no differences according to protocol (Flag-Ida or HAM). Allogeneous stem cell transplantation could have been performed in 1st CR in two patients (one from group 1. and another from group 2.) One of them died due to relapse, the other one is in CR since then. The combinations seem to be relatively safe. Induction related death rate was low (1 MDS/AML patient, thrombocytopenic bleed). 9 other patients had severe neutropenic sepsis (2 with fatal outcome). Pulmonary syndrome, which may follow Velcade+ARA-C had not been documented. Other adverse events did not differ from the pattern observed with standard induction therapies. The addition of bortezomib to induction therpay might imrpove responsiveness in different cohorts of refractory AML, without significant additional risk.

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THERAPEUTIC REGIMENS INCLUDING CLOFARABINE IN ADULT PATIENTS WITH RELAPSE OR REFRACTORY ACUTE LEUKEMIA

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Background. Clofarabine is a new generation purine nucleoside antimetabolite effective in the treatment of Relapse or Refractory Acute Leukemias either in children and in adults. *Aims*. The aim of our study was to demonstrate the effectiveness and the tolerability of a treatment regimen including Clofarabine or Clofarabine plus Cytarabine in adults with AML and ALL refractory to standard treatment. *Design and Methods*. In 2008 at the Department of Hematology of the University of Pise (Italy) we treated 11 adults with refractory leukemias with chemotherapy regimens Clofarabine based. Patients' diagnosis were as follows: 7 with AML and 4 with ALL. Of them, 4 patients were treated with Clofarabine as second-line therapy, 6 patients as third-line therapy and 1 patient as fourth-line therapy. Clofarabine based regimens were as follows: -Cycle A - 7 patients (4 AML, 3 ALL): Clofarabine 20 mg/mg days 1-5; -Cycle B - 3 patients (AML): Clofarabine 40 mg/mq days 1-5, Cytarabine 1 gr/mq days 1-5. Cycle A and B were repeated every 3 to 5 weeks based on response to the previous one (CR, PR or non-response) and were started when hematopoiesis was fully recovered. Patients were allowed to receive a maximum of 2 cycles of induction therapy or until a CR, CR with incomplete platelet recovery (CRp) or partial response (PR) was achieved. A CR required normalization of the marrow (5% blasts) and peripheral counts with no circulating blast cells, a neutrophil count ≥$1x10^9$/L and platelet counts ≥$100x10^9$/L. A PR consisted of a blood count recovery as for CR, but with persistence of 5% to 25% marrow blasts. A CRp had criteria similar to a CR, but without recovery of platelets ≥$100x10^9$/L. 6 patients received just one cycle of therapy and 5 patients received two cycles. During the treatment, transient liver dysfunctions were common in most patients. Myelotoxicity was grade III-IV in all patients; neurotoxicity never occurred. Mucosytis grade III-IV occured in 3 patients and also bacterial sepsis in 3 patients (colonized before the treatment). *Results*. Our results have shown an overall response rate (CR+PR) to Clofarabine higher than 70%: 8 out of

11 patients responded to treatment, one patient was a non-responder and 2 patients died of sepsis before any possible marrow evaluation. Out of 8 responding patients, 3 obtained a CR and 5 a PR. Of the 8 responders, a fully ablative allogenic transplant was performed in 2 CR patients and in 5 PR patients. One is still in CR 6 months far from treatment. *Conclusions*. Our preliminary results show that Clofarabine is a very effective and well tolerated drug for adult patients with Refractory Acute Leukemia and allowed us to obtain good response in high risk patients. Furthermore Clofarabine based regimens provided to obtain response in chemorefractory patients allowing thus to perform subsequent fully ablative allogenic transplant without additional toxicity.

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HIGH DOSE CYTARABINE, CLOFARABINE AND GEMTUZUMAB OZOGAMICIN (CLAC-MYL) IN RELAPSED OR REFRACTORY AML PATIENTS

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Background. Clofarabine has been shown to be effective in AML patients, both as single agent and mainly in association with high dose cytarabine. *Aims*. On the basis of these reports, we conducted a preliminary study combining clofarabine, high dose cytarabine and gemtuzumab ozogamicin (Mylotarg) in AML patients who relapsed or failed to respond to at least two induction therapies. *Design and Methods*: we treated 10 patients affected by relapsed/refractory AML with a regimen including clofarabine at 22,5 mg/m^2 daily on days 1-5, followed after three hours by cytarabine at 1 gr/m^2 daily on days 1-5, with the addition of gemtuzumab ozogamicin 6 mg/m2 on day 6 (CLAC-Myl). *Results*. Among the ten patients, four were in first relapse, three in second relapse, three with resistant disease. The mean age was 53.2 years (range 33-68 years), the white blood count at the accrual was 31.500 mcc (range 2140-153.000). 5/10 patients achieved a complete remission, 1/10 obtained a partial response with less than 10% bone marrow blasts, 3/10 had resistant disease and died of their AML, 1/10 died of complications during the aplastic phase (multiorgan failure in a woman 62 years old, at the third relapse after allogeneic bone marrow tranplantation, diagnosed with liver GVHD before starting the treatment). The most frequent non haematologic adverse events were vomiting (4/10), diarrhea (5/10), transient liver toxicity (2/10 grade 3-4), infections microbiologically documented (7/10), febrile neutropenia (3/10). Comparing with other salvage strategies, in this small cohort of patients we did not observe a significant delay in bone marrow recovery (median time to ANC recovery 25 days), and the addition of gemtuzumab seemed not to increase the haepatic toxicity. Interestingly, the three non-responding patients were all primary resistant AML Among the six responding patients, 4 received a further consolidation cycle with clofarabine at 22,5 mg/m2 and cytarabine at 1 gr/m^2 day 1-4, and underwent allogeneic bone marrow transplantation, one relapsed after 6 months, and one not eligible for transplant procedures is still in complete remission with a follow up of 6 months. *Conclusions*. Our very preliminary results suggest that the CLAC-Myl regimen is effective in this particularly poor prognosis category of patients, with safety data consistent with previously reported salvage therapies. Further studies are warranted.

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GEMTUZUMAB OZOGAMICIN, FLUDARABINE, ARA-C AND CYCLOSPORINEA (MFAC) REGIMEN AS INDUCTION THERAPY IN RELAPSED OR REFRACTORY CD33+ ACUTE MYELOID LEUKEMIA PRIOR TO ALLOGENEIC HEMATOPOETIC STEM CELL TRANSPLANTATION: A SINGLE-CENTER EXPERIENCE

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Background. Gemutzumab ozogamicin (GO) has been used as a single agent, as well as in combination with conventional chemotherapy in CD33+ acute myeloid leukemia (AML). *Design and methods*. We report 26 patients with CD33+ primary refractory or relapsed AML receiving the MFAC regimen (GO, fludarabine, ara-C and ciclosporin) directly followed in aplasia by reduced intensity conditioning (RIC) for allogeneic hematopoetic stem cell transplantation (HSCT). *Results*. Early treatment assessment revealed an overall response (OR) of 65.4%. Grade III/IV hepatic toxicity after MFAC occured in 30.8% of the patients. The median time to allograft was 22.5 days. 77% of all patients proceeded to allogeneic HSCT. Grade III/IV hepatic toxicity after allografting was observed in 25%, including one patient with veno-occlusive disease (VOD). When summarizing the sequential regimens, CTC grade III/IV hepatic toxicity (including hyperbilirubinemia and transaminitis) was seen in 16 patients (=61.5%). Median follow up was 8.2 months (range 0.3 - 39.7 months). Estimated twelve-month overall survival (OS) was 52.4%. discussion MFAC directly followed by RIC for allogeneic HSCT seems to be active and feasible, but with an increase in hepatic toxicity.

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A FLOW CYTOMETRIC IMMUNOBEAD ASSAY FOR THE DETECTION OF PML-RARA FUSION ONCOPROTEIN IN ACUTE MYELOID LEUKEMIA

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Acute promyelocytic leukemia (APL or AML-M3), a subtype of acute myeloid leukemia, is one of the most lethal forms of acute myeloid leukemia when not diagnosed and treated expeditiously. Genetically, APL is characterized by a translocation between chromosomes 15 and 17, (t(15;17)(q22;q21), which fuses the retinoic acid receptor α (RARA) gene on chromosome 17 with the PML gene on chromosome 15. Depending on the position of the PML breakpoint, a long (bcr1) or a short transcript isoform (bcr3) is produced. The PML-RARα fusion protein arrests the maturation of myeloid cells at the promyelocytic stage, which leads to an increased proliferation of promyelocytes. Most APL patients are treated with all-trans-retinoic acid (ATRA) in combination with chemotherapy, to avoid side effects of ATRA and to deplete the leukemic cells. ATRA activates the retinoid receptor RAR (present as wild type RARα and as part of the fusion protein) which causes the promyelocytes to differentiate and mature, which deters them from proliferation. Using this therapy. a complete remission can be obtained in most APL patients. At present, diagnosis of APL is based on the detection of the t(15;17) translocation by karyotyping, FISH or PCR. However, these techniques are laborious and demand a specialized laboratory. To circumvent these restrictions and to decrease the time for detecting the fusion protein, a novel research-based flow cytometric bead assay, using the BD' Cytometric Bead Array (BD Biosciences), was developed using a bead-bound anti-PML antibody and a PE-conjugated anti-RAR_ detection antibody. This research-based immunobead assay detects the presence of the PML-RAR_ fusion protein within 4 to5 hours in cell lysates of blood or bone marrow. The antibody-binding sites on the fusion protein were carefully selected to recognize all variants of the PML-RARα fusion protein. The assay is specific and sensitive, detecting less than 10% of the APL cell line NB4 (bcr1) in a background of normal PBMCs and WBCs. Additionally, the immunobead assay had 100% concordance (16/16) with PCR for detection of the fusion protein in individuals newly diagnosed with AML-M3 (either bcr1 or bcr3). We conclude that this novel immunobead assay can be used for fast and easy detection of PML-RAR_ fusion protein in AML-M3 patients. The assay is sensitive, independent of the PML breakpoint region, fast (completed within 4 to 5 hours) and can easily be performed in a standard laboratory using routine flow cytometry. Future studies are needed to demonstrate the full clinical utility of this assay.

1404

AN 'INTENTION-TO-TREAT' FACTOR IN 299 AML PATIENTS - SINGLE CENTER EXPERIENCE

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Background. Initial intention of acute myeloid leukemia patients' treatment is not always kept during the course of therapy. There are many factors influencing the clinicians' decision-making about changes of intended treatment. Neither these changes nor their effect on patients' survival have been analyzed generally. *Aims*. The aim of analysis was to compare the initially intended option of consolidation treatment (based on prognostic factors, performance status, comorbidity, CR achievement etc) with subsequently realized one and explore the effect of this decision and its parity or disparity with realized treatment on treatment outcomes. *Design and Methods*. Data of 299 consequently curatively treated AML patients (1996-2008; APL were excluded) in Olomouc were ana-

lyzed; M:F = 45.5% : 54.5%; 50.5% of pts were younger (<55); cytogenetic risk distribution : good prognosis 10.7%, intermediate 48.5%, poor 31.4% (9.4% NA). 55.9% of pts were intended to be consolidated with chemotherapy, 10% of pts with autologous transplantation and 34.1% of pts with allogeneic transplantation. *Conclusions.* There was significantly higher proportion (87.9%) of younger pts in groups intended to be transplanted when compared with patients intended to be consolidated with chemotherapy (21%; $p<0.001$). We found significant difference in distribution of cytogenetic risk among groups of pts according to intention-to-treat. An intended treatment has been kept in comparable proportion of pts (48-56%) of all groups. We found the highest proportion of untreated (without consolidation) pts in the group intended to be consolidated with chemotherapy and the lowest one in the group intended to be transplanted with autologous stem cells. Both the highest incidence of changes in treatment course and the best survival were found in the group of pts intended to be treated with autologous transplantation. However, there was the highest proportion of pts with good cytogenetic prognosis and younger pts in this group, too. The treatment outcome and survival were worse in pts intended to be treated both with chemotherapy and allogeneic transplantation. The pts both intended to and treated with autologous transplantation did have comparable survival with pts intended to be treated with autologous transplantation and treated with another treatment option subsequently. The pts both intended to and treated with allogeneic transplantation did have better survival when compared with pts intended to be treated with allogeneic transplantation and consolidated with another treatment option finally. The pts both intended to and treated with (any) transplantation did have better survival when compared with pts intended to be transplanted and consolidated with chemotherapy. The worst outcome we found in pts intended to be treated with allogeneic transplantation and consolidated with chemotherapy only.

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1405

A SIMPLE MOLECULAR PROFILE AT DIAGNOSIS MAY PREDICT THE PROBABILITY OF ACHIEVING COMPLETE REMISSION IN NON-M3 AML PATIENTS: A PRELIMINARY ANALYSIS ON 85 PATIENTS.

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Background. FLT3 mutations and expression of NPM, WT1, BAALC and other genes may exert a relevant role in AML . *Design and Methods*: A molecular profile was performed in 85 untreated non M3 AML patients receiving induction chemotherapy with the aim of predicting CR rate and long term outcome. Expression levels were obtained by Real-Time-PCR upon normalization on Abl expression. *Patients*. Median age was 60 years (17-80). Sixty-one patients (72%) had *de novo* AML, 24 (28%) had a secondary disease. The karyotype was favourable (FK) in 2 patients , intermediate (IK) in 64, including 59 patients with normal karyotype (NK), unfavourable (UK) in 17. In 2 patients could not be evaluated. FLT3 (85 patients): 12 (14%) had ITD and 6 (7%) had exon 17 mutations. WT1 (83 patients): in 10 (12%) expression was < 100, in 10 (12%) between 100 and 500, in 17 (20%) between 500 and 1000, in 46 (56%) >1000. NPMA (81 patients): in 56 (69%) expression was ≤1000 (66% with NK, 4% with FK, 25% with UK); in 9 (11%) between 1000 and 10 000 (78% with NK, 22% with UK); in 16 (20%) was > 10000 (94% with NK, 6% with UK). NPM B (81 patients): in 66 (81%) expression was ≥ 1000 (67% with NK, 3% with FK, 30% with UK); in 8 (10%) between 1000 and 10 000 (88% with NK, 12% with UK); in 7 (9%) was >10000 (100% with NK). BAALC (84 patients): in 34 (41%) expression was <1000, in 28 (33%) between 1000 and 10 000, in 22 (26%) >10 000. *Results*. 11/12 patients with FLT3 ITD achieved CR (91%) relapse (58%). Four out 6 with FLT3 mutations in exon 17 reached CR (67%) relapse (25%). Level of WT1 expression did not correlate with CR rate. 31/56 (55%) with NPM A 1000 achieved CR (15 relapsed); 5/9 (55%) with NPM A between 1000 and 10 000 reached CR (2 relapsed); 13/16 (81%) with NPM A > 10 000 reached CR (4 relapsed). 34/66 (51%) with NPM B 1000 achieved CR (15 relapsed); 8/8 (100%) with NPM B between 1000 and 10 000 reached CR (5 relapsed); 7/7 with NPM B > 10 000 reached CR (2 relapsed). 29/34 (85%) with BAALC < 1000 achieved CR (28 had NK; 11/29 relapsed); 11/28 (39%) with BAALC between 1000 and 10 000 achieved CR (11 relapsed); 11/22 with BAALC > 10 000 reached CR (5 relapsed). The highest CR rates have been reported in patients with BAALC <1000, NPM A >10 000 (13/14, with 4 relapses); BAALC <1000, NPM A >10 000 , without FLT3 mutations (11/11, with 1 relapse); BAALC <1000, NPM B >10 000 (6/6, with 1 relapse). *Conclusions.* A simple molecular analysis at diagnosis based on BAALC and NPM expression may predict the probability of achieving CR. A longer observation is needed to evaluate whether this will be associated with improved outcome also.

1406

FLT3 TYROSINE KINASE DOMAIN (TKD) MUTATIONS IN PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML)

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Background. Although the point mutations involving TKD of the FLT3 gene are less frequent than internal tandem duplications (FLT3/ITD), they still occur in about 10% of AML cases. These changes most commonly affect codons Asp835 and Ile836 and their prognostic impact is not as yet fully clarified. Aims: To study the frequency of FLT3/TKD mutations and their prognostic significance in our patients with AML. *Design and methods*. A cohort of 298 newly diagnosed adult AML cases was studied. The median patients' age was 54.0 years (range: 18.3-81.7), the male/female ratio was 148/150. The initial median WBC count was $18.2x10^9$/L (range 0.4-488.0). The median follow-up was 8.3 months (range 0-153.7). To detect mutations in the TKD of the FLT3 gene, exon 20 was amplified by RT-PCR. The resulting products were digested with EcoR V and separated on 3% agarose gel. Undigested bands were directly sequenced in order to specify the mutation. *Results*. Out of 298 AML patients, 30 (10.1%) harboured FLT3/TKD mutation. It was found in 10/56 (17.9%), 11/129 (8.5%) and 5/66 (7.6%) patients with a favourable, intermediate and poor-risk cytogenetic profile, respectively. The prevalence of FLT3/TKD mutations decreased with the age of patients. In the group of patients<30 years, 7/41 (17.1%) had the mutation, among patients between 30 and 60 years 15/161 (9.3%) were mutated and in the group over 60 years only 8/95 (8.4%) harboured the mutation. The most frequent was the Asp835Tyr change, which was found in 15 (50%) patients, followed by Asp835Val and Asp835Glu substitution (4 patients each), Asp835His and del836Ile (3 patients each). One patient carried del(835-837)/ins mutation. According to the FAB classification, FLT3/TKD mutations were most frequently present in subtypes M4 (9/49 patients), M5 (3/17) and M3 (6/37). On the other hand, none of 12 patients with M0 subtype harboured the mutation. Patients with FLT3/TKD mutation had a significantly higher initial WBC count when all patients were analyzed together (p=0.002), as well as when APL and FLT3/ITD-positive patients were excluded (p=0.005). Patients with Asp835Tyr substitution had higher initial WBC count when compared to patients carrying any other FLT3/TKD mutation (p=0.047). Out of the 268 patients without FLT3/TKD mutation, 147 (56.3%) reached complete remission (CR), while among 30 mutated cases, 19 (63.3%) achieved CR (p=0.231). When 186 patients without APL or FLT3/ITD were evaluated separately, 91/163 (55.8%) unmutated reached CR and 16/23 (69.6%) mutated cases reached CR (p=0.106). The incidence of relapse was slightly higher in FLT3/TKD mutated patients (11/19 cases relapsed; 57.9%) compared to non-mutated ones (61/137; 44.5%; p=0.137). This trend was confirmed when APL and FLT3/ITD-positive patients were excluded from analyses (11/16; 68.8% mutated cases relapsed vs. 43/84; 51.2% unmutated ones relapsed; p=0.098). The overall survival (OS) was not influenced by the presence of the FLT3/TKD mutation either when all patients were analyzed together (p=0.618) or when APL and FLT3/ITD-positive patients were excluded (p=0.699). *Conclusions*. We have confirmed the prevalence of FLT3/TKD mutations in about 10% of AML patients. In our group of patients, these mutations seemed to cause higher initial WBC counts. Insignificant tendencies towards higher CR rates and to more frequent relapses were noted for the mutated patients, so that the resulting OS was unaffected by FLT3/TKD mutations.

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1407

METRONOMIC APPROACH FOR TREATMENT OF ACUTE MYELOID LEUKEMIA (AML): DOSE INTENSITY DOES NOT ALWAYS MATTER!

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Background. Recent developments in AML therapeutics are remarkable, however their applicablility in economically less-resourced countries is limited as many patients cannot bear the cost of conventional chemotherapy and supportive care and die untreated. For them we need to develop novel therapeutic approaches which can control leukemia, require less supportive care and are affordable. In one such attempt we have treated patients with acute myeloid leukemia who were financially challenged and/or had underlying organ infections making them unsuitable for standard chemotherapy with oral metronomic therapy based on Etoposide, 6-thioguanine and Prednisolone. *Aim.* To test the efficacy of metronomic chemotherapy in patients with *de novo* acute myeloid leukemia who were not eligible for standard chemotherapy. *Methods*, In this retrospective study, consecutive patients diagnosed to have adult de-novo acute myeloid leukemia between january- november 2008 at a tertiary care center were analyzed. We enrolled patients who were not eligible for standard chemotherapy due to financial constraints or documented organ infections and were willing for oral metronomic therapy. Treatment included 28 days cycle of chemotherapy with once daily administration of tab. 6-thioguanine 40 mg, tab Prednisolone 40 mg/m^2 and cap Etoposide 50 mg/m^2 for 21 days on outpatient basis. Supportive care with antibiotics, antifungal, packed red blood cell and platelet transfusion was given as indicated. Response assessment was done at the end of first cycle. Those patients who responded were given either post remission therapy with high dose ara-c or continued on oral metronomic therapy till finances were arranged for post remission treatment. *Results*. Ten patients (male: female 1:1) with median age of 35 years (range 16-57 years) with confirmed diagnosis of AML were analyzed. Four patients had probable fungal pneumonia with respiratory insufficiency at presentation. Median time between onset of symptoms and diagnosis was 2 months (range 1-5 months). Median presenting total leucocyte count was 18,950/cumm (range 1250-1,85,000/cumm). Favorable cytogenetics i.e. t(8;21) and inv(16)was found in 2 patients. Remaining patients had normal cytogenetics. Median number of oral chemotherapy cycles were 1 (range 1-6). At the end of first cycle, 8/10 patients were in complete morphological remission. Among patients who did not achieve hematological remission one patient could be started on conventional chemotherapy as her fungal pneumonia improved on oral metronomic therapy. At median follow up of 6 months (range 2-9 months) all patients continue to be in remission. The remarkable feature was that 6/10 patients received treatment as outpatient and only who required antifungals were admitted. The need for packed cell transfusion and platelets was minimal - median 1 (range 0- 2) and median 2 (1-5) respectively. This is quite lower than what is required with standard induction therapy. *Conclusions*. Metronomic therapy for treatment of de-novo adult AML seems to be effective and required much less supportive care in this small retrospective analysis form a developing country. Patients who have underlying infections at presentation, which limit the application of full dose induction chemotherapy or who have financial constraints can be considered as potential candidates for this treatment.

1408

THREE CASES OF THERAPY-RELATED ACUTE MYELOID LEUKAEMIA IN PATIENTS WITH MULTIPLE SCLEROSIS: THE ROLE OF ARSENIC TRIOXIDE IN THE TREATMENT OF SECONDARY APL

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Multiple sclerosis (MS) is an autoimmune disease characterised by a relapsing-remitting course caused by immune activity directed against central nervous system antigens. Treatment of MS include the use of immunosuppressive chemotherapy with Mitoxantrone (Mtz), a DNA-topoisomerase II inhibitor that induces DNA cleavage and may lead to chromosome aberrations. Therapy-related acute myeloid leukaemia (t-AML) is a well-recognized complication in cancer or in autoimmune disease treated with agents targeting topoisomerase II . We report three cases observed in our Institution of acute myeloid leukaemia (2 APLs; 1 M2-AML) developed in patients affected by primary progressive MS (PPMS) following treatment with Mtz. The first patient, a 40-year-old-man affected by PPMS since 1995, was unsuccessfully treated with β interferon from January 2001 to December 2004. In January 2005, he started Mtz therapy at dose of 16 mg (8 mg/m^2) every 3 months until June 2007 (total dose 160mg). In that period blood tests showed Hb 10.1g/dl, WBC 1.4x10^9/L and PLT 89x10^9/L and morphology, molecular and cytogenetic analysis of bone marrow were compatible with a diagnosis of a typical APL (FAB M3). The second patient, a 48-year-old-female with PPMS since 2004, after unsuccessful treatment with β Interferon (January 2005 to November 2005), was treated with Mtz 20mg every 3 months (from December 2005 until June 2008; total dose 200mg). While she was receiving the last course of Mtz, blood tests showed pancytopenia; the bone marrow examination, molecular analyses and imunophenotyping were consistent with a diagnosis of APL. Both patients were treated according to AIDA protocol in induction (all-trans retinoic acid plus idarubicin) and achieved hematologic CR (HCR). Considering the cumulative anthracycline dose (Mtz for MS and idarubicin for APL induction), further chemotherapy was discarded and both patients were given consolidation with ATRA plus Arsenic Trioxide (ATO) for a total of 16 cycles according to protocol reported by Estey et al. (Blood 2006). Either patients achieved molecular remission (MR) after 1 cycle of ATRA plus ATO and are presently in MR after eight and one months from achievement of HCR, respectively. The third patient (a 50-year-old-male) was affected by PPMS since 1997. In October 2005 he started treatment with Mtz 18mg every three months. (toal dose 216mg). Four months after completion of Mtz therapy, blood and bone marrow morphological and immunological analyses disclosed 50% of blast cells compatible with a diagnosis of AML-M2. Chromosome analysis, demonstrated 46;XY with (3) and (21) deletion. Molecular studies showed a t(16;21) involving AML1 and resulting in a AML1-ETO2 gene fusion. Patient was treated with two cycles of induction chemotherapy according to the FLAG protocol, achieving complete remission. Consolidation therapy consisted of 2 courses of high dose of Ara-C). He is presently in HCR eight months after the end of consolidation therapy. After consolidation therapy the patient maintained HCR The occurrence of therapy-related acute myeloid leukemia after exposure to DNA-topoisomerase II inhibitors such as Mtz is well known; however, the emerging problem of sAML cases after MS raises important issues for optimal treatment of both MS and sAML. The therapeutics subcommittee of the American Academy of Neurology has recently recommended the use of Mtz only for patient affected by PPMS. As to sAPL, ATO plus ATRA seems a valid option to consolidate HCR and could also be considered front line treatment as an alternative to ATRA plus chemotherapy.

1409

INTENSIVE TREATMENT OF ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA OR MYELODYSPLASTIC SYNDROME: A SINGLE CENTER EXPERIENCE

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Background. Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) peak incidence is in the seventh decade of life. The outcome of elderly patients (pts) with AML or advanced MDS is dismal because of the unfavourable characteristics of the disease and the frequent co-morbidities. Intensive chemotherapeutic programs, with or without haematopoietic stem cells transplantation (HSCT), are not usually offered to pts older than 60-65 as most of them are considered too frail to tolerate the side effects of such treatments; alternatively, they receive palliative and supportive care, or experimental targeted molecules. We here review data from elderly pts with AML or MDS, treated at our Center with an intensive approach. *Aim*: retrospective evaluation of feasibility and efficacy of different intensive treatments administered to AML/MDS elderly pts, at our Center. *Design and Methods*. period March 1999-Jenuary 2009, 52 pts, median age 69 (range 65-78), PS 0-2, renal, hepatic, cardiac and pulmonary function were within normal limits. Diagnosis (WHO): RAEB1 2, RAEB2 9, MDS/MPD 1, AML MD 23, AML 17. Cytogenetic risk (47 pts): high 7, intermediate 38, low 2. All pts received at least one cytarabine-containing induction cycle. Pts in CR were addressed to post-remission treatments according to their age, clinical condition, prognosis and donor availability. *Results*. CR rate was 63.4% (33 pts) after 1 or 2 cycles without significative difference comparing subgroups of pts according to diagnosis and cytogenetics. Induction mortality was 13.4% (7 pts). Post-remission treatment: 15 pts received ≥2 cycles of standard or high-dose chemotherapy, 18 pts

(54.5%) received an HSCT. HSCT (20 pts, 2 with residual disease after induction): 15 autologous (AUTO), 1 allogeneic from a sibling donor (SIB), 2 from an haploidentical familiar donor (HAPLO). Relapses: 19 pts (57.5%), 18 received salvage treatments, 10 (55.5%) obtained a second CR. At last up-date 18 pts were alive (34.6%), 13 in CR, with a median follow-up of 696 days (range 86-2677). Median survival of all pts from start of treatment was 408 days (range 20-2657). *Conclusions*. Different intensive approaches proved feasible and effective in our elderly pts with AML or poor prognosis MDS. A significative rate of long term survivors free from disease was documented. Our results suggest that transplantation can be a therapeutic option for selected elderly pts. In our opinion, palliative and supportive care should be offered only to pts with serious co-morbidities; alternatively, these pts could be enrolled in experimental trials with new targeted molecules.

1410

FLOW-CYTOMETRIC IMMUNOBEAD ASSAY FOR THE DETECTION OF PML-RARA FUSION PROTEINS IN ACUTE MYELOID LEUKEMIA

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Acute promyelocytic leukemia (APL or AML-M3) is one of the most lethal forms of acute myeloid leukemia when not diagnosed and treated in time. Genetically APL is characterized by a translocation between chromosomes 15 and 17, t(15;17)(q22;q21), which fuses the retinoic acid receptor α (RARA) gene on chromosome 17 with the PML gene on chromosome 15. Depending on the position of PML breakpoint, a short (bcr1) or a long transcript isoform (bcr3) is produced. The PML-RARα fusion protein arrests the maturation of myeloid cells at the promyelocytic stage which leads to the increased proliferation of promyelocytes. Most APL patients are now treated with all-trans-retinoic acid (ATRA) in combination with chemotherapy, to avoid site effects of ATRA and to deplete the leukemic cells. ATRA activates the retinoid receptor RAR (present as wild type RARα and as part of the fusion protein) and causes the promyeloctes to differentiate and mature which deters them from proliferation. Using this therapy, a complete remission can be obtained in most APL patients, of which few relapse. Since treatment of APL is specifically aimed at the fusion protein, detection of t(15;17) or the presence of PML-RARα is necessary to predict treatment response. At present diagnose of APL is based on the detection of t(15;17) by karyotyping, FISH or PCR. However, these techniques take relatively long and demand a specialized laboratory. To prevent the need of specialized laboratories and to decrease the time for diagnosing APL, a novel flow cytometric bead assay (CBA) was developed using a bead-bound anti-PML antibody and a PE-conjugated anti-RARα detection antibody. This CBA detects and quantifies the presence of the PML-RARα fusion protein in cell lysates of blood or bone marrow samples of APL patients within 4-5 hours. The antibody binding sites on the fusion protein were carefully selected in order to recognize all variants of the PML-RARα fusion protein, independent of the breakpoint position. The assay is specific and sensitive and detects less than 10% of the APL cell line NB4 (bcr1) in a background of normal PBMCs and WBC's. Moreover, lysates of PBMC's of AML-M3 patients (either bcr1 or bcr3), previously diagnosed by PCR techniques, generated robust and specific signals in the assay. We conclude that this novel immunobead assay can be used for fast and easy diagnosis of AML-M3 patients which makes it possible to include these patients at an early stage in the right treatment protocols, much faster than by use of current techniques. The CBA is sensitive, independent on the PML breakpoint region and fast (completed within 4-5 hours) and can easily be performed in a standard diagnostic laboratory using routine flow cytometry. Furthermore, as this assay involves PML-RARA protein rather than RNA or DNA, it will measure the presence of cells that are sensitive to ATRA therapy. Futher clinical studies will reveal whether the new CBA is sufficiently sensitive for monitoring of minimal residual disease and detection of early relapse.

1411

THE PERCENTAGE OF MYELOPEROXIDASE-POSITIVE BLAST CELLS IS A GOOD PROGNOSTIC FACTOR IN ACUTE MYELOID LEUKEMIA (AML) IN ELDERLY PATIENTS

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Background. The percentage of myeloperoxidase-positive blast cells (MPO+) has considered to be important for the diagnosis of AML while some recent studies have shown the prognostic significance of MPO-positivity in AML (T. Matsuo et al, Leukemia 2003;17:1539-1543). Aims: to examine whether the percentage of MPO+ blast cells is a useful prognostic factor for AML patients. *Design and Methods*. 61 elderly patients suffering from AML, were treated in our Clinic with MAC protocol (combination of mitoxantrone and cytarabine) from January 2002 to March 2008. Our population consisted of 33 patients with de novo and 28 patients with secondary AML, classified according to FAB as follows: M0 (4), M1 (11), M2 (24), M4 (7), M5 (7), M6 (5), M7(1), hybrid-leukemia (2). Their mean age was 70,48 (±4,33) yrs and M/F ratio was 38/23. Cytogenetic analysis was performed in 57/61 cases: 6 failed to produce metaphases, 40/57 revealed intermediate risk abnormalities (thirty with normal karyotype, five with trisomy 8, five with other abnormalities) and 11/57 cases had adverse risk abnormalities. MPO positivity was assessed with flow cytometry analysis on blast cells at diagnosis, and it was confirmed with cytochemistry. Patients were categorized into four groups according to MPO-positive percentage among the leukemic blast cells: Group A: <25%, Group ': 25-49%, Group C: 50-74%, Group D: 75-100%. The characteristics of the four groups (number of patients, existence of secondary leukemia, cytogenetics (Adverse Risk/AR, Intermediate Risk/IR, Without Metaphases or not done/WM) and the status of the disease after therapy (Complete Remission/CR, Refractory disease/RD) are shown in Table 1.

Table 1. Characteristics of patients.

GROUP	n	IR	AR	WM	sAML	CR	RD
A	17	8/17	5/17	4/17	5/17	9/17	8/17
B	10	5/10	1/10	4/10	4/10	3/10	7/10
C	7	5/7	2/7	0/7	3/7	5/7	2/7
D	27	22/27	3/27	2/27	16/27	14/27	13/27

Statistical analysis was done with Kaplan-Meier (log Rank test) and multivariable interpretation Cox. *Results*. 1. Patients with low MPO survive much less than the patients with high MPO: Overall Survival in Groups and ' was 7,77 and 8,27 months vs 36,10 and 20,79 months in Groups C and D respectively (p value <0.05). The duration of disease-free survival (DFS) is much greater in patients with high MPO than patients with low MPO: DFS in Groups C and D was 31,87-20,75 months vs 5,32 and 5,0 months in Groups A and B respectively (p value <0.05). The above findings prove bad prognosis in patients with low percentages of MPO+ blasts in myelogram at the time of diagnosis and indicate the importance of the MPO assessment at the time of the diagnosis in elderly patients with AML.

1412

HYDROXYUREA + VINDESINE IN THE TREATMENT OF ELDERLY AML PATIENTS UNFIT FOR INTENSIVE CHEMOTHERAPY

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Background and aims. In order to improve the dismal results obtained in elderly patients with Acute Myelogenous Leukemia (AML) not eligible for Intensive Chemotherapy (IC), we tested the addition of Vindesine (VND) to standard palliative chemotherapy with Hydroxyurea (HU). *Design and methods*. From 12/2001 to 12/2007, 38 elderly patients [M/F 24/14, median age 77.6 yrs, interquarile range (IR) 74.0-81.6] with newly diagnosed AML and not eligible for standard IC were enrolled into the study. Twelve patients had a previously documented myelodysplastic phase, 4 patients had a PS (WHO) > 2 and 5 patients had a documented infection at onset. Median Hb, WBC, PLTS and marrow blasts were 9.0 g/dl, 15.1x10^9/L, 63x109/l and 54%, respectively. Clinical criteria for exclusion from IC were age >75 yrs (21 patients), cardiological disease (7 patients), PS >2 (4 patients), renal disease (3 patients) or other organ failures (3 patients). The primary endpoint was to achieve a phase of stable disease (WBC <10x10^9/l for > 2 months during treatment). After diagnosis, patients were observed weekly and the association of HU (initial dose 1500 mg/day) and VND (5 mg iv every 15 days) was started in the presence of WBC >10x10^9/l or a doubling time shorter than a week. Results Median time from diagnosis to treatment was 13 days (IR 3 - 49). Three patients (7.9%) achieved a morphological and/or cytogenetic Complete Remission (CR), 2 (5.3%) an haematological improvement (HI), 12 (31.5%) a stable disease and 21 (55.3%) showed a disease progression. As to the 3 patients in CR, 1 with an abnormal karyotype (47 XY,+13) at diagnosis, achieved both morphologic and cytogenetic CR that lasted 14 months; the other 2 patients with normal karyotype at diagnosis achieved CR lasting 4 and 16 months, respectively. Documented infections (14 broncopneumonic episodes, 4 abscesses and 1 bacterial sepsis) were the most common complications during treatment. Median time of hospitalization was 10 days (IR 5 - 20). Median overall survival was 108 days (IR 55 - 262), with 14/38 patients (36.8%) surviving > 6 months and 4/38 (10.5%) surviving > 12 months. Median survival of responding patients (CR + HI + stable disease) was 298 days (IR 206 - 333), median survival of patients with progressive disease was 68 days (IR 48 - 133) (p=0.0017). After a minimum observation period of 18 months, no patient is still alive, 5 patients were lost to follow-up and 33 died, 24 from disease progression and 9 in stable disease from infections (5), acute myocardial infarction (2), haemorrhage (1). *Conclusions*. The addition of VND to HU seems to ameliorate the response rate (CR + HI) as compared to HU historical controls, but it does not seem to affect overall survival. The role of VND in this subset of frail patients remains unclear and further studies with different doses and associations might be tested.

1413

PROGNOSTIC VALUE OF MULTIPARAMETER FLOW CYTOMETRIC ASSESSMENT OF MINIMAL RESIDUAL DISEASE IN ADULT ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Most patients receiving current induction treatment for acute leukemia enter in complete remission (CR). However, many patients will eventually relapse later due to the presence of resistant to chemotherapy leukemic cells, which are undetectable by conventional light microscopy and referred as minimal residual disease (MRD). Multiparameter flow cytometry (MFC) has been shown to be a useful approach for evaluation of MRD based on the detection of aberrant phenotypes on the leukemic cells. The aim of the study was to detect residual blast cells with aberrant antigen expression by MFC in patients with acute myeloid leukemia (AML) in CR and to determine whether the level of MRD would be predictive of subsequent overt relapse and overall survival. *Design and methods*. Thirty-three patients with AML were monitored for MRD after the achievement of CR. Among them, 21 were males and 12 females (median age 37 years, range 19-67). Median follow-up time was 24 months (range 1-125,7). At diagnosis, MFC allowed for defining a "leukemia immunophenotypic fingerprint" for each patient by staining leukemic cells with several combinations of monoclonal antibodies conjugated to fluorochromes. CellQuest and FACS Diva (Becton Dickinson) softwares were used for acquisition and analysis of flow cytometric data. Blast cells were gated according to CD45/SSC properties. MRD studies during remission were performed on erythrocyte-lysed whole BM samples using antibody combinations defining the specific "leukemia immunphenotypic fingerprint", at completion of induction therapy and at completion of consolidation therapy. Relapse rate, overall survival (OS) and event free survival (EFS) were investigated in relation to the influence of MRD presence. For statistical analysis, Kaplan-Meier was used. Results: Eleven of the evaluated patients (35%) relapsed after an average period of 16 months [ranging 4,8 - 29 months]. The mean MRD value in the group of relapsed patients was 3,2 x 10-4, SE 1,1 x 10-4 compared to a mean of 0,8 x 10-4, SE 0,5 x 10-4 in patients who were still in remission after a mean period of 20 months of follow up [ranging 1,5 - 55,5 months] (p=0,049). A threshold discriminating MRD(negative/low) from MRD(high) cases was set at 3,5 x 10-4 residual leukemic cells, a level that allowed optimal sensitivity and specificity for prediction of EFS but also OS. The mean EFS in patients with negative/low levels of MRD was estimated as 41,2 months compared to 18,4 months in patients with high MRD (log rank test, p = 0.02). Similarly, high MRD levels significantly correlated with poorer OS - 2-years survival was estimated at 57% with a mean OS of 20 months, compared to patients with undetectable or low MRD levels, characterized with a 2-years relative survival of 100% with a mean OS not reached after a follow up of 31,9 months [range 1,4 - 125,7 months] [log rank test, p=0.04]. *Conclusions*. Our results suggest that MRD detection during therapy of adult AML is a negative prognostic indicator for (OS) and (EFS), the independency of which has to be confirmed after prolonged follow-up time and increased number of cases.

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1414

CAN ESTIMATION OF WT-1 IN PERIPHERAL BLOOD HELP US IN MONITORING RESIDUAL DISEASE OF PML/RARα-POSITIVE AML PATIENTS?

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Background. Monitoring of residual disease (MRD) is now a gold standard in the treatment of AML patients. MRD in many of AML patients is estimated by expression of AML-specific genes in bone marrow aspirates. Unfortunately, bone marrow aspiration is a painful procedure for the patients and is a limiting factor for more frequent monitoring of MRD in the patients. *Aims*. We tested whether estimation of Wt-1 in peripheral blood could partially substitute PML/RARA estimation in bone marrow. *Design and Methods*. Expression of PML/RARA and Wt-1 was performed by quantitative real-time RT-PCR. The results were referred to expression of gene ABL. *Results*. In 31 PML/RARA-positive AML patients the quantitative estimation of Wt-1 in peripheral blood was found to be at least as sensitive as PML/RARA one in bone marrow. Also the course of the residual disease in peripheral blood monitored by Wt-1 expression correlated with the MRD course monitored by PML/RARA in bone marrow very well. Monitoring of MRD by Wt-1 in peripheral blood can thus partially substitute estimation of PML/RARA in bone marrow. Of course, when a considerable increase in Wt-1 expression above the upper limit is noticed, the estimation of PML/RARA in bone marrow is performed. *Summary*. According to our results, monitoring of the residual disease by Wt-1 expression in peripheral blood can help us in monitoring PML/RARA-positive AML patients.

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LEUKEMIA IN PREGNANCY; 9 CONSECUTIVE CASES IN 9 MONTHS FROM A SINGLE INSTITUTION IN SAUDI ARABIA

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Background. Cancer incidence during pregnancy is 0.07% to 0.1%. Leukemia e.g. AML, ALL, and CML are often seen in women of childbearing age. So the management of these disorders may be complicated by a coexistent pregnancy. There is no standard approach for this clinical dilemma - in part because of variables such as; the type of malignancy, the seriousness of the symptoms; the patient's personal beliefs. In many cases, the diagnostic work up has to be altered because of the pregnancy. Often the treatments available have varying risks to the fetus.

In most situations, the course of the pregnancy does not seem to be affected by the presence of leukemia and the course of the malignancy does not seem to be affected by the pregnancy. Most leukemias don't seem to behave differently during pregnancy. *Aims*. The aim of this report is to summarize the type and treatment outcome of 9 consecutive cases of pregnant patients with Leukemia who were referred and evaluated at our Prince Sultan Hematology & Oncology Center (PSHOC) at King Fahd Medical City (KFMC) within a short period of 9 months.*Design and Methods*. Between May 20007 till January 2008 a total of 9 consecutive cases of leukemia were referred, diagnosed and treated in our institution of PSHOC at KFMC, Riyadh, Saudi Arabia. The clinical, laboratory, and treatment outcome information of theses cases were summarized and analyzed along with addressing the legal and religious issues related to these cases. Results: In the specified period a total of 9 consecutive pregnant women with suspected hematological malignancies were referred to our center over a short period of 9 months. 6 cases were diagnosed as AML (2 cases AML-M5, 2 cases AML-M2, 1 case AML- M0, and 1 case as AML-NOS but patient was Philadelphia +ve for BCR-ABL p210? Blast crisis of CML) and 3 cases as Ph +ve CML. The median age was 27 year. High WBC was the main laboratory presentation in all of these cases. Our approach for AML management during pregnancy consisted of; first trimester AML to terminate the pregnancy due to high teratogenic effect of chemo in this period (in one case of AML M0 who died later because of disease & sepsis) and early delivery and symptomatic management during the third trimester using leukopheresis and hydroxyurea (in one case who had AML M2 with t(8;21) but CD56+ she received 3+7 followed by one HIDAC then allogeneic BMT from MRD at KFSHRC in Riyadh). We had 3 cases of AML during second trimesters and our approach to use 3+7 as induction followed by 2+5 as a consolidation. The last patient was diagnosed in the post partum period and she received the standard treatment protocols. *Conclusions*. Leukemia complicating pregnancy is not uncommon medical problem in our society where there is a high fertility rate and large number of young age population. The management of leukemia during pregnancy is challenging but is achievable. Using our approach in AML pregnant patients we were able to achieve a high remission rate 83% (5/6 patients) with an acceptable mortality and morbidity of 17% (1/6 patients). 50% (3/6 patients) underwent Allogeneic HSCT.

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1416

THE ROLE OF CYTOGENETICS AND MOLECULAR GENETICS IN THE MANAGEMENT OF ACUTE MYEOID LEUKEMIA. THE EXPERIENCE OF THE HEMATOLOGY CLINIC CLUJ-NAPOCA, ROMANIA

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Background. The overall prognosis of acute myeloid leukemias (AML) remains poor despite progress achieved over the past decade. Cytogenetics define three risk groups (good, intermediate and poor), based on the presence or absence of certain chromosomal abnormalities. Molecular genetics may further subdivide the group of patients with normal karyotype. *Aims*. Between October 2007 and October 2008, thanks to a collaboration between our institution and the University of Ulm, Germany, funded by the German José Carreras Leukemia Foundation, we analyzed systematically from a cytogenetic and molecular genetic point of view our adult AML patient population. *Design and Methods*. There were 40 patients (23 men, 17 women) aged 21-79 years (median 48 years). Besides the routine cytological, cytochemical and immunophenotypical analysis, karyotyping was applied in all cases and in those cases with normal karyotype the mutational status of the NPM1 and FLT3 genes was assessed. Results. Four cases (10%) were in the cytogenetically defined good-risk group (1 case with t(15;17), 2 cases with t(8;21), 1 case with inv16). Twenty cases (50%) were in the intermediate-risk group (2 cases with trisomy 8, 2 cases with trisomy 22, 16 cases with normal karyotype). Sixteen patients (40%) were included in the poor-risk group (13 cases with multiple abnormalities, 1 case with -5, 1 case with -7, 1 case with inv(3). The molecular analysis in patients with normal karyotype revealed the presence of the NPM1 mutation alone in 4 patients, the association of NPM1 and FLT3 mutation in one patient and the presence of mutated FLT3 alone in another 4 patients. Out of the 40 patients, 34 were treated with curative intent. In 3 patients with NPM1 mutation as sole abnormality, all-trans retinoic acid (ATRA) was added to chemotherapy. In 17 patients (50% of those treated with curative intent) complete remission (CR) was achieved. Based on cytogenetic and molecular data, we divided the patients into 2 broad prognostic groups: favorable cytogenetic/molecular profile, comprising patients with low-risk cytogenetics as well as those with normal karyotype and the NPM1 mutation as sole molecular abnormality and a second group of unfavorable cytogenetic/molecular profile, comprising patients with poor-risk cytogenetics, as well as those with intermediate-risk cytogenetics except those with NPM1 mutation as sole abnormality. When comparing the treatment outcome between these two groups, there was a significantly better response and survival in the favorable cytogenetic/molecular profile group. Three out of four patients harboring mutated NPM1 and treated with chemotherapy + ATRA achieved a complete remission. None of the patient with a mutated FLT3 gene entered complete remission. *Conclusions*. This study represents the first attempt in our center towards a modern, systematic categorization of AML cases. Our results emphasize the bad prognosis of cases with unfavorable cytogenetics and the presence of the FLT3 mutation as well as the favorable prognosis of patients with good-risk cytogenetics and the NPM1 mutation. Complex, cytogenetic and molecular diagnosis is essential in the 21st century, considering the fact that targeted small molecules against several mutated sites may soon become available.

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EXPLORING WILMS TUMOR GENE (WT1) IN ACUTE MYELOID LEUKEMIA - INITIAL EXPERIENCES

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Background. The Wilms tumor gene (WT1) that is normally expressed only in podocytes, is expressed in peripheral blood of at least 75-80% of acute leukemia patients. It may contribute to the development of acute leukemia. There are some suggestions in the literature, that the higher the WT1 expression, the less likely to achieve complete haematological remission (CHR), therefore the prognosis is worse. Patients showing WT1 expression at diagnosis can turn into WT1 negative at CHR. After the reappearance of WT1 transcripts, relapse is expected, which makes detection of WT1 transcripts eligible for testing minimal residual disease (MRD). *Aims*. We aim to demonstrate the potential usefulness of WT1 to establish the quality of remission in AML patients and for the early identification of patients at high risk of relapse. *Design and Methods*. Peripheral blood (PB) samples were collected at diagnoses, after induction and consolidation chemotherapy (1-5 days after the achievement of a neutrophil count 0.5x109/l) from 26 AML patients. The median follow-up was 6 month (mean 7.4, range 0.1-24). We measured AML patients' WT1 expression with Applied Biosystems 7500 Real Time PCR. The assay of the gene is based on TaqMan reaction, using the Applied Biosystems Hs00240913-m1 assay. The expression of WT1 was normalized with that of the housekeeping gene GAPDH. No WT1 expression was detected in healthy controls. *Results*. There was no correlation between WT1 expression and WBC count at diagnoses. We did not find correlation between the amount of WT1 transcript at diagnoses and survival time, FAB and cytogenetic risk subgroups or FLT3 mutations. The worst survival time was found, when the degree of WT1 expression did not fall after chemotherapy. (The median survival time was 5 month, range 1-14). Better survival time was observed (median 8.5 month, range 6-13), when the amount of WT1 transcripts substantially decreased after chemotherapy. The disappearance of WT1 transcripts was detected only in one patient. This patient is in complete remission (CR) since 11 month. In all of the patients, who reached a favorable WT1 value after chemotherapy and achieved CR and later relapsed, increased WT1 expression was detected well before the signs of relapse was observed. *Conclusions*- Our results show that detection of WT1 expression is a possibility to estimate the prognosis and to follow up AML patients more accurately. Consistent and repeated quantitative analysis of WT1 expression may provide prognostic information and early identification of patients at highest risk of relapse.

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FLAG-IDA IN THE TREATMENT OF REFRACTORY/RELAPSED ACUTE LEUKEMIA: A SINGLE CENTRE EXPERIENCE

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Background. Relapsed or refractory adult acute myeloid leukaemias (AML) have poor prognosis. The strategy for treating these patients is by reinduction chemotherapy followed by allogeneic stem cell transplantation, provided that the toxicity of the salvage regimen is acceptable. High or intermediate dose cytarabine has been reported to be effective in the salvage treatment of AML; addition of the purine analogue fludarabine to cytarabine increases the rate of accumulation of cytarabine in leukemic blasts and the response to chemotherapy may be improved by the addition of idarubicin, an anthracycline that is less susceptible to multidrug resistance. AIMS Based on these considerations, we evaluated the efficacy and the toxicity of FLAG-IDA in a series of 95 refractory/relapsed AML patients. *Design and Methods*. Sixty-five patients (68%) were in first relapse and 30 (32%) were refractory to conventional chemotherapy. The patient group included 51 males and 44 females with a median age of 41 years (range 15-63). All patients were treated with fludarabine (30 mg/m^2/day iv for 5 days), cytarabine (2 gr/m^2/day iv for 5 days), idarubicin (10 mg/m^2/day iv for 3 days) and G-CSF (5 mcg/Kg/day subcutaneous 24 h after completing chemotherapy and until neutrophil regeneration). *Results*. The overall CR rate was 55% (52 of 95): 39 of 65 (60%) in relapsed and 13 of 30 (43%) in refractory patients; there were 6 (6%) deaths during therapy: 2 due to cerebral hemorrhage and 4 to infection. In patients achieving remission, the median time to reach an absolute neutrophil count (ANC) more than 0.5x10^9/L and 1x10^9/L was 21 (range 16-26) and 24 days (range 20-28) from the start of chemotherapy, respectively. Platelets levels of more than 20x10^9/L and 100x10^9/L were achieved in a median time of 24 (range 19-26) and 32 days (range 28-39) days, respectively. Fever higher than 38.5°C was observed in 77 of 95 patients (81%): 45 had fever of unknown origin and 32 documented infections. Non hematological side effects consisted mainly of mucositis (75/95 or 79%) and transient liver toxicity increase (40/95 or 42%). All 52 patients who achieved CR received a second course with FLAG-IDA, and 27 received allogeneic stem cell transplantation, 8 patients received autologous stem cell transplantation, 9 were judged unable to receive any further therapy, and 8 refused other therapy. The median overall survival (OS) for all 95 patients was 4 months (range 2 - 86); for all 52 responders patients, the disease free survival (DFS) and OS were 9 (range 3-86) and 11 (range 7-86) months, respectively; particularly, the 28 patients who received allogeneic transplantation had a DFS of 53 (range 7 - 86) months. *Conclusions.* In our experience, FLAG-IDA treatment seems to improve a good clinical outcome and its duration in patients with poor prognosis. It has shown to be a well-tolerated regimen in refractory/relapsed AML enabling patients with CR (55%) to undergo further treatment, including transplantation

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SERUM VITAMIN D LEVELS ARE ASSOCIATED WITH PROGNOSIS IN HEMATOLOGICAL MALIGNANCIES

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Background. Vitamin D, a steroid hormone produced in skin, acts through a nuclear transcription factor to regulate many aspects of cellular growth and differentiation. The major circulating metabolite of vitamin D is serum 25-OH vitamin D. Vitamin D is a membrane antioxidant, and observational studies have indicated that inadequate vitamin D levels are a risk factor for certain types of cancer. Recently, it has been reported an associated of low serum levels of 25-OH vitamin D with increased breast cancer risk and with a poor prognosis (ASCO 2008, abstract 511). *Aims*. We examined vitamin D levels in a cohort of 105 patients with hematological malignancies at different stage of their disease, seen between June and November 2008. *Design and Methods*. 25-OH vitamin D was measured by radioimmunoassay. Normal levels ranged from 12 to 40 ng/ml. *Results*. Mean serum 25-OH vitamin D level±standard deviation (SD) was 22.9 ng/ml±10.5 ng/ml. Fourteen patients had vitamin D deficiency, while 4 were above the normal range. Lower vitamin D levels were associated with higher malignant cell burden as indicated by the correlation observed between the stage of the disease in acute leukemias and levels of vitamin D. The difference was significant between patients with long-term disease-free survival and those tested at diagnosis (p=0.001) or those tested at the time of relapse (p=0.05) (Figure). Similarly in patients with Philadelphia-positive leukemias, the study of molecular residual disease showed a correlation between molecular response and levels of vitamin D (p=0.01). In patients with progressive disease, vitamin D levels were not significantly different among myeloid and lymphoid malignancies. In acute leukemia patients tested at the time of diagnosis, we did not find any correlation between vitamin D levels and age. No difference was noted when vitamin D was drawn in summer (June-August) versus autumn (September-November) months. Vitamin D levels were also not associated with gender or with the index of corporal mass (IMC). However, the two patients presenting with under weight at diagnosis (IMC < 18.5 kg/m^2) showed low vitamin D levels (15 and 16 ng/ml). *Conclusions*. Vitamin D deficiency appeared uncommon in hematological malignancies, but lower levels appeared related to the stage of the disease, response to therapy, and therefore aggressiveness of the disease. Whether lower vitamin D levels has other explanations remains a challenge, like the exact role of vitamin D in malignant cells and its potential need for a therapeutic or preventive approach.

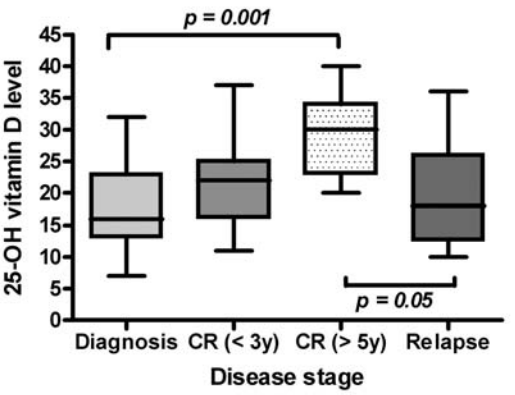

Figure.

1420

EARLY BONE MARROW BLAST CLEARANCE BY INDUCTION CHEMOTHERAPY PREDICTS SUPERIOR OUTCOME AND DISEASE FREE SURVIVAL IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML)

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Background. Risk assessment in AML using pretreatment characteristics may be improved by incorporating parameters of early response to therapy. It is reported that the early blast clearance in the bone marrow after induction therapy is related to the outcome of the *disease(Kern W et al, Blood 2003;101:64-70*). *Aims*. Assessing bone marrow response to therapy on day 16 after the induction therapy and correlating it to achievement of complete remission and long term outcome in AML. *Design and Methods*. 46 patients with AML (29 Males, 17 Females) were treated with MAC (combination of mitoxandrone and cytosine-arabinoside) in our Clinic from January 2002 to March 2008. Their median age was 71 (60-81) years and their mean age 70,1(±4) years. According to the FAB classification they were classified as M0(3), M1(9), M2(17), M4(4), M5(4), M6(7), hybrid-diphenotypic leukemia(2). 21/46 patients presented with secondary AML(sAML). Leukocytosis (>50x10^9/L) was noted in 6/46 patients and leukopenia (<5x10^9/L) in 23/46 patients. Cytogenetic analysis was performed in 43/46 patients: 3/43 cases failed to produce metaphases, 29/43 cases revealed standard risk abnormalities (21/29 patients presented with normal karyotype-intermediate prognosis) and 11/43 cases had poor risk abnormalities (unfavourable prognosis). Bone marrow examinations were carried out on day 16 after induction therapy. Both cellularity and the amount of residual leukemic blasts of the bone marrow were evaluated in all cases. Patients were classified in the

following groups: Group A: aplastic bone marrow, Group B: blasts 0-4%, Group C: blasts 5-9%, Group D: blasts 10-24%, Group E: blasts 25-49%, Group F: blasts >50%. The number of patients in every group, their cytogenetics (intermediate cytogenetics/IC, unfavourable cytogenetics/UC, without metaphases or without cytogenetics/WC), the presence of sAML, the outcome after the induction therapy, achievement of complete remission(CR) or presence of residual disease(RD) in every group are shown in the table. *Results.* 1.Patients from groups A and B, having aplastic bone marrow or bone marrow in remission on day 16, present higher percentages of CR compared to those from the other groups who present higher percentages of RD. 2.Patients from groups A and B present significantly better overall survival compared to those from the other groups: the median survival was 22(±2,49) and 15,33(±2,35) months for groups A and B and 7,88(±2,59), 9,85(±5,1), 9,42(±3,05), and 7(±1,99)months for groups C, D, E and F respectively (p<0.05). *Conclusions*. Despite of the small number of patients of this study, it is clear that the early bone marrow blast clearance on day 16 after induction therapy in elderly patients with AML is a very important prognostic factor for achievement of complete remission and long term outcome in AML

Table 1. Characteristics of evaluated patients.

GROUP	n	IC	UC	WC	sAML	CR	RD
A	5	5/5	0/5	0/5	2/5	4/5	0/5
B	10	9/10	1/10	0/10	3/10	7/10	2/10
C	4	3/4	0/4	1/4	3/4	1/4	1/4
D	11	8/11	3/11	0/11	5/11	3/11	8/11
E	6	3/6	2/6	1/6	3/6	1/6	5/6
F	10	1/10	5/10	4/10	4/10	0/10	10/10

1421

CD2+ ACUTE PROMYELOCYTIC LEUKEMIA IS ASSOCIATED WITH A POOR PROGNOSIS AFTER CHEMOTHERAPY±AUTOLOGOUS TRANSPLANTATION

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Background. The classical form of acute promyelocytic leukaemia is characterised by hypergranular promyelocytes with absent CD34 and HLA-DR expression and the long or bcr1 form of the PML/RAR transcript. CD34 expression has been associated with the hypogranular M3 variant form of APL and frequently with expression of CD2 and the short or bcl3 PML/RAR transcript. Some but not all published series report a worse prognosis in CD34+ CD2+ APL. *Design and Methods.* We conducted a retrospective review of patients presenting with APL to examine their laboratory features at presentation and correlate remission rate and long term survival with CD2+ expression. Results: 12 patients presenting with APL between 1992 and 2008 were identified from an electronic database. Median age was 28 years (range 10-72). Immunophenotyping was available for 11 patients. 6/11 patients were CD2+ APL, 4/6 patients co-expressed CD34 and 3/4 were CD34+HLA-DR-. 5/6 CD2+ patients had the short/bcr3 APL/RAR isoform. The remaining CD2+ patient was not analysed. All CD2- patients were CD34-HLA-DR- and expressed the long/bcr1 isoform. The most commonly administered treatment was the PETHEMA regimen (n=9). 3/6 CD2+ patients and 6/6 CD2- achieved CR with induction therapy. All six CD2- patients remain in CR1 after a median 40 months (17-140) post diagnosis. 3/6 CD2+ died during induction therapy due to intracerebral haemorrhage (n=2) and DIC-related multi-organ failure. 2/6 CD2+ patients relapsed and received an autologous transplant in CR2. One patient died 6 months after presentation and the other relapsed. One CD2+ patient received an allogeneic transplant in CR1 and remains disease free at 153 months post diagnosis. *Conclusions*. In our patients CD2+ APL was associated with early death and relapse after conventional chemotherapy or autologous transplantation in our patients. Recognition of this less common phenotype is clinically important in identifying a group of patients at high risk of early death and/or treatment failure. CD2+ patients with an HLA-matched sibling donor may benefit from allogeneic transplant or novel therapies.

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BIPHENOTYPIC AND MIXED ACUTE LEUKEMIA IN PEDIATRIC PATIENTS

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Background. Recent studies have demonstrated that lineage fidelity (myeloid or lympphoid) is not preserved in some cases of acute leukemia. This minority of acute leukemia is represented by biphenotypic or hibrid acute leukemia (BAL) characterized by co-expression of lymphoid and myeloid surface markers in the same leukemic cell and mixed or bilineal acute leukemia (MAL) characterized by two separate clones of myeloid and lymphoid leukemic blasts. The aim of this study was: to corelate the expression of lymphoid and myeloid antigen with clinical, hematological and biological parameters, to analize the diagnostic criteria for BAL and MAL, the impact on the response to treatment and the prognosis. *Design and Methods.* For this study we describe: the clinical features,blast morphology from peripheral blood and bone marrow aspirates, cytochemical stains including myeloperoxidase (MPO), Sudan black B (SBB), periodic - acid Schiff (PAS and the immunological markers (lymphoid and myeloid) expressed by the blasts of four children diagnosed as BAL and MAL at the Oncological Institute in Cluj-Napoca. Results: These four pedriatic patiens studied: two females and two males with ages ranged from 2 years to 13 years (median 9 years) were identified as BAL (patiens no. 2, 3) and MAL (patients no. 1, 4) according to FAB criteria and immunophenotyping. Flow-Cytometric analises was performed for: HLA-DR; CD45; CD10; CD19; CD20; CD22; CD5; CD7; CD13; CD 33; CD34; CD14; CD117; Of the four cases, only two MAL type (cases no. 1, 3) with favorable evolution, achieved a complete remission with intensive myeloid like therapy, if the immunophenothyping has been evidenced precursor B-ALL with myeloid antigenes; In the other cases (no. 2, 4) BAL type we established an unfavorabile evolution: case 2 - death after 17 mounths; case 4 -candidat for bone marrow transplant. Discussion: The cases described as BAL and MAL, with minor incidence 3-4% from all adult and pedriatic acute leukemia, are uncommon types of leukemia, originating more probably from a pluripotent progenitor cell with the potential to differentiate along both myeloid and limfoid lineages.The morphological and cytochemical characteristic features of these cases were: the presence of two distinct blast populations in the same patient: one of small size, with hight nucleus-cytoplasm ratio, condensed nuclear cromatin, small or inconspicuous nucleoli; hand mirror morphology and PAS block positive, resembling lymphoblast and the other myeloid larger with more abundant cytoplasm with or without azurophilic granulations and Auer rods, monocytoid features, cytoplasmic buds, MPO and SBB-posititive blasts. Crucial value in diagnosis of BAL and MAL is holded by the immunophenotyping. According to the scoring sistem (EGIL) a case is considered BAL when point values are greater than two for myeloid and one for lymphoid. From our study result that the immunophenotypic characteristics alone do not predict clinical outcome. Outcome- related prognostic factors are : age, response to treatment, complications, in close corelation with hematological, cytogenetic and molecular aspects. The treatment applied was not unitary, because until now a protocol for these rare forms of acute leukemia with reserved prognostic is not established. At this time we recommend an aggressive and long treatment. In conclussion we stress:-The rarity and the difficulty of differentiation between BAL and MAL.-The complexity of the multidisciplinary investigations in the diagnosis of thiese uncomnom forms.-The essential value of the immunophenotyping in interpretation concomytantly with the cytogenetical and molecular studies.-The immnunophenotyping studies performed in a single laboratory confirm the existence of a distinct subsets.-AML and ALL showing so-called lineage infidelity.-Eradication of BAL and MAL it's possible by association of the intensive therapy with the bone marrow transplantation.

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THE IMPACT OF FLT3 - ITD IN ADULT /ELDERLY ACUTE MYELOID LEUKAEMIA(AML) PATIENTS: A SINGLE CENTER RETROSPECTIVE ANALYSIS

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Background. FLT3-ITD represents one of the most frequent genetic aberrations of AML as they occur in the 20-30% of cases with normal karyotype. Several studies recognized this mutation as a negative prognostic factor although its role in elderly patients is less clear . Aims: We have retrospectively reviewed the frequency and prognostic impact of FLT3-ITD mutation in a consecutive series of adult and elderly (>65y) patients with AML series diagnosed and treated at a single Institution. . *Design and Methods.* Between January 2007 and January 2009 among 60 consecutive AML patients with median age 69yrs were observed at our center. A FLT3-ITD was detected in 11(18,3%) cases. Of these, 9 had intermediate and 2 unfavourable risk karyotype and only 1 patient had concomitant NPM1 mutation. Initial Hb median value was 8.5 gr/dl(range 6.5-12.4 g/dl), median WBC count 43x10^9/L (range 4.- 264 x10^9/l), median PLT count was 51x10^9/L (range 15.-312.x10^9/L). Five patients were treated according to the EORTC-GIMEMA AML12 protocol, while the remaining 6, who were unfit or not eligible for intensive treatment, received differentiating (ATRA+LoDAC) or supportive therapy (HU). Results: Of the 11 FLT-ITD+ cases , 2 are too early, 3 (27%) achieved 1st CR, 6 died during induction. Among patients died during induction, 3 were older than 60y (60,72,80y), and 3 had WBC >/= 40.x10^9/L (42.2, 202., 264x 10^9/L). As of February 2009, 5 patients are alive, with a median OS of 2 months(range 1- 17 mo); 2 are in 1st CCR for 1.5+ and 3+ months, respectively; while a 3rd CR patient (a 72y-old woman), who received as 1st induction ATRA+LoDAC relapsed 14 months after CR achievement, and died during 2nd intensive induction. *Conclusions*. CR rate were disappointingly low in this small series of FLT3-ITD pts, most likely due to concomitant high risk factors (elderly age and hyperleucocytosis). As recently highlighted in a large German study (Buchner et al, 2009) also in our series age, high WBC count and NPM1-gerline status were additional factors negatively influencing outcome. However, differentiating treatment allowed to obtain better survival with respect to intensive chemotherapy. If confirmed in larger series, this observation may suggest that non intensive treatment associated with novel targetd agents represents a valid therapeutic option for elderly FLT3-ITD AML patients.

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THE PROGNOTIC VALUE OF THYMIDINE KINASE LEVEL IN BLOOD SERUM IN PREDICTION OF SURVIVAL POTENCIAL IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Being a cell proliferating enzyme, thymidine kinase (TK) can reflex a speed of tumor cell division, aggressive of leukemia clone and can be useful in prognosis of decease progression and treatment results. Aims. The purpose of the current investigation is determination of blood serum TK value in prediction of survival potential in acute myeloid leukemia (AML) patients. *Design and Methods*. The main epidemiology (sex, age), hematology and clinical features (proliferate syndrome, hemorrhage syndrome, infection complication etc) and TK level in blood serum (measured by radioimmunoassay using 5-125 I-iodedeoxy uridine as a substrate) were defined in 127 AML patients in different phase of course of decease (first acute period - 79 patients, relapse - 48 patients). During the observation time (2003-2008 years) there were documented 40 death incidents among all patients under investigation (21 death incidents in first acute period, 19 - in the time of relapse). Died patients according to the aim of investigation also were divided into the following groups: I - patients, whose death was a result of decease progression (n=14); II - those, whose death was a result of different complications (n=26). *Results*. The TK level in blood serum was significant increased in the time of diagnosis in the patients, died during the observation period in comparison with the TK level in blood serum in the patients, who achieved satisfactory treatment results (43.95±7.46 U/L vs. 14.23±1.46 U/L, < 0.01). There wasn't observed any significant difference in the TK level in the group of the patients in connection with the sex and age. On the contrary, significant difference existed in the TK level in blood serum among the patients, whose death was a result of decease progression (52.28±8.22 U/L) and different complication (11.925±1.165 U/L), (p<0,005). *Conclusions*. Establishing results confirm, that upper TK level in blood serum in the time of AML diagnosis can predict an aggressive course of decease. TK level more, than 30.0 U/L prognoses bad treatment results due to the chemotherapy resistance. TK level more, than 50.0 U/L predicts high risk uncontrolled decease progression and death.

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A DESCRIPTIVE STUDY OF FLOW CYTOMETRIC ANALYSIS OF 151 ACUTE LEUKEMIA PATIENTS : A SINGLE INSTITUTE EXPERIENCE IN EGYPT

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Background. This study was carried out to analyze the proportion of acute myeloid leukemia (AML), acute lymphoblastic leukemia (T / Pro and Pre B-ALL), biphenotypic lineage and Undifferentiated acute leukaemia among 151 acute leukaemia(AL) patients .We also analyzed the coexpression of AML with lymphoid cell surface markers (T / B) and ALL with myeloid cell surface markers. By this method more than 98% of acute leukemia cases can now be precisely allocated to their respective lineages (*Channa J et al.J Coll Physicians Surg Pak 2000;10(5):158-60*). *Aim*. To determine the proportions of each lineage and the coexpression of myeloid and lymphoid cell surface markers for later detection of their prognostic impact. *Design and Methods.* Data of 151 consecutive cases of AL were analyzed in our study between Jan 2005 through Feb 2009. Flow cytometry was performed on all AL cases using the standard protocols. Myeloid associated markers included (MPO, CD 13, CD33, CD117, CD15, CD14 and CD64); T-lymphoid associated markers (Tdt, CD2, CD3, CD5 and CD7); B-lymphoid associated markers (Tdt, CD10, CD19, CD20 and CD22); Lineages non specific markers (CD34, HLADR) and panleucocytic marker(CD45). Florescence labeled antibodies were obtained from (Becton Dickinson, U.S.A) and run on FACSCALIBER using CELLQUEST software. *Results*. Eighty nine patients were AML, 61 were ALL, 3 biphenotypic and 2 undifferentiated AL. Five(6.25%) out of 80 AML patients showed CD19 coexpression ; 7(8.75%) with CD7, 3(3.7%) with CD15.Three(4.9%) out of 80 ALL patients with CD 13 coexpression and 3(4.9%) with CD 33 coexpression. *Conclusions*. Flow cytometry enabled us to determine the proportions of each lineage of acute leukaemia as well as the coexpression of cell surface markers between different lineages. Follow up of the prognostic impact of this coexpression on our patients is still in progress

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SERUM SOLUBLE TRAIL LEVELS WERE DECREASED IN PATIENTS WITH T(8;21) ACUTE MYELOID LEUKEMIA

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Background. TRAIL apoptotic pathway is important in the pathogenesis of leukemia. There are some reports that TRAIL pathway might has some changes in leukemia. Serum soluble TRAIL(sTRAIL) could be detected easily. The levels of sTRAIL in acute myeloid leukemia(AML) with t(8;21) is unknown. *Aims*. To detect the serum sTRAIL levels in AML patient with t(8;21) and investigate its clinical significance. *Design and Methods*. Serum sTRAIL was analyzed by ELISA, compared with normal control and non-t(8;21) AML M2 patients. *Results*. Thirty-eight patients with t(8;21) , 32 normal control and 31 non-t(8;21) AML M2 were enrolled in the study. The levels of serum sTRAIL in patients with t(8;21), non t(8;21) AML M2 and normal control were 450.84±313.91ng/ml, 938.95± 395.08ng/mL and 856.43±298.9ng/mL respectively. The levels of sTRAIL were significantly lower than non-t(8;21) AML M2 (p=0.001) and normal control(p=0.013). *Conclusions*. Our pilot study showed that serum sTRAIL levels in t(8;21) AML were low, it might be related to the pathogenesis of this type of leukemia.

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RESULTS OF TREATMENT IN CHILD ACUTE MYELOID LEUKEMIA IN TUNISIAN PATIENTS

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Introduction. Acute Myeloid Leukemia (AML) is a relatively rare malignancy in the pediatric population, comprising only 15 to 20% of the acute leukemia diagnosed in this age group. It remains a challenging disease with an inferior treatment outcome compared with pediatric acute lymphoblastic leukemia. Despite high-dose cytarabine consolidation and allogenic bone marrow transplantation(BMT), about 40% of the children with AML still died of their disease. *Design and Methods*. In department of hematology(pediatric and adult) of Aziza Othmana Hospital, among 156 children acute leukemia enrolled between January 2003 and June 2008, 26 (16,6%) *de novo* AML (Down's syndrome and FAB M3 excluded) were noted. The median age was 12 years(2.2-17);WBC was > $20x10^9$/L in 11 patients. The diagnosis of AML was done by BM smears and immunophenotyping (CMF) using EGILE scoring. Karyotype was done in all patients and it was informative in 22 cases(85%).According to the Medical Research Council's Trial(MRC): 8 patients were classified into favorable or CBF leukemia,13 into intermediate group and only 1 into unfavorable. All patients were treated according to the French multicenter protocol ELAM02 with a common induction treatment by cytarabine and mitoxantrone than 3 courses of consolidation therapy among them 2 courses with high dose cytarabine. Patients in CR with HLA identical family donor have allogenic stem cell trasnsplantation. *Results*. Twenty two patients (85%) achieved CR after one course and 23(88.5%) after 2 courses. Toxic death and induction failure were both at 7.7%. Fourteen patients had a matched sibling donors, but stem cell transplantation was done only in 6 patients because of problems of feasibility. Two of them died by GVHD but no relapse occurred in this group. Relapse occurred in 8 patients (36%) in median period of 9 months. EFS (18 months)was 65%. When studied separately, the patients with CBF leukemia who were treated by chemotherapy alone and all the other group who were treated by chemotherapy alone or chemotherapy with stem cell transplantation , the EFS (18 months) were respectively 83.5% vs 46.5%. OS(3years) was 59%. *Conclusions*. Despite of the low number of patients, our results seem to be near those reported in large studies, and to improve them more we had to indicate stem cell transplantation to more patients with a better management of graft complications.

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COMPARISSON OF TRANSPLANT PROCEDURES WITH CONVENTIONAL THERAPY IN THE TREATMENT OF ACUTE MYELOID LEUKEMIA- LONG TIME FOLLOW UP

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Introduction. There are three approaches for consolidation of patients (pts) with acute myeloid leukemia (AML) (with exception of acute promyelocitic leukemia). Post remission treatment changes over years mostly by intensification or including new drugs. Those approaches are either allogeneic or autologous stem cell transplantation (SCT) or conventional therapy. AIM: We have analyzed retrospectively 74 pts with AML divided into three groups- conventional group, autologous stem cell transplantation (SCT) group and allogeneic SCT group with respect to relapses, time to progression (TTP) and overall survival (OS). *Design and Methods*. We have treated 110 pts with AML in our clinic from 2001. till 2008. Nine of them had acute promyelocytic leukemia and 27 pts have showed primary resistance. We have enrolled 74 pts into these analysis- 30 pts into "conventional group", 30 pts in the "autologous transplant group" and 18 pts in the "allogeneic transplant group". After one or two courses of standard induction chemotherapy ("7+3"), pts have received three more cycles of consolidation (MAE, MidAC and ICE) and last one was used for mobilization of autologous peripheral stem cells in pts with approved complete remission. Both transplant groups immediately undergone conditioning up to standard BuCy2±IDA regimen and "conventional group" have received maintenance therapy consisted of Thioguanin 160 mg daily per os and Cyto ARA 100 mg subcutaneously daily for 5 days (mostly due to their own rejection of autologous SCT or their age). Prophylaxis of graft versus host disease in allogeneic group is combination of Cyclosporine A and Metothrexate. RESULTS: On intention-to-treat analysis number of relapses was significantly lower in allogeneic SCT group (5,55%, p<0,005) while there were no difference between other two groups (autologous 12/26 - 46% vs 13/30 - 43,3%, ns). TTR was longer in autologous group but not significantly (9,4 range 2-42 vs 8,7 range 1-48 months). There huge significance in OS in allogeneic group compared to others while there is no significance between autologous and conventional group (log rank test-au:al 2,88, p<0,01; con: al 2,69, p<0,01; au:con 0,57, ns) independent from risk groups formed considering to cytogenetics. Median survival in allogeneic group was 37,5 months (range7-86months). *Discussion.* Allogeneic transplant is absolutely superior in treatment of AML pts over other two post remission options. To improve results of autologous SCT more attention should be given on the control of minimal residual disease and also probably by using bone marrow as an origin of stem cells.

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TREATMENT OUTCOME IN UNSELECTED PATIENTS WITH ACUTE MYELOID LEUKAEMIA: THE SINGLE CENTRE EXPERIENCE OF THE WILHELMINEN HOSPITAL

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Background. Acute myeloid leukaemia (AML) occurs with an annual incidence of 2-3/100.000 population, its frequency rising with age. Treatment is based on combination chemotherapy with cytosine arabinoside and anthracyclines and has not changed during the last 30 years. Allografting is a valuable option in younger patients with high-risk features or those relapsing after standard chemotherapy. *Aims*. To assess treatment outcomes in an unselected patient population treated in our institution since April 1999. *Design and Methods*. 144 consecutive patients diagnosed with AML between April 1999 and February 2009 have been included in the study. Median age at presentation was 67.5 years (range, 19.2 - 93.6 years) with a gender distribution of 76 male and 68 female patients. Patients were stratified based on age and performance status and treated according to the current protocols of the "Ostdeutsche Studiengruppe fuer Haemato-Onkologie (OSHO)". Briefly, patients below 60 years of age were scheduled to receive 1 or 2 courses of standard induction chemotherapy with intermediate-dose Ara-C (1g/m^2 twice over 3 hours on days 1,3,5,7) and mitoxantrone (10 mg/m^2 on days 1-3) or idarubicine (12 mg/m^2 on days 1-3). Patients achieving complete remission (CR) after induction therapy received 3 cycles of consolidation chemotherapy using a five-day course of the same drugs as during induction. Patients over 60 years of age considered fit for intensive treatment received induction chemotherapy as described above with two consolidation cycles, whereas for the remaining patients, palliative chemotherapy or supportive care only, was offered. Results: All of the 42 patients below 60 years of age were submitted to standard therapy with curative intent. CR was achieved in 79% of these patients after 1 or 2 courses of induction therapy. Median progression-free survival in this cohort was 44.4 months, with an overall survival of 58% at 5 years. However, 14% of patients did not reach CR after double induction and 7% of patients died during the first cycle, mainly due to infectious complications. Allogeneic bone marrow transplantation was performed in 5 patients, resulting in long-term survival in 4 of these patients. Of the elderly patients among the study population, only 45 (44%) were judged to be in a clinical condition that would allow assignment to a curative treatment approach. CR was achieved in 59% of patients, whereas early deaths occurred in 20% of patients. Progression-free survival (median, 14.4 months) was found to be significantly shorter compared to younger patients, with a 5-year overall survival of only 19%. 57 patients received palliative chemotherapy (administered orally or subcutaneously) or best supportive care only. Median survival in these cohorts was 2.8 and 0.3 months, respectively, showing an only modest, albeit statistically significant impact of palliative chemotherapy on treatment outcome. *Conclusions*. These single institution data reflect the 'real life' spectrum of patients with AML, with the majority presenting at age above 60 years. Results compared favourably with treatment outcomes reported in large clinical trials.

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FOURNIER'S GANGRENE ASSOCIATED WITH ATRA

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Background. Genital ulceration is a rare and poorly understood side effect of all-trans-retinoic acid (ATRA). Fournier's gangrene is the most severe manifestation of this complication. *Aims*. To promote awareness of this complication and to highlight that it may occur within the first week of ATRA therapy, which is earlier than previously thought. To demonstrate this complication does not preclude subsequent use of ATRA. *Case History*. We report the case of a 21-year old male who developed Fournier's Gangrene secondary to ATRA during week 1 of induction therapy for acute promyelocytic leukaemia (APL). Following diagnosis, he was commenced on idarubicin 12 mg/m^2 IV and ATRA 45 mg/m^2 orally, as part of the Spanish protocol. On the seventh day of induction chemotherapy he became febrile and complained of painful scrotal lesions, which had appeared several days earlier. He did not exhibit any other features of the retinoic acid syndrome (RAS). On examination there were several necrotic scrotal ulcers. Microbiology work-up was negative and fever failed to resolve with broad-spectrum antimicrobial therapy. A scrotal biopsy showed an acute inflammatory or necrotic process, consistent with the clinical diagnosis of Fournier's gangrene. The patient proceeded to urgent surgical debridement. Histopathology of debrided tissue confirmed Fournier's gangrene. Following debridement, he became apyrexial and the ulceration resolved completely. He continued on ATRA throughout induction therapy until molecular remission of APL. He received ATRA with subsequent consolidation courses and maintenance therapy, in accordance with the treatment protocol, without recurrence of the ulceration. His leukaemia remains in complete remission to date. Discussion: In the literature, there are reports of scrotal ulcer healing on discontinuation of ATRA and treatment with either topical or systemic steroids. In our patient, this treatment approach was not attempted in view of the need for urgent surgical debridement. The pathogenesis of scrotal ulceration in association with ATRA is not fully understood. Different histology findings are reported varying from acute and chronic inflammation to necrotising vasculitis. All previous case reports have documented scrotal ulceration occurring in week 2 of ATRA therapy. Uniquely in our case, scrotal lesions appeared early during week 1 of treatment. *Conclusions*. Clinicians and patients should be alert to this complication of ATRA therapy, which can occur earlier than previously thought. Following surgical debridement there was complete resolution of the ulceration and we were able to continue ATRA therapy according to protocol without subsequent recurrence. The development of Fournier's gangrene, the most severe complication of ATRA-induced genital ulceration does not preclude the subsequent use of this important therapy in patients with APL.

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ACUTE MYELOID LEUKEMIA AND TYPE II DIABETES MELLITUS IN COMPLETE REMISSION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Bone marrow transplantation has become an important part of the treatment of hematologic disorders, but also of metabolic disorders and autoimmune diseases. Using animal models for autoimmune diseases it has previously been demonstrated that allogeneic bone marrow transplantation can be used to treat autoimmune diseases such as insulin-dependent diabetes mellitus. Aims. To present the case of a patient diagnosed with acute myeloid leukemia (AML) and type II diabetes mellitus, both in remission after allogeneic stem cell transplantation. *Design and Methods*. A.S. is a female AML patient, 54 years old, diagnosed in our institution with AML- M1, normal kariotype (FLT3 and NPM1 wild type) in January 2008. Between January and July 2008 she was treated with 3+7+Etoposide (ICE) induction chemotherapy and 4 courses of high dose Ara-C and Idarubicine consolidation chemotherapy. The patient had previously been diagnosed with type II Diabetes Mellitus and she was under insulin treatment, maintained during the entire chemotherapy schedule. In October 2008 the patient underwent sibling HLA-identical allogeneic stem cell transplantation, with Busulfan+ Cyclofosfamide conditioning. Since then, the patient was under immunosuppressive treatment with cyclosporine. Results. Complete hematological remission was obtained after the induction chemotherapy and was maintained ever since. After the stem cell transplantation the hematological recovery was satisfactory with the persistence of mild thrombocytopenia, anemia and lymphocytosis. The chimerism is 99%. Immediately after the transplantation was done there was no need for insulin treatment, since the blood glucose was constantly under 150 mg/dl. There was an episode of acute hepatic and cutaneous GVHD which resolved after corticosteroid therapy and an increase in cyclosporine dosage. No significant increase of serum glucose was observed despite corticosteroid therapy. Currently, there is a grade I chronic GVHD of the liver and the patient is under cyclosporine treatment, 200 mg/day. *Conclusions*. Our patient's case is a fortunate one where remission of both a hematologic disorder and an autoimmune disease was obtained by allogeneic stem cell transplantation. As autoimmunity seems to play a role in type 2 insulin-dependent diabetes mellitus, it would be interesting to see what happens when immunosuppressive treatment will eventually be tapered and stopped. It is possible that in the near future allogeneic stem cell transplantation will become a powerful strategy for treating patients with hematologic disorders and various intractable diseases, including autoimmune diseases.

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LEUKEMIC INFILTRATION OF RETINA IN AML, A CASE REPORT AND LITERATURE REVIEW

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Background. Leukemic infiltration of the retina without CNS involvement is a rare complication of AML, Here we report a case with retinal infiltration of Leukemic cells with typical fondoscopic features which presented during relapsed disease and was treated with systemic High Dose Cytarabin. The case was a 48 year old male,known case of AML ,M5 subtypewith normal cytogenetic study done through conventional method, who received conventional chemotherapy with 7/3 regimen during remission induction followed by 5/2 as consolidation.He was asymptomatic for 5 months and was following the stem cell transplant primary steps. Afterward he presented with fever and thrombocytopenia and bone marrow study proved relapsed disease.He was admitted again for salvage chemotherapy while he complained of blurred vision.Ophthalmologic consultation showed diffuse red spots with sharp margin and typical features of leukemic infiltration.The CNS cytologic study at the same time was negative as well as MRI of the brain with contrast. High Dose Cytarabin with 3 gr/m2 bid for 3 days together with Mitoxantrone 12 mg/m^2 for D1 and D2 was given as salvage chemotherapy. The visual symptoms were not changed for about 2 months although the patient achieved a complete remission after prolong neutropenia lasting about 38 days and complicated with neutropenic fever and disseminatec Herpes infection. Visual acuity returned to 8/10 after about 3 months without any specific ophthalmologic therapy and no CNS radiotherapy was done either ,due to lack of CNS infiltration. The patient is still in complete remission after 8 months and is able to read with little difficulties. Allogeneic stem cell transplantation from matched related donor is planned for the patient. Leukemic infiltration of the retina is a rare complication for AML and topical treatments are generally not aplicable because of the disseminated nature of the lesions, High dose Cytarabine might be effective in these infiltration as well as in CNS involvement and relapses.

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PRIMARY MYELOID SARCOMA (MS) OF THE STOMACH: A CASE REPORT

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Myeloid sarcoma is an uncommon solid extramedullary accumulation of myeloid blasts. Most often it is diagnosed in the course of acute leukemia, an isolated manifestation is very rare. We report on a 63-year-old male patient who underwent examination for melena and significant anemia. A tumor of the small gastric curvature with a size of 4 cm was discovered. Although malign lymphoma was diagnosed in the first place a comprehensive histopathological and immunhistological examination revealed a manifestation of acute myeloid leukemia. Immunhistologically, the malign cells displayed positivity for CD33, CD34, CD43, CD117, TdT, Lysozym, ASD and MPO. In line with the diagnosis of MS negativity for CD2, CD3, CD20, CD30, CD56, CD61, CD68, CD79, Glycophorin C and S100 was shown. In FACS-analysis, positivity for CD13,

CD33, CD34, CD45, CD11b, HLA-DR and MPO could be observed. Neither peripheral blood nor bone marrow presented signs of acute leukemia. The patient received two courses of intensive AML-type chemotherapy according to the protocol of the German AML Cooperative Group. Although there was just a moderate decrease of tumor size chemotherapy was continued with cytarabine and mitoxantron. The following examinations did not show any change in tumor size but a still viable tumor. Operation, radiotherapy and combination of both were discussed to continue treatment. At this time FACS-analysis and light microscopy of the bone marrow showed for the first time an infiltration with myeloid blasts. These cells were marked by the same antigen pattern as those blasts initially isolated from the gastric tumor. Cytogenetic examination revealed a complex aberrant karyotype with an involvement of 15 chromosomes. The antileukemic treatment was continued with chemotherapy according to the FLAG-Ida protocol. After regeneration of hematopoiesis bone marrow blast count had increased from 50% to 90%. Subsequently the patient was transferred to another center to receive allogeneic bone marrow transplantation. Isolated gastric manifestation of MS is an outspoken rariety with just a few cases published world-wide. Thus its diagnosis is a challenge for clinicians and pathologists. In almost all reported cases primary MS heralded the imminent development of acute leukemia. Therefore, they should be considered as the initial manifestation of a systemic disease rather than a localized process. Prognosis is generally poor. Allogeneic bone marrow transplantation seems to offer best chances for prolonged survival or cure. On this account, MS should be considered in the differential diagnosis of gastric tumors to avoid incorrect treatment of a potentially curable condition.

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DOES INTENSIVE ANTI-LEUKAEMIC THERAPY REALLY IMPROVES THE PROGNOSIS FOR PATIENTS SUFFERING FROM ISOLATED MYELOID SARCOMA?

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Introduction. Myeloid sarcoma /MS/ represents the tumour mass made from blasts, or immature myeloid cells, which can appear as solitary tumours, or multiple changes in different tissues and organs. Acute myeloid leukaemia /AML/ develops within 10.5-11 months in almost 90% patients with isolated MS who initially do not receive any treatment. The optimal therapy has not been defined yet. Chemotherapy, stem cell transplantation /SCT/, radiotherapy, surgical resection, or combination of these methods has been used, from case to case. Aim: Study goal is to estimate the efficacy of anti-leukaemia therapy in patients with isolated MS, overall survival and period until systemic disease develops. *Design and Methods*. This study included patients with isolated MS, who had been diagnosed at Institute of Haematology of Clinical Centre of Serbia, from 2002 until 2008. Group was included patients diagnosed for MS based on WHO criteria and who did not have signs of AML, chronic myeloid proliferative disorder or MDS in bone marrow at the point of establishing the diagnosis for MS. *Results*. Average age of our patients was 37.8 years /range: 19-67/. The diagnosis of uterus MS was established for two female patients, as well as the diagnosis of MS localized in the spine, heart, chest, testicles, skull base, tonsils, and lymph nodes, each for one patient. Intensive anti-leukaemic therapy was applied in five patients, whilst initial surgical treatment was performed in four patients. Complete remission /CR/, induced with this initial treatment therapy model, was achieved in all patients who had been treated surgically, and in three patients who had been treated with anti-leukaemia therapy. Currently four patients are alive, and all in CR. Average overall survival in the whole group amounts 27.5 months. Average survival in the group of patients on chemotherapy was 23.3 months, and in the group with initial surgical treatment and monitored afterwards, survival was 31.5 months. One patient of five who had been initially treated with systematic approach, developed therapy resistant AML, and the other died in the course of treatment due to the development of sepsis. SCT had been performed in three patients treated with anti-leukaemia therapy /in two allo SCT and one auto SCT/, and all of them are alive and achieved CR. Two patients among four, initially treated surgically, developed systemic disease, and in other two extramedullar relapse occurred. Only one of them is alive and is in CR, 49 months after completing AML treatment. In our group, average survival until the development of systemic leukaemia was 21.9 months. Average survival in the group of patients who had not developed systemic leukaemia was 29.2 months, and in the group of patients who developed systemic leukaemia average survival time was 25.3 months. *Conclusions*. Our study did not show that anti-leukaemia therapy improved survival in patients with myeloid sarcoma, but further investigations, which would include more patients and long time follow up period, are needed.

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ACUTE MYELOID LEUKEMIA AND BLEEDING DISORDERS AT DIAGNOSIS. A SINGLE CENTRE EXPERIENCE

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Backround. Bleeding manifestations at diagnosis of Acute Myeloid Leukemia(AML) can occur with a frequncy as 50%. Their cause is thrombocytopenia because of marrow infliltration or Disseminated Intravascular Coagulation(DIC) that can complicate acute leukemia, mainly acute promyelocytic type. *Aim*. We searched for incidence of coagulation disorders and bleeding manifestations in our AML patients and whether this could influence their clinical course. *Design and Methods*. Forty AML patients are included in this study and from their records we had their coagulation test results and full blood count at diagnosis and whether they presented with sings of bleeding or not.Platelets $<100x10^9$/L ,INR>1.2,APTT>30 sec and fibrinogen levels <200mg/dl were considered abnormal. *Results*.- This study included 28 men of average age 68 years (range 37-89) and 12 women of average age 65 years (range 45-86). De novo AML was diagnosed in 29 patients and secondary AML in 11 (7 patients had MDS, 2 patients had CMML, 1 patient had PV and 1 had ET). Acute promyelocytic leukemia(APL) was diagnosed in 2 patients. During diagnosis bleeding manifestations were reported in 10 patients (6 patients had thrombocytopenia (15%), and 4 patients had DIC (10%)). Among patients with DIC 2 had APL.Prolongation of INR was recorded in 7 patients while prolongation of APTT in 5, low fibrinogen levels were noted only in AML patients with DIC. There was no episode of severe bleeding manifestation(e.g. cerebral bleeding) that could threaten the life of patients. Four of the male patients are alive with a median overal survival(OS) of 51 months, all of them are younger than 60 years and four women are alive with a median overal survival of 29 months, 3 of them are younger than 60 years. *Conclusions*. Bleeding manifestation at diagnosis of AML is quite often and we search our records for its frequency in our patients. Although this is a small group of patients we record a frequency of 25%. Bleeding is mainly complicating DIC which is a prominent feature of APL. Even when it is present bleeding diathesis does not play an important role in patients OS. Age is an important factor for AML patients survival.

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FLAG-IDA REGIMEN IN THE TREATMENT OF REFRACTORY/RELAPSED ACUTE MYELOBLASTIC LEUKEMIA: SINGLE-CENTER EXPERIENCE

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Background. Conventional chemotherapy is highly effective in the treatment of acute myeloblastic leukemia (AML). About 50-80% of adult patients with *de novo* acute myeloblastic leukemia achieve complete remission (CR) with currently available chemotherapy regimens consisting of antracyclines and cytarabine. However, relapse develops in more that 40% of the cases within two years, and 15-25% of patients fail to achieve complete remission because resistant to treatment or death. The management of cases with primary refractory and /or relapse disease is very difficult and prognosis in this subset of patients after several different chemotherapy combinations is still very poor with a CR rate 33-41%, and median duration of CR 6-24 months. Refractory/relapsed acute myeloblastic leukemia has always been a challenging problem for hematologist. *Aims*. We evaluated efficacy and toxicity profiles of FLAG-Ida combination chemotherapy in patients with refractory/relapsed AML. *Methods and Results*. At the University Hematology Clinic in Skopje, Macedonia, in the period 2006-2008, fifteen patients with refractory/relapsed acute myeloblastic leukemia were treated with FLAG-Ida regiment. Patients were between 16-52 years old, 4 female and 11 male. They were treated with fludarabine 30 mg/m^2, cytosine arabinoside (AraC) 2 g/m2 for 5 days, Idarubicin 10 mg/m^2 for 3 days, and granulocyte colony stimulating factor G-CSF 5 μg/kg from day 0 till neutrofil recovery (ANC >1.0 x10^9/l). Complete remission were achieved in 7 patients (47%), there patients (20%) died of post chemotherapy com-

plications, and 5 failed to achieve complete remission. Out of 7 patients who achieved complete remission, 3 went autologous bone marrow transplantation, 2 went allogeneic bone marrow transplantation, and 2 are being evaluated for the same. Major complication encountered were mucosistis, transient hepatic toxicity, fungal and bacterial infections. *Conclusions*. Our experience confirmed that FLAG-IDA regimen is well tolerated and effective therapy in relapsed/refractory acute myeloid leukemia. The toxicity is acceptable, enabling most patients to receive further treatment, including transplantation procedures. FLAG-Ida is a good choice in cases with refractory/relapsed acute myeloblastic leukemia for salvage chemotherapy and it is wise to consolidate it with hematopoetic stem cell transplantation.

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DIFFERENT MOLECULAR REMISSION ACHIEVEMENT TIME IN INFANTS WITH MLL-REARRANGED ACUTE LYMPHOBLASTIC LEUKEMIA CORRESPONDS TO OUTCOME - REPORT FROM MLL-BABY STUDY GROUP

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Background. MLL-baby protocol, containing all-trans retinoic acid (ATRA), for the treatment of infants with acute lymphoblastic leukemia (ALL) has recently been introduced. Aim. To find out a single time point (TP) for minimal residual disease (MRD) monitoring by RT-PCR of fusion gene transcripts (FGt), that clearly discriminate patients (pts) with different outcomes. *Design and Methods*. To date 35 pts were enrolled into MLL-Baby study. MLL rearrangements were detected in 26 pts (74.5%). Monitoring of FGt was performed in 17 infants with defined MLL translocation partner genes and more than 4 follow-up samples. MRD-negativity was defined as absence of FGt verified by nested RT-PCR with sensitivity 1E-5. Median follow-up in observed group was 25.1 months (range 5.7-65.3). There were 12 pts with MLL-AF4, 2 pts with MLL-MLLT10, 2 pts with MLL-EPS15, 1 patient (pt) with MLL-MLLT1. Bone marrow samples were obtained at the time of diagnosis, at day 15 of remission induction (TP1), end of remission induction (TP2), after one week of first ATRA administration (TP3). The next TPs depended on the arm of treatment. Pts with MLL-AF4 were stratified into high-risk (HR) arm, thus TPs for MRD were scheduled before each HR blocks followed ATRA administration (TP4-TP9). Pts with MLL rearrangements other than MLL-AF4 were recruited into intermediate risk group, where MRD was estimated before each reinduction (TP4-TP6). Written informed consent was obtained in all cases.

Table.

	First group	Second group	P
Total N of patients	12	5	
MLL-AF4	7	5	0.09
MLL-MLLT1	1	0	0.64
MLL-MLLT10	2	0	0.88
MLL-EPS15	2	0	0.88
Median age, mo (range)	7.00 (0.03-10.83)	5.43 (1.70-6.93)	0.17
Initial WBC × 10^6 /uL, (range)	99.35 (19.6-410)	131 (21.5-370)	0.71
≥ 1000 blasts at day 8	2	0	0.88
CNS involvement	3	2	0.97
Number of relapses	2	2	0.39
In CCR	10	3	0.69

Results. All pts were MRD-positive at TP1. 3 pts (two MLL-AF4-positive and one MLL-MLLT10-positive) became MRD-negative at TP2. By TP3 one MLL-AF4-positive and all MLL-AF4-negative pts were MRD-negative. At TP4 other six MLL-AF4-positive pts totally eliminated FGt. The latest TP when pt achieved molecular remission was TP9. It was detected in one pt with successive reduction of MRD value, measured by real-time quantitative PCR (RQ-PCR). 2 pts with MLL-AF4 who never achieved MRD-negativity relapsed at TP8 and at Protocol II (TP10). Alternatively, MLL-AF4-positive girl who had achieved molecular remission at TP4, converted to MLL-positive status at TP8 and subsequently relapsed at TP9. Another relapse that occurred in well-responded group was in MLL-EPS15-positive boy during maintenance therapy. *Conclusions*. Retrospectively, based on achievement of MRD-negativity and outcome, we divided pts into 2 groups. Detailed pts characteristics are presented in table 1. First group included 12 pts who achieved molecular remission by TP4, where 2 relapses occurred (MLL-AF4-positive and MLL-EPS15-positive). Thus 70-months RFS was 83%±10%. Among second group, consisted of 5 MLL-AF4-positive pts that had not achieved molecular remission by TP4, there were 2 relapses. In 3 others consecutive reduction of MRD value till the undetectable level was observed. RFS was 60%±21%. Hence, absence of FGt by TP4 for the pts with MLL-AF4 and by TP3 for the other MLL rearrangements allows us to distinguish low risk pts.

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BAX / BCL-2 EXPRESSION IN CHILDHOOD AND ADOLESCENTS' ACUTE LYMPHOBLASTIC LEUKEMIAM

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The mitochondrial'mediated pathway of apoptosis is regulated by the bcl-2 family of antiapoptotic (bcl-2, bcl-xl, mcl-1) and proapopotic proteins (bax, bad, and bak). Although over-expression of bcl-2 associated with poor outcome in hematological malignancies and high bax levels related with a good prognosis in AML, studies in ALL yielded conflicting results. Western blotting studies determined the level of bcl-2 in childhood ALL and showed a correlation between high bax expression and increased relapse probability. However, it has been suggested that the bax/bcl-2 ratio may be more important than either marker alone in determining apoptosis. Aim. The estimation of Bax, Bcl-2 and Bax /Bcl-2 expression ratio in childhood ALL compared with benign cytopenias and solid tumors as well as the correlation of these values with ALL prognostic factors. *Design and Methods*. Sixty nine children were enrolled in the study. Bone marrow from 19 children with ALL at diagnosis, 19 months-18years old and 16 children with ALL in remission on day 33, were studied. Twenty children with benign cytopenias and 14 children with solid tumors without bone marrow involvement were studied as control groups. Five hundred nanograms of extracted RNA were used for cDNA synthesis (Reverse Transcription System, Promega). Real-Time RT-PCR was performed using commercial primer sets (Quantitect Primer Assay, QIAGEN) and SYBR Green (Quantitect SYBR Green, QIAGEN). The cell line HL-60 was used for the creation of a standard curve including GAPDH. Duplicate reactions were set up for each sample and were run in ABI PRISM 7700 Sequence Detection System. Bax, bcl-2 expression and Bax/Bcl-2 ratio were studied within the ALL group at diagnosis in correlation with prognostic factors such as age, White Blood Count, haemoglobin levels and the risk group according BFM ALL-2000 treatment protocol. Analysis was also performed between the ALL group at diagnosis compared with ALL patients in remission and the control groups, using the SPSS program. Results. The analysis within the ALL group at diagnosis did not show any statistical significant difference for the values of bax, bcl-2 and bax/bcl-2 ratio in relation with the age, haemoglobin, leucocytes values and the risk group. The comparison between ALL at diagnosis and the control groups did not reveal any significant difference although the levels of bcl-2 were much higher in ALL compared with the solid tumors. Statistically significant higher values of bax /bcl-2 ratio (0.713 vs 0.17 p=0.046) were estimated in the older children (>10 years) within the ALL high risk group compared with the younger ones. Within the ALL high risk group the bax/bcl-2 ratio was statistically higher at diagnosis compared to remission (0.34 vs 0.0365, p=0.028). *Conclusions*. The bax, bcl-2 and bax/bcl-2 expression ratio did not show any statistical difference in relation with ALL prognostic factors. Nevertheless, significantly higher values of bax /bcl-2 ratio were estimated for high risk patients at diagnosis compared to remission and for older children versus younger ones. Higher levels of bcl-2 were estimated also in ALL compared with the control groups. The prognostic implications of these data are under investigation.

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C677T AND A1298C MTHFR POLYMORPHISMS ROLE IN GRADE 3-4 TOXICITY AFTER CHEMOTHERAPY WITH HIGH DOSE METHTREXATE (HDMTX)

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Background. Polymorphisms in genes affecting drug transport, metabolism, or drug targets may directly influence drug response. Thus, MTHFR polymorphisms could potentially modify the therapeutic effects of any drug that interferes with folate transport or metabolism, such as methrotexate (MTX), as well as play an important role in MTX mediated toxicity. More over, a relationship has also been described between MTHFR polymorphisms and the incidence of ALL. *Aim*. The aim of this study was to determine whether the combined effect of HDMTX treatment and decreased activity of MTHFR associated to C667T and A1298C polymorphisms could play any role in MTX related toxicity. Patients & *Design and Methods*. DNA from peripheral blood and/or bone marrow samples of 26 ALL patients (15 adults and 11 children) were analysed for the MTHFR C677T and A1298C polymorphisms using Q-PCR and Melting Curve analyses to determine their genotype. All patients were treated according to PETHEMA LAL-AR/2003 (adults) and LAL-AR-N/2005 (paediatric patients) protocols, with high dose methrotexate (HDMT) at 3mg/m^2 for adults and 5 mg/m^2 for pediatric patients. Grade 3-4 renal, lung, cutaneous and mucous toxicities, as described by the WHO Common Toxicity Criteria v2, were then recorded for each patient. *Results*. Grade 3-4 HDMTX related toxicity was detected in six (23%) patients, all of them adults, carrying a given polymorphism. Their genotypes were: C667T in 66% (2 heterozygous and 1 homozygous), A1298C in 50% (2 heterozygous) and 16% double heterozygous.This group was then compared to the group of adults that did not develop any toxicity or grade 1-2 toxicity. This group's genotypes were: C677T in 66% (3 heterozygous and 1 homozygous), A1298C in 33% (1 heterozygous) and 22% double heterozygous. Three of the patients (15%) did not have MTHFR polymorphisms. The overall incidence of MTHFR polymorphisms in the ALL population studied was 88% compared to 66% in the healthy population used as control. *Conclusions*. No significant association was found between the different polymorphisms and grade 3-4 HDMTX related toxicity. However, if the adult population is analysed separately, a higher involvement of the A1298C polymorphism can be seen in relation to toxic effects (50% in those patients who developed toxicity in contrast to 33% in those who did not). With an incidence of HDMTX related toxicity of 23%, which varies widely in the literature, and with no MTHFR polymorphism associatd in particular to toxicity, we doubt whether MTHFR polymorphisms should be routinely performed in ALL patients at diagnosis as a predictor of severe adverse effects to come. A lower incidence in the A1289C homozygous genotype was found in comparison to northern Eurpean populations. A higher incidence of MTHFR polymorphisms was found in the ALL population when compared to healthy donors, making us think further studies should be carried out to determine a potential role in ALL pathogenisis in particular, ant tumorigenesis in general.

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T-CELL RECEPTOR AND IMMUNOGLOBULIN GENE REARRANGEMENT PATTERNS IN POLISH PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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Background. Using current treatment protocols, it is possible to cure approximately 70% of children with T-cell acute lymphoblastic leukemia (T-ALL). Leukemia relapse remains the main cause of therapy failure. This reflects significant biological and clinical heterogeneity of this disease. T-cell receptor (TCR) gene rearrangement patterns in T-ALL are known to reflect the maturation stage arrest of the malignantly transformed thymocytes and hence provide data on the biological subclassification of this malignancy. Additionally, identification of the TCR gene rearrangements is a prerequisite for RQ-PCR-based minimal residual disease (MRD) monitoring, which provides insight into the therapy response and prognosis in T-ALL patients. *Aims*. We aimed at identification of TCR gene rearrangement pattern in Polish T-ALL patients. *Design and Methods.* The study group consisted of 48 consecutive children with T-ALL treated at the centers of Polish Pediatric Leukemia and Lymphoma Study Group (PPLLSG). Detection of TCR and immunoglobulin (Ig) gene rearrangements was performed with heteroduplex PCR analysis using primers and protocols established by BIOMED-1 and BIOMED-2 Concerted Actions. The analyses comprised the rearrangements in TCRG, TCRD, TCRB loci and incomplete IGH gene rearrangements. Additionally, we screened patients for SIL-TAL1 rearrangements that are specific targets for MRD monitoring because of their unique junctional sequence. Results: We have detected 166 clonal TCR/Ig gene rearrangements in total, 3.5 per patient on average (range 0-7). At least one rearrangement was detected in 43 patients (90%) and at least 2 rearrangements in 42 patients (88%). No rearrangements were detected in 5 patients (10%). A total of 76 TCRG rearrangements were detected in 40 patients (at least one in 83% of T-ALL) and at least two rearrangements were found in 34 patients (71%). Twenty four TCRD rearrangements were identified in total; at least one rearrangement was detected in 19 patients (40%) and two TCRD rearrangements were detected in 5 patients (10%). At least one TCRB gene rearrangement was detected in 36 patients (75%), at least two rearrangements were detected in 16 patients (33%). SIL-TAL1 rearrangements were detected in 4 patients (8%). There were 7 incomplete IGH rearrangements detected in 7 patients (15%). Conclusion: The incidence of particular Ig/TCR gene rearrangements in Polish children with T-ALL is comparable to the data reported in literature, which indicates that there are no particular distinct ethnical Polish features of the immunogenotype as compared to other nations. Our results also prove that the BIOMED-1 and BIOMED-2 protocols enable detection of Ig/TCR gene rearrangements in most pediatric T-ALL patients, which form basis for introduction of routine MRD monitoring in childhood T-ALL in Poland.

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THE MTHFR POLYMORPHISMS AND SUSCEPTIBILITY TO CHILDHOOD ALL

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Background. Folate plays an important role in DNA methylation, synthesis and repair. Reduction of 5,10 methylenetetrahydrofolate - a donor for dUMP to dTMP- is catalyzed by methylenetetrahydrofolate reductas (MTHFR). Two common polymorphisms (C677T and A1298C) in the gene coding for MTHFR reduce MTHFR enzyme activity and have been shown to associate with the susceptibility to malignancies. Aim: Studies on the role of these two polymorphisms in the susceptibility to acute lymphoblastic leukemia (ALL) led to discrepant results. Moreover assessment of the prevalence of the two most common polymorphisms of MTHFR has not been studied in the Iranian population so far and it is presented for the first time. *Design and Methods*. Using PCR and RFLP analysis, we studied the prevalence of the C677T and A1298C MTHFR genotypes in 103 pediatric ALL patients and 160 age-sex matched control. We calculated Odds ratio of MTHFR genotypes to examine if one or both of these polymorphisms are associated with childhood ALL. Results: No significant association between two common polymorphisms of MTHFR and risk of ALL were observed. The other result was the high prevalence of 1298CC which was significantly higher than that reported for most Asian and European population. Conclusion: Our findings suggest that the MTHFR C677T and A1298C gene variants do not have a major influence on the susceptibility to pediatric ALL, but despite of absence of significant association, the frequency of MTHFR 677TT among cases was lower comparatively to control supporting previous indications that it might confer protection against ALL.

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CLINICAL USEFULNESS OF ARRAY COMPARATIVE GENOMIC HYBRIDIZATION FOR DETECTION OF CHROMOSOMAL ABNORMALITIES IN CHILDHOOD PRECURSOR B-ACUTE LYMPHOBLASTIC LEUKEMIA

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ETV6-RUNX1, generated by the chromosomal translocation t(12;21)(p13;q22), is the most prevalent fusion gene in pediatric precursor B-ALL. Secondary abnormal changes are commonly found in these groups, occurring in some 60-80% of ETV6-RUNX1 cases. However, not all ETV6-RUNX1 positive patients have identifiable secondary chromosomal changes, as found by standard routine cytogenetics. We report here multiple gains and losses that are undetectable by cytogenetics but identifiable by high resolution array comparative genomic hybridization (array-CGH). A 6-year-old boy presented for uncontrolled epistaxis, was diagnosed for precursor B-ALL. ETV6-RUNX1 rearrangement is positive by FISH and RT-PCR. Karyotype was analyzed from the diagnostic bone marrow as follows: 46,XY,add(1)(p13),del(2)(q33q37),del (6)(q15),add(11)(q22),add(12)(p11.2),del(15)(q26)[2]/46,idem,add(1)(p22)[4]/46,idem,-2,der(5) t(2;5)(p11.2;p11.2),+mar[3]/46,XY[21] FISH with a dual-color extra signal probe showed a ETV6/RUNX1 fusion and non-translocated ETV6 deletion. The array-CGH using 244K whole genome microarray provided comprehensive information about genomic alterations. The patient had a deletion of 4Mb on 12p13, encompassing the ETV6 gene. Array-CGH exhibited a gain of 6q14.3, from 90651516-91067469 base pairs and a loss of 11q13.4-q23.2, from 75277556-115420958 base pairs. These regions contains gene for BACH2 and ATM respectively. Loss of ATM or gain of BACH2 has not been reported in precursor B-ALL. Losses of 3q3q26.1, 6q26 and 14q32.3 were also found but these regions are known to be copy number variations. The results of array-CGH are consistent with conventional karyotype partially, but additional chromosomal abnormalities were found. This case shows genomic array-CGH may become a powerful tool to discover subtle chromosomal imbalance changes in patients with malignancies.

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TREATMENT RELATED MORTALITY IN INFANTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA DURING ALL TRANS-RETINOID ACID CONTAINING MLL-BABY THERAPY

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Background. Different international study groups have reported adverse prognostic factors and unfavorable events negatively influenced on clinical outcome in infants with acute lymphoblastic leukemia (ALL). Besides high proportion of early relapses, treatment related toxicity also resulting in poor survival in this age group. *Aim*. To estimate treatment related mortality rate during induction and post induction therapy in infants treated with ATRA containing MLL-Baby Protocol. To determine main cause of treatment related deaths in this cohort of patients. *Design and Methods*. MLL-Baby Protocol intended for infants' ALL treatment is recently applied therapy regimen in Russia and Belarus. Preliminary data indicates that this treatment approach results in significant early relapses prevention. Between July 2003 and November 2008, 35 babies with newly diagnosed ALL were treated with MLL-Baby Protocol, which consisted of short 7-14 days ATRA courses in daily doses 25 mg/m2 and conventional chemotherapy. Among this cohort of patients, 19 infants were stratified to intermediate risk-group (ImRG) and 16 to high risk-group (HRG) schedules according to the type of MLL-rearrangement and remission status on day 36/43. *Results*. Three patients (8.5%) died during remission induction treatment prior to start of first ATRA administration. All of them have had high initial WBC count (73; 612 and 1250 /μl); were younger 6 months (17 days, 2 and 3 months old); had MLL-rearranged BI-ALL: two with t(4;11)/MLL-AF4 and one with t(11;19)/MLL-ENL. All of these patients were dexamethasone poor responders. Two children with initial WBC > 600 /μL died on days 52 and 58 due to severe infection associated with prolonged neutropenia and hypocellular bone marrow without signs of normal regeneration. Youngest patient died on day 9 from septic shock. Three patients (9.4%) out of 32 who have achieved complete remission (CR) died within first month after induction therapy completion. All of them were diagnosed with MLL-rearranged BI-ALL: two had t(4;11)/MLL-AF4 and one - t(11;19)/MLL-ENL and were stratified to HRG and ImRG arms respectively according to MLL-Baby stratification criteria. One 8 months old patient from ImRG has died from ATRA-syndrome developed during second course of ATRA, one died from severe infection and one from other reason. *Conclusions*. 1. Although, the proportion of treatment related deaths was rather high: 5 (14.3%) cases in 35 patients, ATRA associated death has been registered in one case (2.8%). This death might be prevented by timely ATRA discontinuation and ATRA-syndrome treatment. Our data demonstrates acceptable safety profile of ATRA administration in infants with ALL. 2. More frequently, 4 out of 5 patients died from severe infections (11.4%). Young age - below 6 months, presence of MLL rearrangements, profound immunosupression have maintained poor response to standard infectious prophylaxis and treatment. Dexamethasone in daily dose 6 mg/m^2, also seems to adversely influence on the life threatening infections rate in our patients.

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CLINICAL UTILITY OF MINIMAL RESIDUAL DISEASE IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: 7 YEAR EXPERIENCE IN A PEDIATRIC HEMATOLOGY-ONCOLOGY UNIT

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Background. Rearrangements of the immunoglobulin heavy chain (IgH) and the T-cell receptor (TCR) genes constitute a universal target for minimal residual disease (MRD) detection in childhood acute lymphoblastic leukemia (ALL). Aim: The aim of the present study was to evaluate the clinical utility of MRD in children diagnosed with ALL, within a 7-year period. *Design and Methods*. Totally 716 polymerase chain reactions (PCRs) were performed using DNA extracted from bone marrow samples of 91 patients (8 PCRs per patient). Patients were diagnosed with B-cell ALL (N=85) and T-cell ALL (N=6). Sequential monitoring of MRD was performed at diagnosis, at the end of induction and at regular time points during chemotherapy and after treatment completion. Seven VH primers (VH family) were used to detect rearrangement of the IgH gene (CDRIII region) by PCR. In patients with T-ALL nested PCR was performed to detect rearrangements of the TCRα, TCRβ and TCRγ genes. Results: Rearrangement of the IgH was detected in 69/85 (81%) patients with B-ALL, at diagnosis. The commonest clone at diagnosis was VH3 (72%). In 3/16 patients without identified rearrangement at diagnosis, positive PCRs were detected during chemotherapy treatment, prior to relapse. PCR was negative at the end of induction, as well as, in most samples during and after treatment in 59/69 (85%) patients with identified IgH rearrangement at diagnosis. PCR remained positive at the end of induction in 10/69 patients. However, 4/10 had negative PCRs in all subsequent samples and were in clinical remission. Furthermore, in 8/69 patients, with negative PCR at the end of induction, PCR positivity reappeared 1-12 months after treatment completion. These 8 patients were in clinical remission, with all followed samples being negative for MRD. Finally, 9/85 (10%) patients with B-ALL relapsed. Six of them had positive PCR at diagnosis, at the end of induction and in most samples during chemotherapy treatment. In the other 3/9 patients that relapsed, IgH rearrangement was not detected at diagnosis, but in most samples prior to the relapse. In the 6 patients with T-ALL, rearrangement of the TCRδ gene was identified in most cases (4/6). All these patients reached clinical remission. *Conclusions*. Rearrangement of the IgH by PCR is a significant tool for MRD monitoring and prognosis in acute lymphoblastic leukemia. In the present patient cohort, persistence of PCR positivity, at the end of induction and/or during treatment, was associated with clinical relapse. PCR negativity at the end of induction is usually related to favorable outcome.

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THE PROGNOSTIC SIGNIFICANCE OF MINIMAL RESIDUAL DISEASE IN ADULT PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. The results of the large prospective MRD studies in adulthood ALL indicate that MRD analysis gives highly significant prognostic information superior to other standard criteria (age, sex, and WBC) in distinguishing patients at high, intermediate and low risk of relapse. *Aims.* to determine whether MRD investigation is valuable in predicting outcome in adult ALL patients (except mature B phenotype). *Design and Methods*. This study was carried out on 57 adult ALL patients, 44 cases (77.2%) were males and 13 cases were females (22.8%). The median age of the cases in our study was 22 years with a mean of 24.4+ 6.8 years (ranges from 18 to 49 years). MRD was assessed by 4-color flowcytometry on bone marrow samples collected during three time points in the first 24 months of treatment. The relationship between MRD status and clinical outcome was investigated and compared with age, sex, immunophenotype, and initial TLC. Results: There was no statistically significant difference in cumulative overall survival according to age and sex (p=0.579& 0.518) however, with initial TLC <30000 in B lineage and in T-lineage <100000, the differences were of statistically borderline significance (p=0.089). There was statistically significant correlation between cumulative overall survival at 2 years and response to chemotherapy (p=0.0007) as well as GMALL risk stratification (high-risk vs. low risk group) (p=0.018). The cumulative disease free survival at 2 years was 34.1% for B-lineage ALL (35 cases) and 61.6% for T-lineage ALL (18 cases). The difference was statistically significant (p = 0.038). Disease free survival (DFS) rates for MRD-positive and MRD-negative patients and log-rank testing established that MRD positivity was associated with increased risk of relapse at all time points (p<0.05) but was most significant after induction. The difference was also statistically significant (p=0.009) in case of MRD after consolidation between low risk and high risk. Correlation between cumulative overall survival at 2 years and MRD was also statistically significant (p<0.001). *Conclusions*. The association of MRD results and DFS was independent of and greater than other standard predictors of outcome and is therefore important in determining treatment for individual patients. Flowcytometric immunophenotyping is the method of choice for detection of MRD in our institution especially post-induction.

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RISK ADAPTED CHEMOTHERAPY TREATMENT FOR ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: LONG TERM FOLLOW UP

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Background. ALL is a heterogeneous disease, the application of risk adapted treatment protocols in now a well established strategy. The aim of this study was to asses the five years leukemia free survival and overall survival of our first risk adapted adult ALL protocol initiated in 1999. *Design and methods*. Hundred and fifteen patients were included in this study conducted at the Medical Oncology Department - NCI .Cairo in the period between 1999 to 2004.The diagnosis was ALL in all patients. The patients were stratified according to their prognostic factors into standard, high, and very high risk groups. Mature B phenotype were excluded and treated with a separate protocol. The treatment plan included: Prephase for patients with high TLC and/or organomegaly. Induction phase I: Four drugs: Vincristine, Doxorubicin, L-Asparaginase and prednisone with intrathecal MTX. Patients that attained CR were subjected to cranial irradiation with 24 Gy and intrathecal MTX for four injections. Phase II induction with Cyclophosphamide and Cytarabine. Consolidation phase I: Vincristine, Doxorubicine and prednisone with Triple intrathecal. Phase II consolidation: Cyclophosphamide, Cytarabine and Etoposide with triple intrathecal. Maintenance therapy: two years with 6 mercaptopurine and methotrxate. For patients with high and very high risks, one cycle of high dose cytarabine and mitoxantrone (HAM regimen) was added between induction and consolidation. Very high risk patients with available donor were referred to transplantation in CR1.Informed consents were signed by all cases. Results: The median age was 25 years (range 16to60). The study included 73 males and 42 females. CNS involvement at presentation was reported in 14 cases (12.2%). Immunophenotyping were pro B (7%), C. ALL &Pre B (56.5%) and T phenotype (20.9%).The BCR-ABL fusion gene transcript was positive in 15 cases. Forty five patients (39.1%) reported to have standard risk, while 55 (47.8%) and 15 (13%) were high and very high risk respectively. Complete remission was achieved in 76.5% (n = 88) while 23.5% (n=27) showed no CR. The CR rate of the standard risk group was 88.9% versus 70.9% and 60% for the high and very high risk respectively (p=0.029).The median survival for all patients was 14 months (95% CI, 9.2 to18.8). Survival at 60 months was 28.24%, it was 34%, 21% and 20.1% for the standard, high and very high risk respectively (p=0.017). There was significant difference in survival between patients with pro-B, pre-B&CALL, and T phenotype (p=0.0019). The median time to progression was 16 months (95% CI, 13.5to 18.5). At 60 months 35.2% were still in remission. Time to progression was 44, 12 and 14months for the standard, high and very high risk groups respectively (p=0.047). Time to progression between patients with Pro-B, pre-B&C-ALL, and T phenotype were 3, 17 and 16 months respectively (p=0.0007). *Conclusions*. The CR rate, LFS and survival of the standard risk are satisfactory while those of the high and very high risk are still in need to be improved , whether we can achieve this by higher post remission chemotherapy, targeted therapy or stem cell transplantation remains to be investigated.

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PARASITIC INFECTION IN EGYPTIAN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Parasitic infections continue to be a major public health problem in developing countries. Immunosuppressed patients are at increased risk of serious complications associated with some parasitic diseases. Aims: To determine the frequency of some endo-parasites among children with acute lymphoblastic leukemia (ALL) in relation to controls. Subjects: The study included 234 children divided into 117 children with acute lymphoblastic leukemia their age ranged from 2-14 years and 117 immunocompetent children with matched age and sex as control group. *Design and Methods*. Laboratory study included examination of stool using five different staining techniques, urine, cerebrospinal fluid (CSF) and serological tests. *Results*. The overall percentage of parasitic infections was significantly higher among children suffering from ALL (90.6%) as compared to the controls (58.1%) [X2 32.37, p<0.001]. Protozoa recorded the highest percentage of infection among the leukemic children (66.7%) as compared to controls (44.4%) [X2 11.70, p<0.001]. Followed by microsporidial infection (60.7% vs. 11.1%) [X2 62.47, p<0.001], then by coccidial infection (53.8% vs. 41.9%) and helminthes infection (2.6% vs. 12%) [X2 7.68, p<0.006]. Microsporidia was the only parasite detected in the CSF in 54.7%. Neither Toxoplasma gondii by Giemsa stain, nor Acanthameba was detected in the sediment of the CSF. Higher percentage of infection was found among children living in rural areas and among those who followed bad personal hygiene. *Conclusions*. There were a high percentage of parasitic infections among leukemic children mainly protozoa, adding to their morbidity. Routine examination of CSF for the presence of microsporidia is recommended in such cases especially those having neurological manifestations.

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QUANTITATIVE ASSESSMENT OF WT1 EXPRESSION BY REAL TIME PCR IN ALL AS PROGNOSTIC MARKER

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Background. WT1 gene was first identified in patients with Wilms tumor ,coding for a zinc finger transcription factor located on chromosome 11p13,which is involved in differentiation and proliferation of hematopoietic precursor cells as well as some other tissues like kidney, ovary, heart, etc. It has been reported to be expressed in almost all hematological malignancies including ALL,AML ,CML and MDS, being a useful marker in minimal residual disease (MRD) detection and leukemia management. *Aims.* To assess the efficacy of WT1 as a predictor for

relapse in acute lymphoblastic leukemia. *Design and Methods*. This study was conducted on peripheral blood samples from 30 ALL patients (17adults=6.6% and 13children=43.3%; 19 males=63.3%and 11females=36.6%) were analyzed for the expression level of WT1 in mRNA using quantitative real time reverse transcriptase QR-PCR (using Taqman fluorescent probes; ABI prism 7700 Applied Biosystems). Pretreatment and post-induction samples were obtained, only 20 patients (66.6%) could be followed up . Eight samples from normal individuals matching in age and sex were used as control. For exact quantification of gene expression (WT1) , an endogenous reference (housekeeping gene ABL) was used to correct for differences in the amount of total RNA added to the reaction ,for compensation of different RT efficiencies and for compensation of PCR inhibitors in the sample. Standard curve were plotted and the exact quantities were estimated relatively as ratio of target to housekeeping gene. *Results*. Samples from patients newly diagnosed and others from relapsed patients(only 3 out of 20 who were followed-up 15%)proved to have significantly higher WT1 expression levels (p<0.01) compared to samples from patients in complete remission(CR)(85%)orhealthy individuals(controls).Our study revealed that rising ofWT1 expression could be used as an indicator of forthcoming relapse. *Conclusions*. WT1 represents a candidate prognostic molecular marker in acute lymphoblastic leukemia .Additional studies are needed in order to assess the potential role of WT1 in the monitoring of the patients response to treatment and in the detection of hematological relapse prior to its occurrence.

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DEPOCYTE TREATMENT FOR LYMPHOMATOSIS MENINGITIS - ROMANIAN WORKING GROUP FOR ADULT ACUTE LEUKEMIA' EXPERIENCE

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DepoCyte, a liposomal formulation of cytarabine with a prolonged half-life for intra-thecal (IT) administration has been shown to be safe and effective in the neoplastic meningitis. After legal availability of liposomal cytarabine in Romania (covered by National Health Insurance), Romanian Working Group for Acute Leukemia Study (RWGALS) have begun an observational study on patients with lymphomatous meningitis. Liposomal cytarabine was given to the 15 patients, aged 28-62 year, 50 mg as a single agent, on a different schedule (weekly, every-2-weeks, monthly or more rarely), concurrent with 16 mg systemic dexamethasone for 4 days, in order to reduce the incidence of the latter toxicity. Concurrent high doses systemic chemotherapy with agents that penetrate the blood-brain barrier as well as concurrent radiation therapy that potentiate the neurotoxicity associated with liposomal cytarabine were avoided, but conventional doses of chemotherapy were allowed for 3 patients with relapsed nonhodgkin lymphomas with central nervous system (CNS) disease and one patient with lymphoblastic phase of chronic myeloid leukemia (CML). Three patients with acute lymphoblastic leukemia (ALL) received Depocyte monthly as SNC prophylaxis schedule. One patient received Depocytes once a month as prophylaxis for SNC relapse after match related donor (MRD) allotransplant for B-ALL with meningitis leukemia. He had a very good tolerance with any adverse event at 10 months after stem cell transplant (SCT). A 28 year old patient with lymphoblastic blast-phase of CML with blasts cells meningitis had a very good liquor clearance of blasts cells and showed a very good tolerability with 10 times of Depocyte IT weekly administrations. Two patients with B-ALL experienced severe cytopenia but the relationship with Depocyte was not clear because they received in the same time systemic conventional chemotherapy. Depocyte had any influence on the diffuse large B cell lymphoma (DLBCL) cerebral tumor for a 55 year old female with SNC DLBCL relapse, and just before her death, IRM showed leptomeningitis. A patient had severe neurological adverse event and shown a CNS tuberculosis after 3 IT Depocyte administrations. Three patients had moderate intensity headache the day the drug was administrated. Death related disease appeared at 3 patients during Depocytes prophylaxis for SNC lymphomatousis disease. Liposomal cytarabine 50 mg given IT is an attractive alternative, considering the ability to reduce the frequency and total number of IT treatments and the potential to further efficacy improvement of the current CNS prophylaxis regimen and even curative regimen. Concurrently administration with corticosteroids reduces the incidence of arachnoiditis

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CEREBRAL THROMBOEMBOLISM IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Cerebral Venous Thromboembolism (CTVE) is an uncommon but severe complication in children affected by Acute Lymphoblastic Leukemia (ALL). Several risk factors have been described, the incidence may vary according to the risk profile of the patient. Objectives: 1. To know the incidence of the CVTE within the patients affected by ALL and treated in Hospital Sant Joan de Deu, Barcelona between January 2002 and December 2008. 2. To identify the risk factors associated to CVTE. *Aims*. A retrospective analysis of the children diagnosed of ALL between January 2002 and December 2008 in our Hospital. CVTE and their risk factors were reviewed. *Results*. During the referenced period 123 patients were diagnosed of ALL. Three of them (2%) presented CVTE during the induction treatment period. The mean age was 9.3 years. Two were ALL-B and one T. Two of them were treated according to the protocol of the Sociedad Hemato-Oncológica Pediátrica Española (SHOP) 1999 and one according to SHOP protocol 2005. All three belong to the ALL high risk patient group. The differences between the two protocols is the dose of L-Asparaginase (15.000 UI/m2/24 hours from day +15 to day +24 in SHOP 99; and 10.000 UI/m^2/48 hours from day +15 to day +33 in SHOP 2005). The most frequent symptom was a persistent headache (100%). Two patients had episodes of seizures associated. All patients were diagnosed by computerized tomography. Two of three patients had thrombosis of transverse sinus and the other longitudinal sinus thrombosis. Two patients had also lesions with hemorrhagic component. The basic study included the most important genetic risk factors for thrombosis (V Leiden factor, deficiency of Antithrombin III (AT III), Protein C and Protein S deficiency, Prothrombin Mutation G20210A). All three patients had low levels of AT III in the moment of the diagnosis. However one out of three was heterozygote for V Leiden Factor. The treatment included removal of L-ASP; infusion of frozen plasma and AT III; treatment of seizures with phenytoin (then GABA or Valproate); Low Molecular Weight Heparin (LMWH) (1 mg/kg/day if hemorrhagic component was found or 1,5mg/kd/day if not). After acute treatment further management was individualized depending on the clinical situation. Neurological recovery was complete in all of them, but two died 2 months and 10 months later after induction due to infectious complications. *Conclusions*-. The incidence of CTVE in children affected by ALL and treated in our Hospital is according to other publications. All the CVTE diagnosed during the ALL treatment appeared during the induction period. The presence of persistent headache during induction period is highly suspicious of CVTE. The treatment with plasma, antithrombin and LMWH was effective in all cases.

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RESULTS OF TREATMENT WITH HYPER-CVAD IN ADULT ACUTE LYMPHOCYTIC LEUKEMIA: SINGLE CENTER EXPERIANCE

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Background. Hyper-CVAD is a highly active regimen which has improved the outcome in adult ALL compared to conventional regimens, with complete remission (CR) rate of 80%, and a long-term survival rates ranging from 30 to 45%. *Aims*. To evaluate the efficacy of Hyper-CVAD in adults with de novo ALL, treated in the Institute of Hematology, CCS. *Design and Methods*. From November 2006 to November 2008, 17 newly diagnosed adult ALL patients, were treated with standard Hyper-CVAD regimen. Treatment consisted of four cycles (cy) of hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone, alternating with four cy of methotrexate (MTX) and ara-C, together with intrathecal CNS prophylaxis (MTX and ara-C on days 2 and 7 of the first 3-4 courses), and supportive care with antibiotic prophylaxis and granulocyte colony-stimulating factor therapy. Oral POMP (6-MP, vincristine, MTX, prednisone) maintenance was administred for at least 2 years. *Results*. Pretreatment patients characteristic were as follows: median age of 29 years (range: 18-55), 2 patients were ≥50 years old; 11/17 were male. Leucocytosis of more then 50x10^9/l was registered in 6/17 patients. CNS disease at diagnosis was present in 1/17 patients; mediastinal mass was not registered. FAB L1 type was present in 5/17, L2 in 10/17 and not classified/mixed in 2/17 patients. The T-cell

immunophenotype was present in 5/17, B in 11/17 and byphenotypic (B and T) in 1/17 patients. Myeloid marker positivity was registered in 4/17 patients. There was not mature B ALL. Philadelphia (Ph) positive disease was present in 3/17, and t(4;11) in 2/17. 10/17 of patients had a high risk for relapse according to Hoelzer prognostic model. Risk stratification for CR duration was assessed according to M.D.Anderson Cancer Center (age≥45 years, WBC≥50x10^9/L, ECOG score 3-4, Ph-positivity, French-American-British L2 morphology, > 1cy to achieve CR, day 14 bone marrow blasts > 5%) for 9 patients. Low and intermediate risk had 3 and 6 patients, respectively. Overall CR rate was 94% while one patient had resistant disease. There were not deaths during induction therapy. After a 24 months-long follow-up 9/17 patients remained alive, 8 of them in first CR, with median CR duration of 7.5 months (range 3-22) and median survival time of 12 months (range 8-23). Eight patients relapsed: two between the 4th cy and 5th, four between 8th cy and start of maintenance therapy, one during maintenance and one after allogenic SCT. This last patient co-expressed initially myeloid markers positive (CD13, CD113) and received 7 cy of Hiper-CVAD before transplantation. AML relapse occurred after allogenic SCT. Eight patients died: one due resistant disease and 7 after leukemia recurrence. Their median survival time was 11 months (range 6-23). Ph-positive patients had a CR of 100%, with median CR duration of 6 months and median survival of 12 months. One patient is still a live, without being transplanted. *Conclusions*. Hyper-CVAD is a highly aplicatable regiment for ALL treatment with acceptable toxicity profile.

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CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: A SINGLE CENTER EXPERIENCE

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Background. Childhood Acute Lymphoblastic Leukemia ALL, is the most common malignancy in children. The survival rates for ALL have been improving steadily during the past four decades, and reaching 80% in developed countries. This improvement can be attributed, in part, to intensification of therapy using agents previously shown to be effective in the treatment of ALL, in addition to the risk-adapted treatment, taking into account clinical features and biologic features, which are used to maximize cure rates while minimizing long-term side effects. *Aims*. To study the outcome (overall survival and event free survival for Acute Lymphoblastic Leukemia (ALL) in children at Sheikh Khalifa Medical City (SKMC). *Design and Methods*. All children age 0-13 years that were diagnosed with ALL and started on treatment at SKMC between 2000 and 2008, were studied retrospectively with respect to their clinical, immunological, and cytogenetic features at presentation and their relation to the treatment outcomes. Kaplan-Meier and Cox Proportional-Hazards regression were used for statistical analysis. *Results*. A total of 44 patients were eligible for the study, Mean age at diagnosis was 5.2 years (1-13.4 years) and median age 4.3 years. There were 23 males and 21 females with ratio of 1.1:1. 37 (84%) had B-lineage and 7 patients (16%) had T-lineage ALL. All patients received risk adapted treatment according to Children Oncology Group (COG) risk categorization which included 20 (45.5%) and 24 (54.5%) patients in standard and high risk groups respectively. The mean duration of follow up was 2.6 years (3 months- 7.5 years) and median of 2.45 years. All patients achieved remission at the end if induction. 34 patients had evaluation for early response based on the bone marrow study on day 7 or/and day 14 of induction, Only 4 (12%) patients had slow early response (SER) and the remaining 30 (88%) patients had rapid early response (RER). 5-year Overall survival probability (OS) was 90.9±8.3 and 5-year Event Free Survival (EFS) was 91.5±6.7. There were no statistical significant differences in both OS and EFS among different risk groups including the age, gender, or lineage. *Conclusions*. According to our data, children with acute lymphoblastic leukemia who were and being treated at SKMC. Although the total number of patients who were eligible for the study is small, however the survival analysis indicates that the OS and EFS are superb and this result is comparable to the major pediatric oncology center. The lack of significant influence of the COG and/or NCI risk factors on the survival probable is due to the small number of the cohort or/and the risk adapted treatment those patients have received.

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PREVALENCE OF BONE DISEASES IN PEDIATRIC HEMATOLOGY/ONCOLOGY PATIENTS

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Background. Osteopenia and osteoporosis are known to occur frequently in children with cancer at diagnosis, during and after completion of chemotherapy. Moreover, many studies in the literature have reported pathological fractures during and after therapy, osteonecrosis and skeletal abnormalities. Many therapeutic regimens in cancer treatment carry the risk of causing or favouring the development of osteoporosis. *Aims*. To assess the prevalence of bone disease among paediatric oncology in our institute. *Design and Methods*. A total of ninety seven patients were enrolled in the study age range from 1- 16 years old. Fifty children with acute lymphoblastic leukaemia (ALL), ten patients with AML, twelve with NHL and twenty five children with other malignancies during 2007 and 2008. These patients were treated and followed at the King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia. All patients were assessed clinically. Blood and urine samples were obtained for the determination of biochemical and hormonal profiles included PTH, 25 OH vitamin D3. Bone maturation was assessed by radiological bone age. Bone mineral density (BMD) by DEXA was determined patients. Bone formation markers (bone-specific alkaline phosphate, CTX and osteocalcin) and bone resorption markers were analysed for patients whom had BMD and referred for treatment. Results: High prevalence of reduced low bone mass (LBM) among the majority of screened patients. BMD is reduced in early phase of disease. Patients with ALL had significantly reduced lumbar volumetric (-0.77) and femoral areal and volumetric BMDs (-1.02 and -0.98, respectively). In patients with other malignancies, femoral areal and apparent volumetric BMDs were significantly decreased (-0.70 and -0.78, respectively). Half of the patient associated with hypovitaminosis D. *Conclusions*. High prevalence of osteopenia among patient with oncology disorders. A follow up of BMD during and after chemotherapy is highly indicated. Causes of osteoporosis are multifactorial: related to disease nature, antineoplastic treatments, physical inactivity, and non healthful lifestyle. Prevention can be achieved with early diagnosis and treatment also through supplements by vitamin D and calcium, encouraged exercise and sun exposure.

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THE ROLE OF DYNAMIC RESEARCH OF SERUM FERRITIN LEVEL IN CHILDREN WITH ACUTE LEUKEMIA

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Background. Failure of iron methabolism are described for children with acute leukemia. Enhanceable maintenance of iron in the organism of patients can be related both to initial disturbances of ironkinetics processes and by the phenomena of diserithropoiesis, or red blood cells transfusions . The iron overload can influence on activity of all organs and systems of organism in a whole. *Aims*. to investigate the serum ferritin level (SFL) of children with acute leukemia in the dynamics of leukemia process taking into account the phenomenon of diserithropoesis (morphological changes in erythroid elements and bone marrow syderoblasts number), amount of red blood cells transfusions , life-span of patients and reasons of their death. *Design and Methods*. 128 children with acute leukemia are inspected (ALL - 98, AML - 30). Clinical, morophological, byochemical, immunoenzyme and statistical methods were used. *Results*. At 60% of children the debut SFL was higher as compared to normative level (r<0,01). In the dynamics of leukemia process the SFL rose also. In the I acute period of disease this index was 338,2±10,4 ng/ml, in the II acute period - 794,5±21,6 ng/mL and in the III acute period - 1274,4±43,8 ng/ml. Direct cross-correlation dependence is revealed between SFL - number of bone marrow sideroblasts (r= + 0,51), SFL - diserythropoiesis manifestation, especially in the period of hemopoiesis renewal (r=+ 0,37), and SFL - red blood cells transfusions number (r= + 0,47). SFL did not influence on life-span of children (r= - 0,19). However, for children with debut SFL 864,1±18,3 ng/mL different manifestations of cardiac - vascular failure were detected and duration of these patient's life was 27,7±2,2 years, that in 2,1 time shorter as compared to children with SFL less than 200 ng/ml. *Conclusions*. Serum ferritin level in children with acute leukemia is the marker of diserythropoiesis and criterion of prognosis of cardio-vascular complications . This research is needed for timely diagnostics of haemopoiesis failure and providing of prophylaxis measures of complications in course of acute leukemia in children.

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ACUTE NEUROTOXICITY SECONDARY TO TREATMENT OF PEDIATRIC ACUTE LIMPHOBLASTIC LEUKEMIA

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Background. Central nervous system (CNS) prophylaxis is a central element in the treatment of childhood acute lymphoblastic leukemia (ALL). However, acute neurotoxicity has been reported during this treatment, although the exact incidence of neurotoxicity complications is difficult to determine (3.7-7.8%). *Aims*. To know the incidence of acute neurotoxicity in patients affected by ALL and treated in a single University Hospital in Barcelona between January 1993 and December 2008. To identify the drugs associated to this toxicity. *Design and Methods.* A retrospective study was performed to describe severe neurological complications (grade III-IV) in children treated for ALL in a single center during a 15-year period. The patients were treated according to four sequential trials of the "Sociedad española de hematología y oncología pediatrica" (SHOP). *Results.* During this period 211 children were diagnosed with ALL at our institution. Eleven of them (5.23%) presented acute neurotoxicity. The median age was 5 years. Nine cases were ALL-B and one ALL-T (7 high risk, 2 very high risk and 2 standard risk patient). Neurological symptoms occurred in induction (n=4 cases), consolidation (n=3) and continuation (n=4) treatment and included: transient neurological deficits in 4 patients, seizures in 4 cases, severe headache in 1 and polyradiculoneuritis in 2 patients. Cranial computed tomography and magnetic resonance imaging showed CNS thromboembolism in 3 of the patients (2 of them with secondary hemorrhage), suggestive images of leucoencephalopathy in other 3 cases and CNS stroke in 1 of them. The related drugs were intrathecal methotrexate as well as systemic high-dose methotrexate in six of the patients, asparaginase in three (thrombo-hemorrhagic events), and vincristine in two of them (polyradiculoneuritis). In most children the symptoms improved in 24-48 hours; however four patients had a poor outcome with persistence of neurological damage. *Conclusions*. The incidence of acute neurotoxicity in our series is similar to that previously reported. Drugs involved in neurological complications were methotrexate, asparaginase and vincristine. Drug neurotoxicity is a rare but severe complication of treatment of children with ALL that in some cases can have devastating sequelae.

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IMPACT OF CYTOGENETICS ON THE OUTCOME OF ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: RESULTS FROM A SINGLE INSTITUTION

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Background. Except for the t(9;22), the impact of cytogenetics on prognosis in adults with acute lymphoblastic leukemia (ALL) is much less clear than in childhood ALL. *Aims*. To assess the prognostic significance of cytogenetics in a relatively large series of adults with ALL from a single institution. *Design and Methods.* From January 1977 to July 2008, cytogenetic analysis was carried out in 206 adults with ALL at our hospital. The median age was 34 yrs (range, 15-90) with a ratio M/F of 1.48:1. Most patients were treated with intensive chemotherapy PETHEMA regimens (LLA-93, LLA-96 and LLA-2003). Complete remission (CR) was achieved in 84%. Fourteen patients underwent allogeneic stem cell transplantation in first CR (7 ALL-Ph+ and 7 with other high-risk characteristics, such as hyperleukocytosis or slow response to induction therapy). Results: Ninety eight patients (47%) had inadequate cytogenetic analysis, either by absence or poor-quality metaphases. Of 108 patients with evaluable karyotype, 35 (32%) were normal (NK), whereas 73 patients (68%) had a clonal cytogenetic abnormality. The most frequent abnormality was t(9;22) (n=24), followed by t(8;14) (n=7) and del(9p) (n=6). The remaining 36 patients had t(4;11) (n=4), t(14q11) (n=2), del(6q) (n=2), abn(11q23) (n=1), complex karyotype (n=1) or other numerical (n=7) or structural abnormalities (n=10). Nine additional patients were classified according to the grade of ploidy as low hypodiploidy (<39 chromosomes) (n=3), low hyperdiploidy (47-49 chr.) (n=1) and high hyperdiploidy (>60 chr.) (n=5). The impact on the outcomes was assessed in 99 patients who received intensive treatment. Patients with NK or del(9p) showed a better outcome (standard-risk group) than patients with the rest of abnormalities (high-risk group). Overall Survival (OS) of the standard-risk group was significantly longer compared with the high-risk group (median OS 37 vs. 12 months, respectively, p=0.048). Relapse-free survival was also statistically different between these cytogenetic groups (52 vs. 11months, p=0.023). Conclusions: Cytogenetic abnormalities were found in 68% of adults with ALL, being t(9;22) the most frequently observed. According to karyotype, two risk groups were identified: the standard-risk group, which included patients with normal karyoptype and del(9p), and the high-risk group (all other abnormalities).

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ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN ASSOCIATED WITH 1P36 DELETION SYNDROME

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Background. Deletion of material from chromosome 1p (1p36 region) is common in neuroblastoma. It is possible that a tumor suppressor gene may be present in this region, though no specific gene has yet been identified. Monosomy 1p36 is a recently delineated contiguous gene syndrome, which is now considered to be the most common subtelomeric microdeletion syndrome. From the recent literature it appears as if 1p36 deletions account for 0.5-1.2% of idiopathic mental retardation. The deletions can be detected by high resolution cytogenetic studies in a

minority of patients, and fluorescence *in situ* hybridisation (FISH) is required in most. 1p36 deletion syndrome is characterized by distinct craniofacial features, associated with developmental delay/mental retardation, hypotonia, muscle hypotrophy, seizures, brain abnormalities (ventricular enlargement), optic nerve atrophy, deafness and heart defects. *Design and Methods*. We diagnosed in June 2000 a 1 year old girl with acute lymphoblastic leukemia, L1 FAB, B common CD10+. At that time we cannot perform cytogenetic and molecular diagnostic. She was treated with INTERFANT protocol and she obtained first complete remission (CR1). In 2004 she relapsed with the same morphological and immunophenotypic aspect and she was treated according COOPRALL 97 protocol obtaining CR2. The follow-up relieved a moderate mental retardation, ventricular enlargement on MRI and optic nerve atrophy with secondary cecity. We consider at that time these complications as secondary effects after intrathecal chemotherapy. Four year later, in 2008, she relapsed again and we performed complete diagnostic in Gaslini Institute, Genoa and we observed the same morphological and immunophenotyping aspect. The cytogenetic analysis showed hipoploidy associated with complex cariotype and 1p36 syndrome - 44XX, del(1)(p36), -7, -12, add(12)(p13), -15, del (15)(q15)+mar. The patient failed to obtain CR3 with FLAG protocol and died secondary to pulmonary and cerebral aspergillosis. *Conclusions*. The 1p36 deletion syndrome wasn't reported until now in association with acute leukemia, but is common in patients with neuroblastoma. It is associated with poor outcome with standard chemotherapy and further studies are required to a better understanding of malignant process.

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TREATMENT RELATED ADVERSE EVENTS IN ADOLESCENTS AND YOUNG ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA TREATED WITH PEDIATRIC PROTOCOLS

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Background. Historically, adolescents and young adults (AYA) have a worse outcome than children that had been attributed to higher incidence of T-immunophenotype and less favourable cytogenetics. Recent reports suggest that AYA with acute lymphoblastic leukemia (ALL) have far superior outcomes when treated on more intensive pediatric regimens. Current BFM based pediatric protocols are characterized by dose-intensive use of non-myelosuppressive drugs such as vincristine, L-asparaginase, corticosteroids and continuous antimetabolite-based maintenance. However, toxicity increases with age and children >10 years have a higher incidence of side effects especially of glucocorticosteroids and L-asparaginase. *Aims*. To assess treatment related adverse events and tolerability of current pediatric BFM based ALL protocol applied in AYAs. *Design and Methods*. Four male patients with newly diagnosed B-lineage ALL aged 17-20 years old at diagnosis treated with intensive chemotherapeutic regimen according to the high risk arm of the ALL-BFM 2000 protocol in a single Department of Pediatric Hematology-Oncology from 2006-2008 were enrolled. AYA accounted for the 22% of patients with ALL treated in the Department during this period. No patient had Ph+ ALL. *Results*. Three out of the four patients presented delayed methotrexate (MTX) excretion at the indicated by the protocol dose in the high-risk blocks and received reduced doses at subsequent administration. All received rescue therapy with carboxypeptidase G2. One patient presented substantially elevated triglyceride levels (up to 2385 mg/dL) correlated with asparaginase administration at the induction and subsequent treatment phases that returned to normal with dietary restrictions. Other metabolic disorders such as drug-related diabetes mellitus were observed in one patient after steroid administration. Three patients presented high levels of both SGOT and SGPT during different treatment phases, with maximum values 1025U/l and 2068U/l, 819U/l and 918U/l, 741U/l and 345U/l respectively for each patient, the third patient with concomitant elevation of both γGT and bilirubin. Infectious-inflammatory complications were also observed. One patient was diagnosed with pulmonary aspergillosis and subsequently a severe serositis occurred that responded only to corticosteroid administration. Another patient presented a severe pneumonitis that necessitated admission to Intensive Care Unit. The oldest patient in this study developed severe aseptic femoral necrosis due to high dose dexamethasone in intensification blocks and a severe peripheral neuropathy due to vincristine. No deaths occurred during induction or in first remission. One patient had resistant ALL, with early extramedullary relapse after matched sibling donor bone marrow transplantation and succumbed to the disease. *Conclusions*. Although older patients seem to have less tolerance to L- asparaginase, steroids and vincristine compared to children, treatment-related side effects are generally manageable. The administration of pediatric regimens to adolescent and young adults although offering better outcomes compared to protocols designed for adult patients, poses certain difficulties over treatment toxicity and tolerance that may result in significant protocol modifications or delays in the treatment completion. Further prospective studies could reassess the treatment of adolescents and young adults as well as the tolerability and efficacy of pediatric dose-intensive protocols.

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OUTCOME OF THE >55 YEAR OLD PATIENTS WITH ACUTE LEUKEMIA - RESULTS OF THE ROMANIAN WORKING GROUP OF ACUTE LEUKEMIA STUDY

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Acute leukemia patients aged more than 55 years are a challenging field for the therapeutical approach. On the one side many of them have a good condition for intensive chemotherapy, on the other side the curative treatment like myeloablative stem cell transplantation is prohibitive for these patients. We conducted a retrospective study on the outcome of the acute leukemia patients aged more than 55 years in charge on the last 5 years. We had 213 acute myeloblastic leukemia (AML) patients and 116 acute lymphoblastic leukemia (ALL) patients. Median age for ALL patients was 64 (the upper limit was 83 years) and for AML, median age was 68 (the upper limit was 82 years). Intensive chemotherapy or low dose chemotherapy were chosen according to the comorbidities of the patients. Any patient received stem cell transplantation. 58% of the AML patients received conventional intensive chemotherapy and 40% low doses chemotherapy. 68% of the ALL patients received conventional intensive age adapted chemotherapy and 28% palliative treatment with vincristine and prednisone, or mercaptopurine and low doses of metotrexate. 40% of the AML patients and 67% of the ALL patient receiving conventional intensive chemotherapy had complete remission (CR). Only 5% of the ALL patients and 7% of the AML patients with low doses chemotherapy achieved CR. All patients with ALL Ph+ achieved CR with chemotherapy and low dose imatinib (100-200 mg/d). ALL Ph+ patients were only 5, due to the fact that genetic approach for acute leukemia diagnosis was not routinely performed. EFS for ALL patients was average 8 months and for AML patients 5 months. OS for ALL patients was average 14 months and for AML patients 11 months. 72% of all deaths were infections related and disease related in the same time and only 25% were vital organ failure and age related in the same time. Five percent of the aged patients had a second solid malignancy (genital, gastrointestinal). From all more than 55 years old patients, 96 requested transfusion supplies, and 75% requested growth factor or erythropoietins treatment. Seventy percent of aged patients showed severe depression and requested psychological assistance. Romanian National standards have upper age limit for SCT at 55 years. Outcome of the more than 55 year old acute leukemia patients wiould be improved by new drug formulation or target new drug. A high incidence of psychological disturbance characterize the third age acute leukemia patients.

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PREDECTION OF EARLY RELAPSE OF ACUTE LYMPHOPLASTIC LEUKIMIA BY INFRARED SPECTROSCOPY

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Background. Despite advances in the treatment of childhood acute lymphoplastic leukemia (ALL), the risk of relapse remains about 30%. The presence or absance of residual disease or early relapse has been assesed by flow cytometry and PCR. *Aims*. To assess early relapse or minimal residual disease by Fourier transform infrared spectroscopy (FTIR) *Design and Methods*. Adeeb a known case of Acute biphenotypic leukemia (myeloid and B lineage), off chemotherapy for one month which had been re-admitted to Pediatric wards at King Abdulaziz University Hospital, Jeddah, KSA, because he had progressive weakness in both lower limbs, neurologic examination showed bilateral symmetri-

cal ascending paraplegia with intact cranial nerves. The patient has been assessed clinically and laboratory. Repeated CSF and bone marrow examination were normal and negative for blasts. MRI brain and spine were normal, given a differential diagnosis of transverse myelitis or Gillian-Barre' syndrome. Patient diagnosed at that time as leukemia in remission. Bone marrow samples were collected from this case at different events to be studied by Fourier transform infrared spectroscopy (FTIR). The first sample while the patient in remission at the end of his treatment (Adeeb I).The second sample after the patient presented to pediatric ward with complete paralysis of the lower limb (Adeeb 2), 2 months later he had symptoms suggestive of increased intracranial tension, another workup revealed combine BM & CNS relapse B-lineage ALL, a third sample was sent. A fourth sample analysed after the patient had induction and two cycles of chemotherapy and radiotherapy. All samples were kept at -80oC and lyopholized prior to FTIR measurements. The lyopholized samples were dispersed in potassium bromide (KBr) to form 1% concentration discs.

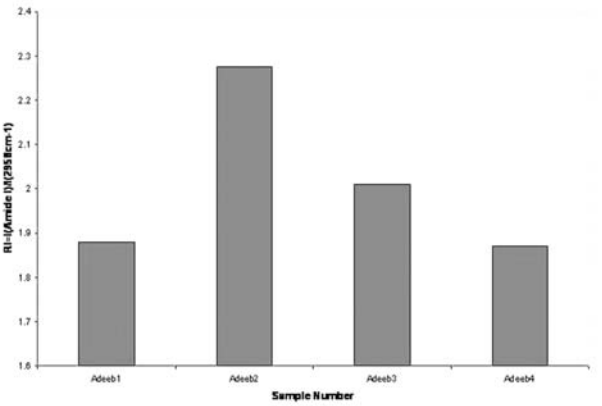

Fig.2. Variation in I(Amide I)/ I(2958cm-1) Ratio from Adeeb 1,2,3 and 4 bone marrow samples. The data points are the average of three different measurements on each sample

Figure.

Results: Fourier transform infrared spectroscopy (FTIR) was explored as a means to distinguish newly diagnosed of acute leukemia from disease free bone marrow samples. Characteristic bands alterations were identified in both healthy and diseased samples arising from cellular protein, lipid and DNA. There were specific changes that affected the secondary structure of proteins appeared in the FTIR spectra confirmed with the second derivative analysis. The overall protein structure in the control sample consists primarily of α-helix, whereas in ALL sample it has a relatively high proportion of anti-parallel β-sheet proteins constituents presumably due to leukemia. Different absorbance's ratios for specific bands were calculated and plotted versus the patient samples. There are significant fluctuations in the ratios under investigation which can be attributed to the changes in the biomolecular structure between normal and leukemic samples Our results indicate that the absorbance of amide A and B in the range 3340-3000, the lipid/protein ratio and the phosphate/amide II ratio are all yielding statistically significant differences parameters, that it can be used as a biomarker in differentiating acute leukemia (samples 2 & 3) from leukemia free bone marrow (sample 1 & 4), also a good predictor of an early relapse, as seen in Figure 1 (samples 1 & 2). *Conclusions*. This study provides evidence that FTIR spectroscopy is a valuable tool in detecting early relapse and minimal residual disease. Several interesting and consistent spectral differences between patient and control spectra may be considered as a promising basis for a future study with large number of samples and as a routine study in predicting minimal residual disease.

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INCREASING *DE NOVO* SYNTHESIS OF CERAMIDES INCREASED NILOTINIB INDUCED APOPTOSIS IN HUMAN MEG-01 CHRONIC MEGACARYOBLASTIC CELLS

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Background. The sphingolipids are a family of membrane lipids with important structural roles in the regulation of the fluidity and subdomain structure of the lipid bilayer. These molecules have significant roles in cell growth, proliferation, apoptosis, cell migration and senescence. Many sphingolipid-regulated functions have significant and specific links to various aspects of cancer initiation, progression and response to anticancer treatments. It is very well known that accumulation of intracellular ceramides induce apoptosis while the conversion of ceramides to some other metabolites result in resistance to anticancer agents. Nilotinib is a novel anti cancer agent used for the treatment of chronic myeloid leukemia. *Aims*. In this study, we tried to examine the possibility of increasing sensitivity of Meg-01 cells to dasatinib by increasing intracellular concentrations of ceramide through exogenous addition of C:8 ceramide to growth medium. By this way de novo synthesis of ceramide is induced and ceramide accumulation is provided. *Methods*. The Ph (+) human Meg-01 cells were exposed to increasing concentrations of nilotinib and a ceramide analog (C8:ceramide) and combination of both. IC50 values of nilotinib and C8:ceramide was calculated from cell proliferation plots obtained 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. Results: IC50 values of nilotinib and C8:ceramide were found to be 2.2 nM and 70 μM in Meg-01 cells. There were 19-, 22-, 45-, and 82% decreases in cell proliferation in 0.01-, 0.1-, 1-, and 10 nM nilotinib applied Meg-01 cells, respectively, while the combination of these doses with 70 μM C8:ceramide resulted in 72-, 76-, 80-, and 88% decreases in cell proliferation, respectively, as compared to untreated controls. There were 1.9- and 3.62 times increases in caspase-3 enzyme activity in 1- and 10 nM nilotinib applied cells while their combination with 70 μM C8:ceramide resulted in 9.67- and 12.54 times increases comparing to untreated controls. 70 μM C8:ceramide alone resulted in 2.1 times increase in caspase-3 enzyme activity. Similar results were obtained for changes in MMP. There were 1.1- and 1.2 times increases in MMP in 1- and 10 nM nilotinib applied cells while their combination with 70 μM C8:ceramide resulted in 22.3- and 26.1 times increases comparing to untreated controls. 70 μM C8:ceramide alone resulted in 10 times increase in MMP. *Conclusions*. By these results we have shown for the first time that increasing de novo synthesis and accumulation of C8:ceramide resulted in significant sensitivity of Philadelphia positive chronic megacaryoblastic cells to nilotinib.

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DETECTION OF BCR-ABL BREAKPOINTS IN SAMPLES OF CML PATIENTS UTILIZING THE QUANTITATIVE RT-PCR ASSAY COMBINED WITH THE MELTING POINT ANALYSIS USING SYBR GREEN

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Background. Chronic myeloid leukemia (CML) is a model of a disease with targeted and tailored therapy. Monitoring of CML patients, treated by tyrosine kinase inhibitors, is based on evaluation of hematological, cytogenetic and molecular response. Quantification of BCR-ABL fusion gene transcripts in leukemic blasts are measured by quantitative real time PCR with reverse transcription (QRT-PCR). The type of BCR-ABL breakpoint is typically performed by nested reverse transcription PCR (RT-PCR) and visualized on agarose gel. *Aims*. To characterize the most frequent BCR-ABL breakpoint types (b3a2 and b2a2) using a simple melting point analysis with SYBR Green instead of the time-consuming nested RT-PCR simultaneously with quantification of BCR-ABL transcript´s levels by QRT-PCR assay. Methods. Samples from CML patients (n = 162), which have been treated with tyrosine kinase inhibitors, were obtained from 6 hematological clinics in Slovakia. RNA was isolated from PK or BM after separation of WBC and stabilization in RNAlater solution. cDNA was obtained by using TaqMan Reverse Transcriptase system. RQ-PCR was performed by real time PCR system using a specific BCR-ABL TaqMan probes. The quantification of fusion transcripts were evaluated according to ELN guidelines as BCR-ABL/ABL

ratio (NCN values = Normalized copy number). The type of BCR-ABL breakpoint was provided by the denaturation melting point analysis on real time PCR systems using SYBR Green and universal primers for major-BCR-ABL fusion transcripts b3a2 and b2a2 used in QRT-PCR assay (M-bcr_For: 5´TCCGCTGACCATCAAYAAGGA, ABL_Rev: 5´CACTCAGACCCTGAGGCTCAA). The amplification profile was identical as in TaqMan quantification protocol but with additional dissociation step. Control samples were verified by nested RT-PCR and the type of breakpoint was detected on 1.5% agarose gel.

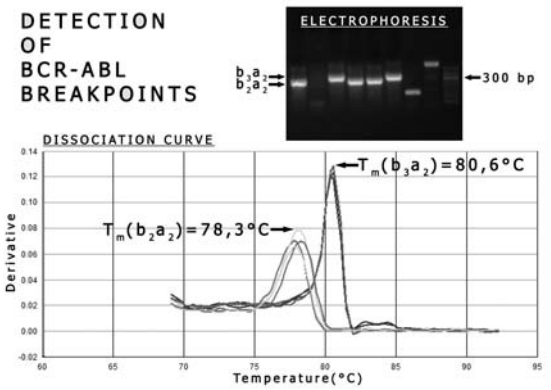

Figure 1. Detection of BCR-ABL breakpoints.

Results. Quantification of BCR-ABL fusion transcripts of CML patients by QRT-PCR has been provided every 3 months and results were expressed in NCN values. During the first period of our study types of BCR-ABL breakpoints were detected by nested RT PCR. Samples (cDNA) with b3a2 and b2a2 breakpoints were used as positive controls in denaturation melting point analysis in the second part of our study. This method utilizing the incorporation of SYBR green during amplification and its release at melting temperature (Tm) was used. From the derivative curve of amplicons, melting temperatures of Tm= 78.3±0.5°C for b2a2 and of Tm= 80.6±0.5°C for b3a2 were obtained. Preliminary results (n=52) were obtained from newly diagnosed and monitored patients with at least value NCN ≥0.005 (average NCN= 1.2). *Conclusions*. We have established a simple method for the detection of most common breakpoint BCR-ABL fusion transcript type (b3a2 and b2a2) by denaturing melting point analysis using SYBR green. This analysis can be in general provided simultaneously with BCR-ABL quantification using TaqMan probes on real time PCR system.

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EFFECT OF IMATINIB MESYLATE ON PLATELET AGGREGATION ABNORMALITIES IN BCR-ABL + CML PATIENTS

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Background. Bleeding and thrombotic events complicate the clinical course of chronic myeloproliferative disorders (CMPDs). A spectrum of platelet aggregation abnormalities have been described in CML. Clonal involvement of megakaryocytopoiesis is regarded as the main origin of these abnormalities. Effects of cytoreductive drugs on platelet aggregation abnormalities have been studied which showed no significant corrective effect. Effect of Imatinib mesylate on platelet aggregation abnormalities has not been studied so far. *Purpose of the study*. Our study aims at assessing the effect of imatinib mesylate on platelet aggregation abnormalities in BCR-ABL positive CML patients. *Design and Methods.* A total of 50 newly diagnosed BCR-ABL positive CML patients and 30 normal healthy volunteers were enrolled in the study. BCR-ABL by RT-PCR and Platelet aggregation parameters by CHRONOLOG aggregometer using arachidonic acid (AA), collagen, adrenaline (ADR), adenosine diphosphate (ADP) and ristocetin as agonists were measured both at diagnosis and after a minimum of 3 months of imatinib therapy. Platelet aggregation parameters measured for each agonist were Degree of aggregation (DOA) and Rate of Aggregation (ROA). DOA was calculated by increase of transmissions for 1 minute/ total maximum transmissions in 2 minutes measurement of platelet aggregation and expressed as percentage. ROA was calculated as the number of 1mm squares under the platelet aggregation curve in 60 seconds. These parameters were calculated for each agonist for all patients and controls and their mean + 2SD were analyzed. *Results*. Forty-eight cases were in chronic phase (CP), accelerated (AP) and blast phase (BP) constituted one each. DOA and ROA parameters in 39/48(81%) cases in CP and both AP and BP cases measured with ADP, AA, and ADP as agonists in the pre-imatinib phase showed a statistically significant lower values compared to healthy controls. 3/48 (6%)CP cases showed hyperaggregation with ADR only and 6/48(13%) showed normal aggregation parameters. In the post-imatinib phase, the abnormal aggregation parameters normalized in 32/39(82%) CP cases. 7/39 (18%) CP, AP and BP cases persistently showed lower values. No significant difference is noted in parameters measured using ristocetin and collagen as agonists when compared among pre and post imatinib phases and controls. *Conclusions*. The normalization of aggregation parameters may be attributed to either partial or complete suppression of clonal abnormal megakaryocytopoiesis and restoration of normal megakaryocytopoiesis by imatinib mesylate therapy in BCR-ABL positive CML.

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ACCUMULATION OF APOPTOTIC CERAMIDES INCREASED APOPTOTIC EFFECTS OF NILOTINIB IN PHILADELPHIA POSITIVE MEG-01 CELLS SYNERGISTICALLY

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Background. Glycolipids, in addition to being essential membrane structural elements, are involved in cell proliferation, differentiation, and oncogenic transformation. Several findings by our and some groups suggest that alterations in ceramide metabolism could be, at least in part, responsible for the acquisition of a multidrug resistance (MDR) phenotype in cancer cells. The roles of ceramides in apoptosis were demonstrated by the finding that alterations in ceramide metabolism, whereby pro-apoptotic ceramide is converted to its non-cytotoxic glucosyle ceramide (GlcCer) metabolite. Several tumor cell lines and clinical samples have been shown to overexpress the glucosyl ceramide synthase (GCS) enzyme, which transfers glucose from UDP-glucose to ceramide and produces GlcCer. Accumulation of GlcCer is a characteristic of some multidrug-resistant cancer cells of breast, ovarian, colon, and epithelioid carcinomas. Nilotinib is a recently developed agent used for the treatment of philadelphia positive chronic myeloid leukemia. *Aims*. In this study, we investigated a strong GCS enzyme inhibitor, PDMP, induced chemosensitization to nilotinib in philadelphia positive human Meg-01 chronic megacaryoblastic leukemia cells. It was observed that a non-toxic dose of PDMP induced apoptosis in Meg-01 cells synergistically in response to nilotinib. *Design and Methods*. The Ph (+) human Meg-01 cells were exposed to increasing concentrations of nilotinib and PDMP and combination of both. IC50 value of nilotinib and IC90 value of PDMP was calculated from cell proliferation plots obtained 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. Results: IC50 value of nilotinib and IC90 value of PDMP were found to be 2.2 nM and 50 μM, respectively, in Meg-01 cells. There were 19-, 22-, 45-, and 82% decreases in cell proliferation in 0.01-, 0.1-, 1-, and 10 nM nilotinib applied Meg-01 cells, respectively, while the combination of these doses with 50 μM PDMP resulted in 67-, 72-, 74-, and 95% decreases in cell proliferation, respectively, as compared to untreated controls. To get more specific results about the synergistic effects of these agents, we performed caspase-3 and MMP analyses. There were 1.08- and 1.91 times increases in caspase-3 enzyme activity in 0.1- and 1 nM nilotinib applied cells while their combination with 50 μM PDMP resulted in 2.45- and 2.59 times increases comparing to untreated controls. 50 μM PDMP alone resulted in 1.42 times increase in caspase-3 enzyme activity. Similar results were obtained for changes in MMP. There were 1.24- and 1.85 times increases in MMP in 0.1- and 1 nM nilotinib applied cells while their combination with 50 μM PDMP resulted in 3.21- and 3.62 times increases comparing to untreated controls. 50 μM PDMP alone resulted in 1.5 times increase in MMP. *Conclusions*. By these results we have shown for the first time that increasing intracellular ceramide concentrations by inhibition of conversion of apoptotic ceramide to anti-apoptotic glucosyl ceramide increased apoptotic effects of nilotinib in human Meg-01 cells.

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GENETIC VARIANTS IN THE CANDIDATE GENES OF THE APOPTOSIS PATHWAY AND SUSCEPTIBILITY TO CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder, characterized by the presence of BCR/ABL fusion gene. It is unclear which cellular events drive BCR/ABL gene translocation or initiate leukemogenesis in CML. Bcl-2 promotes survival of hematopoietic stem cells. Accordingly, apoptosis-related pathway may involve in the leukemogenesis of CML. In the current study, we evaluated 80 SNP markers involved in the pathways of apoptosis (n=30), angiogenesis (n=7), myeloid cell growth (n=14), xenobiotic metabolism (n=13), WT1 signaling (n=7), interferon signaling (n=4) and others (n=5) in 170 CML patients and 182 healthy controls. In a single marker analysis, following SNPs were identified including VEGFA, BCL2, CASP7, JAK3, CSF3 and HOCT1. In the multivariate logistic model with these SNPs and covariates, only BCL2 (rs1801018) was significantly associated with the susceptibility to CML (p=0.05; adjusted-OR 2.16, [1.00-4.68]). In haplotype analyses, haplotype block of BCL2 consistently showed significant association with the susceptibility to CML. Risk allele analysis showed that a greater number of risk alleles from BCL2 SNP correlated to increasing risk of CML (overall p=0.1, OR 1.84, [1.06-3.22] for 3-4 risk alleles vs. 0-1 risk alleles). The current study indicated that BCL2 SNP seemed to be associated with increasing susceptibility to CML.

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IN VITRO ACTIVITY OF TYROSINE KINASE INHIBITORS ON NATURAL KILLER CELLS FROM CML PATIENTS AND HEALTHY CONTROLSB

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The introduction of orally available tyrosine kinase inhibitors (TKIs) for the treatment of chronic myelogenous leukemia (CML) had an enormous impact on the outcome of this disease. However, different resistance mechanisms or intolerance, which may be overcome in the majority of patients by second-generation TKIs, and growing evidence that TKIs can display immunomodulatory or even immunosuppressive effects, are the major obstacles for this therapy. Natural killer (NK) cells have the capacity to detect changes in transformed cells in the absence of inflammatory signals and may therefore be involved in leukemia cell killing. To date, the knowledge regarding the effect of TKIs on natural killer cells is limited. Thus, aim of the study was to analyze the effect of imatinib mesylate and nilotinib on NK cells. The *in vitro* activity of these compounds was tested on NK cells from five CML patients and three healthy controls. Their effectiveness was analyzed regarding NK-specific surface molecule expression, cytotoxicity and interferon-γ release. CD56 expression of NK cells from CML patients and healthy controls remained unchanged after exposure to imatinib and nilotinib at various concentrations. Further, CD16 expression of NK cells was not influenced by nilotinib and and low-dose imatinib mesylate, but exposure to higher concentrations of imatinib mesylate (25, 50 μM) significantly diminished CD16 expression of normal and leukemic NK cells. After a 24-hour incubation with imatinib mesylate (10 μM) and nilotinib (5 μM) the number of viable NK cells decreased: This cytotoxic effect was more pronounced for leukemic NK cells compared to normal NK cells. Natural killing capacity and interferon-γ production of NK cells decreased after treatment with imatinib mesylate to a similar degree in CML-derived and normal NK cells. Notably, the suppressive effect of nilotinib on natural cytotoxicity was less pronounced. In summary, imatinib mesylate and nilotinib at higher concentrations can exert negative *in vitro* effects on the viability and cytotoxicity of NK cells from CML patients and healthy controls.

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EVALUATION OF HUMAN TELOMERASE REVERSE TRANSCRIPTASE (HTERT) EXPRESSION IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA (CML) AT DIFFERENT PHASES OF THE DIDEASE

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Background. Chronic Myelogenous Leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by a translocation between chromosome 9 and 22 called Philadelphia Chromosome. Telomerase- essential enzyme that adds telomeric repeats into the telomeres- maintains integrity of chromosomal ends. Most normal somatic cells do not express hTERT (catalytic subunit of telomerase) but it is expressed in most neoplastic and cancer cells. Aims. Our aim of this study was to evaluate the hTERT expression in patients with CML at different phases of the disease. *Design and Methods*. In this cross sectional study 73 samples of 45 patients with CML were studied. Twenty six of samples were taken from patients in chronic phase before therapy. Tewenty six samples were also taken from theses patients at least three month after therapy. Nine samples were taken from patients in accelerated phase and 12 from patients in blastic phase. RNA was extracted from peripheral blood mononuclear cells and cDNA was made using random hexamers. hTERT expression was studied by qualitative RT-PCR and the results were analyzed using Fisher's Exact test, χ^2, McNemar and Mann-Whitney tests. *Results*. 73% of patients were male and 27% were female. Patients were divided into three age groups; 17-29, 30-40 and 41-75 years. Of 73 samples, 43 samples (58.9%) were positive for hTERT and 30 samples (41.1%) were negative for this gene. In chronic phase (before therapy) 69.2% of the samples were PCR positive, but only 38.5% remained PCR positive after therapy. In accelerated and blastic phases 55.6% and 83.3% of samples were PCR positive respectively. The hTERT positivity was differently significant (p<0.05) among different phases of the disease. *Conclusions*. Our results showed that significant difference between hTERT expression in different phases of CML disease can be used as a useful molecular marker for fallowing up, prognosis and disease progression after treatment

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MOLECULAR MONITORING: AN ESSENTIAL COMPONENT IN THE CLINICAL MANAGEMENT OF PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA TREATED WITH TYROSINE KINASE INHIBITORS

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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 has been introduced since 2002, but for some financial reasons the molecular diagnosis is not routinely available so far. In 2007 using research grants we have set up in our institution, "Fundeni" Clinical Institute, a Molecular Biology Laboratory and here we report our results. *Methods and Results*. A Real-Time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using LightCycler® technology was used to quantify BCR-ABL transcript levels in 207 peripheral blood samples collected from 113 patients with positive BCR-ABL transcript before and in the course of the imatinib treatment. Quantification of BCR-ABL was performed by RQ-PCR assay according to the European LeukemiaNet (ELN) protocol. Total RNA is extracted from 2 x 107 cells by Chomczynski et al protocol. Total amount of extracted RNA is transformed by reverse transcription PCR in to cDNA. Quantification of BCR-ABL transcripts is performed with Abl control gene. Total ABL transcripts were quantified as internal control and results were expressed as the ratio BCR-ABL/ABL. From total investigated cases, 74 patients are at first molecular investigation and 39 are 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%. Multiplex PCR and Nested PCR were performed for transcript type identification and revealed b3a2 and b2a2 BCR-ABL transcripts in 67% of cases and 33% respectively. In one case was identified a dual b3a2+b2a2 BCR-ABL type. Seven patients were resistant to imatinib with the BCR-ABL range of 12-48% during the treatment. For these patients direct sequencing of the ABL KD was performed. We identified some BCR-ABL mutations - T315I, L387M and Q215H, so this assay generate clinically useful information regarding the therapy with new Bcr-Abl inhibitors. *Conclusions*. This study, the first in Romania, indicates that quantitative PCR and screening of mutations in BCR-ABL kinase domain could provide practical indications capable of directing therapeutic interventions for CML patients, so the molecular monitoring must be a essential part in the clinical management of patients with Chronic Myeloid Leukemia treated with tyrosine kinase inhibitors.

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THREE NEW MUTATIONS FOUND IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS RESISTANT TO TYROSINE KINASE INHIBITORS (TKI)

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Background. Point mutations within the ABL kinase domain are the most frequent mechanism for reactivation of kinase activity of the BCR-ABL gene and have been associated with clinical resistance to TKI in CML patients, conferring a poor prognosis. For optimal clinical management of CML patients treated with TKI, early detection of mutations may help in defining alternative therapeutic strategies. Aims: Screening of mutations in exon 6 of the BCR-ABL gene in patients with CML resistant or intolerant to TKI and correlate the presence of mutations with patient outcome. *Design and Methods*. Genomic DNA was extracted from peripheral blood samples from 93 CML patients (5 intolerants and 88 resistants). The study population was classified according to criteria of the Leukemia Net as failure or sub-optimal response to imatinib. Patients resistant or intolerant to imatinib were treated with dasatinib or nilotinib. The study was approved by the Ethics Committee of the University of Campinas and prior to the study informed consent was obtained. The PCR product of BCR-ABL exon 6 amplification was analyzed by D-HPLC, and the patients samples with abnormal D-HLPC profiles were submitted to sequencing, using specific primers. Survival analysis for the whole group and for both groups (mutation vs no mutation) were performed using the Kaplan-Meier method for survival curves and proportional hazards regression model for statistical significance between groups. Overall survival (OS) was calculated from start of imatinib therapy until death or last follow-up. *Results*. Twenty-three out of 93 samples (25%) showed an abnormal elution profile. Sequencing confirmed the presence of a nucleotide change in 19 out of 23 cases. In four cases no mutation was found in sequencing. Mutations identified were: one polymorphism (T315T), T315I, F317L, V339L, M351T, E355G, F359V and three novel mutations: C305R, D325D and I360S. Patients presenting the new mutations located in imatinib contact site (C305R and D325D) had primary resistance or progression after dasatinib treatment. The I360S mutation is located in catalytic domain and was found in a patient with sub-optimal response with imatinib and in a patient with treatment failure that achieved complete hematological response with dasatinib. Overall survival (OS) for whole group was 80% in a median observation time of 30 months. OS was 87% and 56% for patients with no mutation and with mutation, respectively (p<0,0001) (RR=68). *Conclusions*. In this study we screened mutations in exon 6 of BCR-ABL gene and identified 3 novel mutations. In fact, the majority of mutations that confer resistance to dasatinib and imatinib are predicted to involve drug contact residues. In four cases with abnormal profile sequencing, we were unable to identify mutations, probably due to the sensitivity of the method, which requires a relatively high concentration of amplicons with mutation. In the survival analysis, patients presenting mutations had clearly a poor outcome. In conclusion, D-HPLC is a practical and sensitive method for routine clinical monitoring of kinase domain mutations and may be useful for optimizing therapy and decision making for alternative treatment in CML.

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EFFICACY OF IMATINIB DOSE ESCALATION (IDE) IN CHRONIC MYELOID LEUKEMIA (CML) PRETREATED WITH INTERFERON-α (INF-α)

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Background. In case of imatinib (IM) failure or suboptimal response IDE to 600-800 mg daily has been recommended as the first option for management of patients with CML according to guidelines of European Leukemia Net. However there are only a few publications focused on the topic. *Aims.* Our study has been aimed on evaluation of IDE efficacy in INF-α pre-treated patients. *Design and Methods*. A retrospective study was performed. Patient´s risk at diagnosis, presence of additional cytogenetic abnormalities (ACyA), cause of IM resistance and response to therapy were evaluated for influence on IDE efficacy. 4. Results 65 INF-α pretreated CML patients were analysed. Median interval since diagnosis up to IM therapy was 15 months (range, 1-102 months). Median follow-up from diagnosis was 81 months (range, 32-169 months), while the median follow-up from initiation of IM was 60 months (range, 7-98 months). In 14 patients (21,6%) IDE was used for hematologic resistance in 1 patient, cytogenetic relapse or progression in 9 and suboptimal response in 4 patients. In IDE patients median interval since the start of standard dose IM up to dose escalation was 28 months (range, 4-54 months). Previous response to standard dose IM was complete CyR in 8 patients achieved after median 10 months and lasted for median 8 months. The median period of IDE therapy was 9 months (range, 6-14 months). After IDE 5 patients achieved complete CyR and 7 patients major CyR with average duration of 9 and 14 months. However, only 2 patients achieved major molecular response. Majority of the patients were switched to second generation tyrosine kinase inhibitors due to hematologic (2 patients; 14,3%) or cytogenetic resistance (6 patients; 42,9%) and suboptimal response (1 patient; 7,1%). Only 4 patients (28,6%) continue with IDE presently. *Conclusions*. In most INF-α pretreated patients IDE resulted in transient CyR that was shorter than one year. Response to IDE seems to be associated with absence of ACyA and BCR-ABL tyrosine kinase domain mutations and achievement of major molecular response, but with no other variables including Sokal risk or response to standard dose of IM. Larger groups of patients are needed to be analysed in order to assess the actual role of IDE in INF-α pretreated patients with CML.

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MONITORING T315I MUTATION IN CHRONIC MYELOID LEUKEMIA BY ARMS PCR

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Background. The introduction of imatinib mesylate has revolutionized the treatment of chronic myeloid leukemia (CML). Even though experience with imatinib mesylate is limited, clinical resistance has already been observed. In order to overcome imatinib resistance, more potent tyrosine kinase inhibitors such as nilotinib and dasatinib have been developed with demonstrable activity against most BCR-ABL mutations with the notable exception of T315I mutation. More, with the increased use of newer tyrosine kinase inhibitors it has been suggested that the spectrum of mutations may change, possibly selecting the pan-resistant T315I. *Aims*. To assess the prevalence of the T315I mutation in a cohort of Romanian CML patients in order to adapt the treatment at any time the clinical and hematological aspects change. *Design and Methods*. There were 41 CML patients (21 women, 20 men) with a median age of 56,73 years (24-85) diagnosed and treated in our institution between august 1996 and december 2008. At the moment of sampling, 34 patients were in chronic phase, 2 patients in accelerated phase and 5 patients in blastic phase. 32 patients had been treated with tyrosine kinase inhibitors (TKI), for variable intervals (1-60 months, median 21,37). 32 patients had received imatinib, 400-600 mg/day (12 as first line treatment, 20 as second and third line) and 3 patients had received dasatinib (2 as second line and one as third line treatment). The T315I mutation was studied by an ARMS PCR (Amplification Refractory Mutation System - Polymerase Chain Reaction) technique. *Results*. At the moment of sampling 2 patients (0,84%) were in major molecular response (MMR), 1 patient

(0.42%) was in minor molecular response (mMR), 11 patients (4,62%) in complete cytogenetic response (CCyR), 1 patient (0,42%) in partial cytogenetic response (PCyR), 1 patient (0,42%) in minor cytogenetic response (mCyR) and 12 patients (5,04%) were in complete hematological response (CHR).The 2 patients with MMR were both treated with imatinib as the patient with mMR. Among the 11 patients in CCyR, 10 were treated with imatinib and 1 with dasatinib. Of the 12 patients in CHR, 9 were under imatinib and 3 patients were treated with hydroxiurea. The T315I mutation was found in only one patient, diagnosed in 1998 who was in clinical and hematological relapse after several lines of treatment (interferon + Ara-C, imatinib, dasatinib). *Conclusions*. The T315I mutation was a very rare event in our patient population, despite a high proportion of patients who had a suboptimal response after TKI treatment. An explanation could be the fact that few of our patients were treated as first line therapy with TKIs; also, few patients were treated with high-dose TKIs. Therefore it is possible that there was not enough time and selective pressure for the emergence of the T315I mutation. Further follow-up of the patients treated as first line-therapy TKI may result in a higher rate of T315I detection. This aspect is of utmost importance as such CML patients may be the ones who will actually benefit from allogeneic stem cell transplantation.

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NILOTINIB (AMN107) CAN INDUCE COMPLETE CYTOGENETIC RESPONSES (CCYR) AND MOLECULAR RESPONSES IN PATIENTS (PTS) WITH CHRONIC MYELOID LEUKEMIA (CML) RESISTANT OR INTOLERANT TO IMATINIB MESYLATE (IM) . REPORT OF THE COMPASSIONATE USE PROGRAM (CUP)OF NILOTINIB IN MEXICO

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Background. Nilotinib, a potent and highly selective BCR-ABL inhibitor, has been approved in more than 50 countries including Mexico. Nilotinib is indicated for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia (Ph^+ CML) patients (pts) in chronic (CML-CP) or accelerated phase (CML-AP) resistant or intolerant to prior therapy including imatinib (IM). Before the approval of nilotinib by FDA or EMEA a CUP of nilotinib was initiated in Mexico. Aims: To evaluate the rates of cytogenetic and molecular responses to nilotinib in IM-resistant or -intolerant Ph^+ CML included in the CUP of nilotinib in Mexico. *Design and Methods*. The pts included in this program were adults pts with imatinib-resistant or intolerant CML-chronic phase (CP),-accelerated phase (AP), or -blast crisis (BC). IM resistance was considered if pts had failure to standard doses of IM or had loss of response and/or progression and were treated with higher doses of IM (600 mg/day or 800 mg/day) and did not respond to these dose escalation, IM intolerance/resistance was considered if pts with previous failure to standard doses of IM did not tolerate the dose escalation and needed a subsequent dose reduction.Physical examination, EKG, bone marrow aspiration, karyotyping and BCR-ABL mutation screening were performed in all pts before starting nilotinib. All pts signed an informed consent. Nilotinib was administered orally at a dose of 400 mg twice daily (BID). No dose escalation was allowed. All pts were monitored with karyotyping. Patients that achieved CCyR were evaluated with quantitative RT-PCR for molecular evaluation (Quest Diagnostics and Molecular MD) The results of CUP of nilotinib in Mexican patients are reported in this abstract including the cytogenetic, qRT-PCR and mutational analysis in a highly-resistant CML patient population. Results: Between October 2006 and June 2007, 47 pts were included in the nilotinib CUP in Mexico. The median age was 41.7 years (range 22-68) and 20 (44%) were men. Of the 47 pts, 28 (59.6%) had advanced phase CML which includes 7 (14.9%) blastic phase (BP) and 21 (44.7%) accelerated phase (AP) pts. 19/47 pts (40%) were in chronic phase (CP) of CML. Most patients were resistant to IM (86%) and 14% were IM-intolerant/resistant. The median duration since CML diagnosis was 74 months (range 14-183). The median duration of prior IM use was 27 months. All pts had been treated with hydroxyurea, interferon, and/or cytarabine prior to IM. At the time of starting nilotinib 17/47 pts (36.12%) had BCR-ABL mutations (P-loop mutations in 3 pts, IM binding mutations in 5 pts, catalytic domain mutations in 5 pts, and A-loop mutations in 4 pts). Only 3/47 pts (6.38%) had T315I mutation. The median duration of exposure to nilotinib was 403 days (range 19-840). Most pts tolerated nilotinib well. One patient developed long-term myelosupression. At the time of data cut-off (January 31th, 2009), 25 pts (53.2%) remain on therapy. Reasons for discontinuation of therapy were: a) lack of efficacy in 7/47 pts (14.9%) including 3 pts with T315I mutation; b) disease progression in 13/47 (27.5%); c) adverse events in 1/47 (2.1%); d) lost of follow-up in 1/47 (2.1%). The cytogenetic and molecular evaluation was performed after 12 and 18 months of treatment with nilotinib, respectively. The rate of overall hematological response (HR) was 79%. The major cytogenetic response (MCyR) according the phase of the disease were as follows: 57% in CP, 40% in AP and no MCyR was observed in BP. Of the 47 pts, 11 (23.4%) achieved CCyR (7 pts with CP-CML and 4 pts with AP-CML), five patients (10.63%) achieved molecular responses (3 pts with CP-CML and 2 pt with AP-CML) including 2 pts (4%) with complete molecular response (CMR) and 3 (6%) with major molecular response (MMR). *Conclusions*. Nilotinib showed efficacy in IM-resistant or -intolerant CML pts in Mexico regardless of the presence or absence of BCR-ABL mutations. Nilotinib induced complete cytogenetic and molecular responses in some of resistant CP and AP-CML patients.

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KINETICS OF BCR-ABL TRANSCRIPTS IN PATIENTS WITH CHRONIC PHASE CML (CML-CP) TREATED WITH IMATINIB MESYLATE (IM): A PREDICTOR OF RESPONSE AND PROGRESSION FREE SURVIVAL (PFS)

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Aims. To assess whether early kinetics of molecular response to IM therapy is a predictor of PFS, and sustained hematological and cytogenetic responses in newly diagnosed patients with CML-CP. *Design and Methods*. Ninety-five newly diagnosed Egyptian patients with CML-CP were treated with a daily oral dose of IM 400 mg unless otherwise indicated. Conventional karyotyping and fluorescent *in situ* hybridization (FISH) analysis were performed at diagnosis, every 6 months during the first 2 years of treatment, and then annually or as necessary during a median follow-up period (FUP) of 26 months (range 6-64 months). Molecular monitoring by real time quantitative polymerase chain reaction (RT-QPCR) was performed at diagnosis and at regular intervals every 3 months during the FUP. Mutational analysis of the ABL kinase domain was performed by allele-specific oligonucleotide PCR (ASO-PCR). *Results*. The study demonstrated that 98% of patients achieved a hematological response after three months of therapy with IM 400 mg/day. Fifty-nine of 95 patients (62%) showed a 2-log reduction of the BCR-ABL/ABL ratio at 6 months of IM therapy, of whom 49 patients (83%) achieved a complete cytogenetic response (CCyR) and 42 of 59 (71%) of patients achieved a major molecular response (MMR) at 12 months. BCR-ABL transcripts remained undetectable in 22 patients (37%) after 26 months of consecutive measurements and these patients remained in CCyR at the time of data cut-off. Among the 36 patients who did not achieve a 2-log reduction of the BCR-ABL/ABL ratio at 6 months, only 5 patients (15%) achieved a CCyR and MMR by 12 months. There was a statistically significant difference between both groups in the duration of cytogenetic and molecular responses ($p<0.0001$). Failure to achieve a 2-log reduction at 6 months of IM therapy correlated with a decreased rate of PFS at 2 years ($p<0.03$) and with the presence of ABL kinase domain mutations ($p<0.001$). All primary resistant cases (16/16, 100%) and 16/18 (89%) suboptimal IM responders failed to achieve a 2-log reduction of BCR-ABL transcripts after 6 months of IM treatment versus 2/59 (3%) of the optimally responding group ($P < .0001$). Among 15 patients (10 suboptimal responders and 5 with primary resistance), ABL kinase domain mutations were detected in 11/15 (73%) of these patients. *Conclusions*. The present data demonstrate the predictive value of an early molecular response to IM for patients with CML-CP. Less than a 2-log reduction in BCR-ABL transcripts at 6 months of IM treatment could

be considered a cut off level that may predict resistance, lower probability of achieving a CCyR, and decreased rate of PFS at 2 years or suggest the need for IM dose escalation.

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TOWARDS A NEED IN OPTIMIZING CML MANAGEMENT IN SUBOPTIMAL RESPONDERS: DATA FROM THE SCREEN REGISTRY

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Background. The introduction of Imatinib Mesylate (IM) has produced major advances in the treatment of Chronic Myeloid Leukemia (CML). High rates of stable response continue to be reported, with >85% of patients (pts) achieving complete hematologic and cytogenetic responses after 5 years of treatment. However, residual resistant disease after IM treatment may occur in CML and the incidence of resistance increases in the more advanced phases. Given this evidence, IM failure has been defined on the basis of hematologic, cytogenetic and molecular responses at set time points as well as progression and warning signs. Current studies indicate that accurate monitoring of IM response is required to identify CML pts who respond poorly or lose response in order to assess whether a change in therapy strategy could be appropriate. Moreover, a proportion of CML patients still remains who present minimal residual disease to IM therapy and are at high risk of disease progression: they are the suboptimal responders. *Aims and Methods*. To address this clinical topic, we screened a consecutive series of *de novo* 210 pts with CML. All of them were included in the SCREEN registry (Sicilian CML Registry Enterprise) which started on April 2005 and is still open to enrolment, now even in the region of Calabria. The registry was designed in order to create a regional network that will perform the molecular monitoring of IM-treated CML patients and to assure the same standard of care all over the regions. A preliminary data analysis on 140 CML pts, allowed us to identify 26 pts as suboptimal responders. *Results and Conclusions*. Here we will report CML suboptimal responders' baseline features, molecular monitoring of bcr-abl transcript (according to ISS), clinical outcome to tyrosine kinase inhibitor (TKI) therapy and follow-up and, whenever indicated, bcr-abl mutational analysis of this cohort of pts. Our CML suboptimal responders represented 18% of the total enrolled CML population and this data is in accord to the IRIS findings. Of interest, CML suboptimal responders showed a high bcr-abl molecular transcript at diagnosis as compared to normal responders (median bcr-abl transcript 0,73 vs. 0.57 on the ISS). Three out of 26 (11%) suboptimal pts shifted to second line treatment (1 BMT, 2 second TKI). Further, the issue of suboptimal responders who achieved CCyR but failed to achieve MMolR at 18 mo. will be addressed. Our preliminary data strongly support the critical need in identifying and optimizing CML management with second line therapy (high IM doses, second TKIs) in suboptimal responders to IM treatment.

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TREATMENT OF 171 PATIENTS WITH CHRONIC MYELOID LEUKEMIA IN EARLY AND LATE CHRONIC PHASE WITH IMATINIB MESYLATE. EXPERIENCE OF A SINGLE INSTITUTION AT MEDICAL SCHOOL AT UNIVERSITY OF SAO PAULO - BRAZIL

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Chronic myeloid leukemia is characterized by the presence of Ph chromosome where the BCR-ABL chimeric gene is a target for the tyrosine kinase inhibitor imatinib mesylate (IM). Molecular monitoring became the method of choice to follow patients treated with IM. Objective: Compare the complete cytogenetic response (CCR), and major molecular response (MMR) in a population of a single institution (HC-FMUSP), and then correlate with overall survival (OS), event free survival (EFS), and progression free survival (PFS). *Design and Methods*. 171 serial patients with CML-CP were evaluated, and divided in 2 groups: early chronic phase (77/171), define as the interval from diagnosis to the beginning of IM less than 6 mo and late chronic phase (94/171) as more than 6 mo. Cytogenetic studies were done by G-banding karyotyping and molecular monitoring and gene expression were done by QRT-PCR. Mutational analysis was performed by direct sequencing when resistance was identified or progression of the disease. *Results*. CCR was achieved in 71.9% of the patients with a median time of 11.2 months. The ECP patients CCR was achieved in 71.3% and 87.7% at 12 and 60 months what is in agreement with the IRIS study. In the LCP the CCR was 34.8% and 58.9% at 12 and 36 months of treatment. Molecular monitoring was performed only in 86.5% (149/171) of the patients and MMR in the ECP group was 40%, 60% and 74% at 12, 18 and 24 months of treatment, on the other hand, patients with LCP reach a MMR of 15% and 47% at the same time (p=0.005). The best molecular response at 3 months of treatment has a high probability in achieving a MMR when compared to those who do not achieve a 1-log reduction at 3 months (p=0.03). The estimated risk of resistance in the group with the best molecular response was 0%. Mutational analysis detected 6 muation where 5 in LCP patientes.There were no statistical difference between those 2 groups regarding OS, EFS and DFS. Resistance was more frequent in the LCP when compared with ECP (p=0.02). *Conclusions*. For the first time is reported the outcome comparing patients treated with IM before 6 mo and after 6 mo from the diagnosis, demonstrating that within 6 mo is the optimal timeframe for treatment starting regarding CCR and MMR, but not OS, DFS and PFS.

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RETROSPECTIVE EVALUATION OF PATIENTS TREATED WITH DASATINIB FOR PHILADELPHIA POSITIVE LEUKEMIAS: TURKISH EXPERIENCE OF 16 MONTHS

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Background. Dasatinib, *in vitro* 325 fold more potent bcr-abl inhibitor than imatinib, has been registered for use in patients with the diagnosis of CML or Ph$^+$ ALL and AIMS: The aim of this study is to retrospectively evaluate the Turkish patients treated with dasatinib for the diagnosis of either CML or Ph$^+$ ALL under the program of compassionate use. *Design and Methods*. The 130 patients accepted to compassionate use program (sponsored by Bristol-Myers Squibb) between the dates of November 2006 and April 2008 were enrolled into this study. Patients were evaluated according to the age, gender, time from the diagnosis, the reasons for dasatinib treatment, and the stage of the disease, last imatinib doses, last treatment status with dasatinib, adverse events under dasatinib treatment, and response status to dasatinib treatment retrospectively. RESULTS: Of 130 patients, 57 women and 73 man had 48.7±13.7 (18-75) and 45.4±15.7 (19-83) years of age respectively. Median time from diagnosis to dasatinib treatment was calculated as 73.96±52.65 (1-288) months. The disease status at the beginning of dasatinib treatment was as follows; chronic phase of CML in 75 patients (57.7%), accelerated phase of CML in 25 patients (19.2%), blastic phase of CML in 18 patients (13.8%), and Ph$^+$ ALL in 12 patients (9.2%). The reason for initiating dasatinib treatment were grouped as; progression under imatinib treatment in 37 patients (40.2%), resistance to imatinib therapy in 30 patients (32%), intolerance to imatinib in 16 patients (17.4%)and combination of these in 9 patients (9.8%). The last imatinib dose prior to initiation of dasatinib was 613.79±169.93 (300-1000) mg/day. Twenty patients died due to mainly disease progression (12 of 20), and 8 patients were excluded from the program, 5 patients could not start the drug, and remaining 97 patients (74.6%) are still on treatment. There have been no reported adverse events for 90 patients (69.2%); disease progression and grade 1-2 myelosupression were the most frequently reported events. Median duration of treatment with dasatinib was 7.94±4.53 (1-17 +) months at the last follow up. Most of the patients who continued their treatment with dasatinib were under complete hematological response and molecular and cytogenetic statuses of the patients were still under evaluation at the last follow up. *Conclusions*.

Dasatinib treatment in Turkish patients with CML or Ph+ ALL was well tolerated and resulted in favorable outcomes with only mild side effects in most cases during the period of observation of patients with accessible data so far.

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COEXISTENCE OF A JAK2 MUTATED CLONE MAY CAUSE HEMATOLOGIC RESISTANCE (HRES) TO TIROSYN-KINASE INHIBITORS (TKI) IN CHRONIC MYELOID LEUKEMIA (CML)

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Background. Among the classic myeloproliferative neoplasms (MPN), only CML is characterized by the chromosomal translocation t(9;22) resulting in the BCR-ABL oncogene. The discovery that JAK2-V617F mutation is found in virtually all patients with policytemia vera and approximately 50% of those with either essential thrombocytopenia (ET) or primary myelofibrosis (PMF), has refined current diagnostic criteria in MPN. *Aims*. Most systematic investigations showed that JAK2 mutation and BCR-ABL were virtually exclusive and their coexistence has been described in rare patients. We report 3 such cases. *Methods and Results.* Patient 1 was diagnosed of CML, following the discovery of thrombocytosis. She started imatinib 400 mg then increased to 800 mg for HRes, but platelet control was never achieved and hydroxiurea was added. She could not tolerate IFN so started nilotinib for leucocytosis, thrombocytosis and 100% Ph-positivity. Bone marrow (BM) showed granulocytic and megakaryocytic hyperplasia. In six months she was in complete cytogenetic response (CCyR) but the improvement of the counts was transient. We thus researched the Jak-2 mutation that resulted positive. Interestingly the Jak2-V617F was present since nilotinib start, but its expression increased, achieving the maximum concomitantly with the CCyR. Unfortunately the patient died in CCyR because of a cardiovascular event. Patient 2 was diagnosed of PMF because of leukocytosis, thrombocytopenia and splenomegaly. BM was hypocellular with trilinear dysplasia and collagen fibrosis. Ph-chromosome was not detected. After 2 years with no treatment, BM analyzed for a symptom worsening, demonstrated 15% myeloblasts with hyperplastic myeloid series and 100% Ph-positivity. The patient was initially treated with IFN, and then with imatinib for HRes, although a partial cytogenetic response. After an initial improvement, a new progressive increase of leucocytes and platelets followed. BM at this time was consistent with an MPN, with normal karyotype. Imatinib was increased with no improvement and hydroxiurea added, but hematological control remained unsatisfactory and imatinib was stopped. The patient stayed on hydroxiurea, maintaining a CCyR, with insufficient hematologic response for more than one year, until he developed a Ph-negative myeloblast crisis with homozygosis for JAK2-V617F. Going back to older samples, we found that before imatinib the patient had a 50% JAK2 mutated phenotype and after 3 months of therapy, more than 70%. Patient 3 was diagnosed of ET with a normal karyotype. A good control of the thrombocytosis was obtained with hydroxiurea, which continued for 10 years and then the patient refused therapy for the next 3 years of stable disease, until he developed bone pain, leukocytosis, enlarged spleen, with normal platelets. Conventional cytogenetics was normal but FISH disclosed a 47% Ph-positivity. The patient started imatinib, which induced a rapid decrease of leucocytes; however, the platelets raised and hydroxiurea was added. After 3 months the BM showed 7.4% Ph-positive cells. JAK2 was investigated and came back mutated from imatinib start. *Conclusions*. This report suggests the presence of two different clones, one Ph-positive and the other JAK2 mutated and the prevalence of the latter when the former was inhibited by a TKI. The presence of a JAK2 positive clone may be a rare cause of HRes to TKI.

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IMPACT OF HIGH SENSITIVE DETECTION, INCIDENCE AND SIGNIFICANCE OF T315I MUTATION IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE (FIRST STUDY FROM INDIA)

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Background. The onset of BCR-ABLT315I mutations during the treatment of CML with TKIs remains challenging, because this mutation is the most frequently identified in IM-treated patients, and none of the TKIs clinically available to date retain any activity *in vitro*. The fastness, simplicity, and sensitivity of ASO-PCR assays can be used for routine monitoring of BCR-ABLT315I mutation. *Objectives*. To study the high sensitive detection and incidence of T315I mutation in imatinib resistant CML patients. To study the clinical outcome of the imatinib resistant CML patients carrying T315I mutation. *Design and Methods*. One hundred consecutive patients who presented with a haematological diangosis of CML had blood examined by RT-PCR for BCR-ABL transcripts. They were started on treratment with imaitinib by mouth. All patients were monitored for hematologic and molecular responses, time to progression, survival and toxicity Early screening for the T315I mutation was performed by allele specific-oligonucleotide-PCR (ASO-PCR). The ASO-PCR primer sets were designed which were similar to according to the Roche-Lestienne C et al for T315I ABL gene mutations. The sensitivity of ASO-PCR is superior to that of direct sequencing as it could detect one mutant allele in 100~100,000 wild type sequences. Mutated and wild-type alleles were specifically amplified in a 50 ul and mixture 100 ng of gDNA. *Results*. Only 100 Imatinib resistant CML patients (CP-CML 20, Late-CP-CML 30, AP-CML 20 and BC-CML 30) were selected who were treated with imatinib at 400mg to 800mg/day . Median time since diagnosis was 40 months, and progression occurred 20 months after IM initiation, regardless of disease phase. A T315I mutation was detected by ASO-PCR in 30% of resistant patients (CP-CML 01, Late-CP-CML 06 ,AP-CML 08 and BC-CML15). All patients progressed after the 6 to 9 months of T315I positive mutation discovery (1 CP-CML , 3 late-CP-CML and 3 AP-CML).Thirty of five died .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 T315I mutations. Side-effects of imatinib included Anemia (n=30), Thrombocytopenia(n=20) and hypopigmentation of skin (n=10). This mutation seems related to or (partially?) responsible for progression and poor survival. *Conclusions*. ASO-PCR proved to be very economical, sensitive and rapid technique for detection of T315I mutation and is more sensitive than mutation detection by sequencing. The detection of T315I kinase domain mutation was associated with imatinib resistance and poor prognosis

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STABLE RESPONSE TO IMATINIB MESILATE IN CHRONIC MYELOID LEUKAEMIA THREE YEARS AFTER DISCONTINUATION OF THERAPY

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The possibility of stopping imatinib mesilate therapy in patients achieving a complete haematological, cytogenetic and molecular response is still an object of debate. In February 2002 we saw a 76 years old woman presenting a chronic myeloid leukaemia in accelerated phase. Her leukocytes were 191.000 per mm^3 with percentages of 37 neutrophils , 42 neutrophil precursors and 4 blasts. She had the typical finding of Philadelphia chromosome translocation and the resulting BCR-ABL fusion gene. One month later a therapy with imatinib mesilate at a daily dose of 600 mg was started. Hematological, cytogenetic and molecular response was easily obtained in twelve months though a cell line with +8 chromosome appeared. In 2005 she began to experience abdominal pain , nausea and repeated vomiting episodes that usual therapy and the reduction of imatinib dosage could not improve . A progressive body weight loss was present but the patient refused an endoscopic examination and decided to stop imatinib therapy. Her symptoms briefly regressed and the patient in some weeks obtained a condition of very good health. Three years after having stopped the therapy the peripheral blood counts still remain in the normal values with 7,600 leukocytes per mm^3 and normal differential counts . On cytogenetic analysis trisomy of chromosome 8 is still present . On quantitative polymerase chain reaction-based assay BCR-ABL m RNA is not detectable so proving that a complete molecular response is still present.

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T(6;9;22): NEW VARIANT TRANSLOCATION IN CHRONIC MYELOID LEUKEMIA

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Background. Chronic myeloid leukemia (CML) is a myeloproliferative disorder, rarely observed in childhood. It accounts for only 2-3% of all paediatric leukemias and predominates in children older than 4 years, with a F/M ratio of 1,6:1. CML is characterized by presence of Philadelphia chromosome [t(9;22)(q34;q11)] with the fusion of the BCR and ABL genes, resulting in the synthesis of a 210-kilodalton dysregulated tyrosine kinase. This kinase is the target of Imatinib, a tyrosine kinase inhibitor, gold standard in the treatment of CML. In 5-10% of CML are present variant translocations, involving chromosomes other than 9 and 22. Until today it is not established the prognostic significance and the effect on imatinib-response of these cytogenetic variants. *Patients and Design and Methods*. We report a rare case of paediatric CML in a 3 year-old boy with a novel cytogenetic variant translocation. He was admitted in our Institution on December 2008 for massive leukocytosis (WBC 163,540/mmc) and mild anemia (Hgb 10,9 g/dL). On physical examination he presented mild hepato-splenomegaly. RT-PCR on bone marrow specimens revealed the presence of b2/a2 BCR/ABL transcript with quantitative reverse transcriptase-polymerase assay of 49,3 IS (International Scale; Blood 2006; 108 (1): 28-37). FISH analysis revealed a 46,XY, t(6;9;22)(q26;q34;q11) karyotype. *Results*. We started Imatinib therapy (200 mg/die) and we observed a good haematological response (GB 4,170/mmc after 1 month; absence of hepato-splenomegaly) and a sub-optimal molecular response; after 1 and 2 months of therapy RT-PCR revealed a transcript number of 27,5 IS and 18,5 IS, respectively. Side effects of Imatinib therapy were anemia, neutropenia, synthomatic thrombocytopenia that required reduction and temporary discontinuation of drug; non-haematological toxicity was not observed. *Conclusions*. At best of our knowledge, t(6;9;22) is a novel translocation in CML, not described until now. This translocation may influence Imatinib response, because the patient presented a good haematological but a poor molecular response. A further follow-up is necessary to clarify a possible correlation

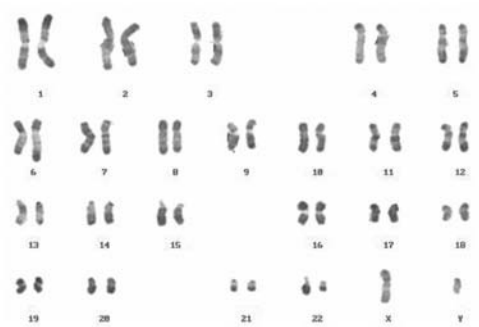

Figure.

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BCR-ABL TYROSINE KINASE MUTATIONS IN IMATINIB MESYLATE RESISTANT CML PATIENTS FROM OMAN: SINGLE CENTRE EXPERIENCE

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Background. Point mutations in the kinase domain of the BCR-ABL tyrosine kinase are a frequent mechanism for reactivation of kinase activity, the most common cause of imatinib resistance. These mutations affect amino acids involved in tyrosine kinase inhibitors binding or in regulatory regions. *Aims*. To investigate and delineate the cause of sub-optimal or poor response to imatinib mesylate in CML patients. *Design and Methods*: We prospectively enrolled and studied 41 cases of CML patients who received imatimib mesylate with a dose ranging between 400-800mg, [Median-400mg] as the first line treatment over the past 7 years at our institution. 8 cases were referred to our laboratory because their response was defined either as failure (n=3 pts) or as suboptimal (n=5 pts) according to recently published recommendations (Baccarani et al, Blood 2006). We performed the RT-PCR amplification of the entire kinase domain of the BCR-ABL mRNA using a 5'BCR primer (5'tgaccaactcgtgtgtgaaactc3') and a 3'ABL primer (5'tccacttcgtctgagatactggatt3'). A nested RT-PCR using the same reverse primer but a different forward primer (5'cgcaacaagcccactgtct3') was done to generate an 863 bp PCR product. Direct sequencing with dye terminator chemistry was performed using PCR-purified products. *Results*. The study group consisted of 41 patients (21 males), with a mean age of 41.9yrs + 15.5(SD), range of 9-70 yrs. All patients were serially followed with regular CBC's, 3 monthly FISH and cytogenetic studies. Complete Haematological Response(CHR) as defined by a white cell count of less than 10X10^9/L, a platelet count of less than 450x10^9/L, no blast in peripheral blood; and disappearance of clinical signs & symptoms with resolution of organomegaly was seen at a median of 21 days[range, 4-35 days]. The CHR was further characterized by cytogenetics [Standard & FISH] and molecular techniques [RT-PCR and RQ-PCR] as complete cytogenetic response (CCyR) - Ph positivity : 0%, Partial cytogenetic response (PCyR) : Ph positivity 1-35%; Minor Cytogenetic Response (MCyR) : Ph Positivity 36-65%; Minimal Cytogenetic Response : Ph positivity 66-90% and No response -Ph positivity >90%. CCyR was achieved in a median [range] of 11.5[2-26] months; MCyR was achieved at a median of 7[4-14] months and MCyR at a median of 5[4-14] months respectively in this study patients. Amongst the 25 evaluable patients, 17 (68%) showed good response, but in 8(32%) patients who showed suboptimal response, we were able to identify two previously reported mutations namely E355G and E279K in one patient each as shown in the figure below. *Conclusions*. This is the first report of mutation analysis in patients receiving imatinib mesylate as initial therapy for CML amongst a cohort of 41 omani patients from a single centre. Interestingly, both these patient had a high Sokal score of >1.2. Thus early detection of an emerging mutant clone may help in decision-making for alternative treatment especially in patients with a high Sokal score.

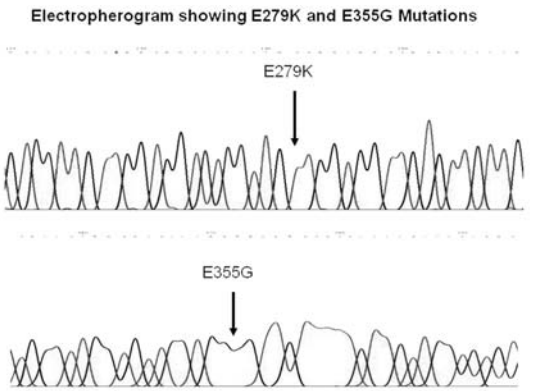

Figure.

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MOLECULAR RELAPSE IN CHRONIC MYELOID LEUKEMIA PATIENTS AFTER IMATINIB DISCONTINUATION: DOES IT ALWAYS NEED THERAPY?

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In patients with BCR-ABL-positive chronic phase (CP) chronic myeloid leukemia (CML) it is generally accepted that molecular responses have prognostic significance. A major molecular response (MMR) has been defined as a ratio of BCR-ABL transcripts compared with a standardized control gene (normally ABL) of 0.1% (equivalent to a 3-log lower level), by quantitative polymerase chain reaction (QPCR). Com-

plete molecular remission (CMR) has been defined as the absence of any detectable BCR-ABL transcript. CML-CP patients who achieve early molecular responses are more likely to experience durable complete cytogenetic responses (CCyR) and are less likely to undergo disease progression, while on treatment. However, some studies have suggested that patients who achieve CCyR may not derive an additional prognostic benefit from achieving a major molecular response. On the other hand, rising BCR-ABL transcript levels may also provide an early indication of loss of response. The clinical significance of rising levels of QPCR in patients who discontinue imatinib while in CMR has not been analyzed in details. About half of these patients showed a molecular relapse, in keeping with the hypotesis that continuous imatinib exposure is essential for inhibiting the neoplastic population. Reappareance of BCR-ABL transcript occurred within few months with the latest described at month 10; however, follow-up of patients who discontinued is still limited in time. In those who recurred imatinib treatment was restarted and all patients proved to be still sensitive to the therapy. We report a case of a patient with CML who started imatinib in association with Pegylated-IFN as front line treatment in 2001, achieving a CCyR whithin 3 months. Peg-IFN was stopped for skin toxicity after 4 months and 2 months later the patient achieved a CMR, that was stable for 45 months before imatinib discontinuation due to intolerance. The patient stayed off treatment in confirmed CMR for 28 months, with undetectable BCR-ABL transcript on BM and PB, in multiple serial samples, until a QPCR analysis on PB disclosed a BCR-ABL/ABL ratio of 0,0208% consistent with a MMR. She refused to start imatinib again, and control samples 3 and 6 months later were again positive at very low leves, with a ratio of 0,0087% and 0,0018% respectively. Our patient is currently off-therapy after 1 year, still in CCyR, and the last BM and PB QPCR remained in the MMR range (0,0314% and 0.0176% respectively). This finding shows two previously unreported phenomenons: first, although most patients who stop imatinib treatment relapse early, late recurrence may occur; second, the presence of minimal residual disease is not invariably associated with the expansion of the leukemic clone even in the absence of TK inhibition. This points to alternative mechanism which can contribute to longterm disease control.

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THE EFFECTIVE THERAPY WITH IMATINIB IN PATIENT WITH CHRONIC MYELOID LEUKEMIA ASSOCIATED WITH HEPATITIS C, CRYOGLOBULINEMIA AND GLOMERULONEPHRITIS

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There is known clinical association between hepatitis C, cryoglobulinemia, glomerulonephritis and hematologic malignancies. We present a patient (pt) with chronic myeloid leukemia (CML) associated with these additional diseases who was successfully treated with imatinib. Case report In a 53-year-old patient in 1999 year nephrotic syndrome and an insufficiency of renal function were found. Furthemore diagnostics showed that these disorders were consequences of hepatitis C, which was resulted in type II mixed cryoglobulinemia with mesangiocapillary glomerulonephritis, neuropathy and a various types of vessel changes including a myocardial infarction. The pt was treated with metylprednisolone, cyclophosphamide, cyclosporine and ribavirin. The therapy with interferon α caused greater proteinuria, higher creatinine level and an appearance of vascular purpura. In January 2006 the pt had one more myocardial infarction. Soon, it was observed the increase WBC number (59,5 G/l) and the spleen enlargement (16 cm). After positive test on BCR/ABL p210 protein chronic myeloid leukemia was recognized. In December 2006 year therapy with imatinib in a dose 400 mg was started. After 6 weeks of therapy complete hematological remission was obtained but the dose of imatinib was reducted to 300 mg because of neutropenia. The major (10% of Ph) and the complete cytogenetic remissions were achieved after 6 and 12 months respectively. After an increasing dose of imatinib dose to 400 mg and next 6 months complete molecular response was achieved. Very important benefits associated with imatinib therapy were normalization of renal function tests, disappearance of proteinuria and vessels changes. We conclude that imatinib therapy is effective and safe in patients with CML complicated by cryoglobulinemia and glomerulonephritis.

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CHRONIC MYELOD LEUKEMIA IN PEDIATRIC PATIENTS; OUR EXPERIENCE

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Background. Pediatrics Chronic myeloid leukemia (CML) is very rare disease (<5% of all pediatric leukemia). There are some different between pediatric and adult CML: origin of the disease, comparable biology, valid prognostic index and treatment strategy. There is dilemma about treatment of pediatric CML with HSCT, which is potentially curative, but with immediate risk, versus tyrosine kinase inhibitors with reducing immediate risk but are generally non curative. Also, the juvenile myelomonocytic chronic leukemia (JMML) is specific form of myeloprolipheration in childhood with frequency of 1-2% of all leukemia, very early appearance of the disease, under 3 month age and associations with some genetic changes in 80% of the patients. Aim. To describe the clinical and diagnostic features and treatment results of our patients with CML and JMML. *Design and Methods*. Date about CML and JMML patients are from the state with two million inhabitants and four hundred thousand child population. In the period from 1998 to 2008 were diagnosed five CML patients with median age of 9, 6 years and 3 JMML patients with median age of 9 months. All of them had significant splenomegaly. Median WBC count was $90x10^9$/l in CML patients and $100x10^9$/l in JMML patients. Philadelphia chromosome was positive in all CML patients and negative in all JMML patients. All three patients with JMML had elevated level of HbF. Results. At the diagnosis, four patients with CML was treated with: one with busulfan, four patients with hydroxiurea, three with interferon. Five years ago, we have a possibility to change the treatment in two patients with Imatinibe masylate. They had acutisation of the disease in acute monocytic leukemia and acute myeloic leukemia, after one and two years of treatment with Imatinibe. Two patients were transplanted from HLA-matched sibling donors and they are in optimal condition 6 and 10 years after transplantations. The patients with JMML were treated with Cytosin arabinoside and 6 Mercaptopurine. One of them died after one year of the treatment, the other one had transformation in acute monocytic leukemia and the third one is still in chronic faze under treatment. All of the patients with JMML had not suitable donor for HSCT.Conclusions. Pediatric CML patients may require a different treatment algorithm then adults. One potential treatment strategy would be to give imatinibe at the diagnosis. If an HLA identical sibling donor is available, transplant the patient after cytoreduction regiment. Additional studies are needed to further define the biology, prognosis and the best course of pediatric CML treatment.

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THE EFFICACY OF IMATINIB MESYLATE TREATMENT PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA PH' POSITIVE (CML) REFRACTORY TO PREVIOUSLY INTERFERON THERAPY (CML) - THE OWN POLISH STUDY REPORT

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Background. Imatinib Mesylate is one of the selective inhibitor of BCR/ABL tyrosine kinase which is present at the most patients with CML and in nearby 20% patients with acute lymphocytic leukemia (ALL). The aim of study : analysis of efficacy and safety Gleevec therapy in 112 CML Ph' positive patients resistant to previously given therapy (102/112pts) i.e. Interferon and others (Hydrea, Mercaptopurine, ARA-C, Vincristine, Epirubicine, relapse after BMT) and 7/59 evaluated pts received Gleevec as the first line treatment. *Design and Methods*. 102 patients were eligible for the 72 months study: -98 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 45.1% (46/102) CP-CML patients after 2-42 months has been lasting 22.3 months. There were maintained the primary hematologic resistance in 2.9% (3/102) patients, and the cytogenetic ones in 22.5% (23/102). The acquired hematologic resistance were in 5.9% (06/102) pts., and cytogenetic ones in 20.6% (21/102) patients. Complete molecular response (CMR) and partial molecular response (PMR) were received in 35.3% (36/102) patients. During study partial cytoge-

netic response (PCR) was observed in 16.7% (17/102) after 4.5 -37.0 mo (median 8.1 mo), which has been lasting 2.0 - 59.0 mo (median 10.3 mo+). Minimal cytogenetic response lasting median 4.0 mo was received in 11.8% pts.(12/102) after 6.0 mo Gleevec therapy. Gleevec was rather well tolerated Side effects (1-4o WHO) were observed at 25.5% (26/102) patients : leucopenia,neutropenia, rash, hypoplasia medullae ossium, thrombocytopenia, bone and abdomen pain, increased AST/ALT level, allergic reactions, fever, hiperhidrosis. Overall survival since Gleevec therapy is 25.5 - 94.0 mo - at CP-CML patients. *Conclusions*. Gleevec is very high well effective drug and good tolerated inhibitor of BCR / ABL tyrosine kinase -Imatinib mesylate can be useful in CP-CML patients as the first and second line therapy and may prolong life survival

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IMATINIB THERAPY IN CHRONIC MYELOID LEUKEMIA. THE ROLE OF PHILADELPHIA CHROMOSOME IN THE THERAPEUTICAL RESPONSE MONITORING

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Background. Imatinib is an effective first line therapy for chronic myeloid leukemia (CML) and has substantially changed its biological and clinical behavior. Durable complete cytogenetic responses (CCyR) were reported in the majority of patients, with a rather benign side effect profile, despite the "off-target" inhibition of several other kinases, including kit, PDGFR and Lck. CML has substantially improved survival with the application of Imatinib, however, the patients with advanced CML such as in the accelerated phase (AP), or black crisis (BC) have a bad prognosis even in the era of tyrosine kinase inhibitors. *Aims*. We analysed the hematological and cytogenetic response of Imatinib therapy in CML, the cytogenetic response monitoring in order to diagnose the AP and BC, the important side effects including secondary malignancy and the toxicity of the therapy regarding the quality of life. *Design and Methods*. We studied 58 CML patients diagnosed and treated in the Timisoara Hematology Department, which started Imatinib therapy during 2003-2007, patients from the west part of Romania. Were included 48 patients in chronic phase and 10 patients with advanced disease. Median age was 48 years (range 34-65). There were 37 males and 21 females. We evaluated hematological and cytogenetic response following NCCI cryteria. In a small number of cases we evaluated the molecular response. Patients started with 400 mg Imatinib every day. Cytogentic evaluation was made by conventional standard cytogenetics at diagnosis, after 6 months, 12 months, and than every year if patient obtained CCyR. *Results*. Ten patients (17,24%) died in BC. 7 patients (12,1%) progressed to AP and are being treated with 600mg or 800 mg Imatinib. 48 patients (82,76%) are still alive, 42 of those are in chronic phase. CCyR was obtained in 34 patients (58,62%). This patients are still monitorised every 6 month by conventional standard cytogenetics and molecular analyssis from the peripheral blood by quantitative PCR (RQ-PCR, TagMan). 16 patients lost CCyR, in 10 of them the progresion to AP or BC was seen in peripheral blood and bone marrow exams. The most frequent side effects were hydrosaline retention 12 patients (20.69%), 2 pts. presented congestive cardiac failure due to Imatinib therapy. No secondary malignancy was seen in the studied group. 6,9% of the Imatinib patients presented abdominal cramps, diarhea, skin fragility. Only 4 patient discontinued the therapy and other 2 pts. received 300 mg Imatinib/day for a while. *Conclusions*. Imatinib therapy was well supported and with litle side effects. Conventional standard cytogenetics monitoring is a highly used evaluation method in the response of patients with Imatinib. It is necessary to complete the response evaluation with molecular examination in order to rapidly diagnose the disease progression.

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EPIDEMIOLOGY OF CHRONIC MYELOID LEUKEMIA IN IRAQI PATIENTS

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Background. Chronic Myeloid Leukemia (CML) is a colonal myeloproliferative disorder with a biphasic or triphasic course and some often, a fatal outcome. The molecular abnormality involve the balance translocation t (9; 22) (q34; q11), and/or the BCR-ABL rearrangement in the peripheral blood or bone marrow cells. Incidence in the U.S. is 1.8/100 000/year. Median age at the diagnosis is ~65 years. In Sweden median age at the diagnosis is ~60 year. This is the only abstract which review the epidemiology of CML in Iraq. *Aim*. to study the natural history of chronic myeloid leukemia in Iraq, through proper epidemiological assessment of data from the central Iraqi registry of CML in the national center of hematology, Baghdad. *Patients' characteristics. T*he study involved 669 patients who had been referred to the national center of hematology in Baghdad/Iraq according to the Iraqi program to treat all the patients with CML in a single center. Between 2002-2006 all patients, who had diagnosed CML, or newly diagnosed; should be referred to our center in Baghdad for evaluation, registration, and prescription of treatment (imatinib). Analysis of patients had been done by teamwork and patients

Table.

region	North of Iraq	Middle Iraq	South Iraq	Total
No. of patients	121	396	82	599
percentages	20.2%	66.1%	13.6%	100%
No. of patients per 100,000	1.85	2.404	1.413	2.079

Age distribution is between 14-70 years, with median age of 37 years. 51.6% were males and 48.4% females. 65% were liberal workers, and 70% have a history of being military employees within 5-10 years of presentation. 15% of our patients were in either accelerated or blast phase of CML. *Conclusions*. This is the major data for CML patients in the Iraqi cancer registry. The age incidence is definitely lower in Iraqi patients. In cover to traditional believe that north and south have increased rate of cancer due to the possible pollution, our data came with increased number in the middle of Iraq. Although a better statistical analysis is important after releasing the total population in Iraq.

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SOKAL RISK INDEX PREDICTS CHRONIC MYELOID LEUKEMIA RESPONSE TO IMATINIB

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Background. The 5-years analysis of the International Randomized Study of Interferon and STI571 (IRIS) has proven a 87% cumulative Complete Cytogenetic Response (CcyR) and an estimated 89% overall survival (OS) for patients treated with Imatinib (IM). Thereafter, quality of response to IM has been recognized as the most important prognostic factor for survival and progression free survival. Although some possible genetic markers are under study, the question of which group of patients (pts) are more prone either to response or to resistance is still unsolved. Sokal Risk Index, elaborated 25 years ago on the basis of clinical criteria and of the different survival-scores of pts treated with conventional chemotherapy, has made its way as one possible prognostic factor for pts on IM treatment. *Aims*. We have analyzed whether Sokal risk formulation predicts responses to IM as evaluated according to the European-Leukemia.Net criteria (ELN).

Table.

Responses to EuropeanLeukemia Net criteria at 12 months p=,007	Optimal Response		Suboptimal Response		Failure	
	n	%	n	%	n	%
Low Risk	26	76	7	21	1	3
Intermediate Risk	21	63	2	6	10	31
High Risk	4	50	0	0	4	50
Responses to EuropeanLeukemia Net criteria at 18 months p=,065						
Low Risk	12	52	8	34	3	13
Intermediate Risk	9	36	6	24	10	40
High Risk	3	38	1	12	4	50

Design and Methods. 83 pts in chronic-phase Ph+ CML received IM 400 mg daily, -among them, as many as 52% had been otherwise treated previously (second line IM). The cases were classified as to their response to treatment, after the recommendations published by the

ELN, as failures, suboptimal and optimal responses. In 80 pts Sokal risk score at diagnosis could be assesed. In 75/56 respectively, responses at 12 and 18 months have been evaluated. *Results*. Sokal risk score was LOW in 42,5%, INTERMEDIATE in 47% and HIGH in 10% of the pts. Percents of responses at 12 months (according to the ELN criteria): failure 36%, suboptimal 24% and optimal 38%. Of note is that no therapeutical changes before this interval (12 months) were undertaken. Distribution of response at 18 months: failure 25%, suboptimal 12% and optimal 63%, the pts considered to be in failure at 12 months have received alternative therapies thereafter. Responses by Sokal risk score at 12 and 18 months are shown in Table 1. *Conclusions*: Sokal Risk Index should be considered a useful prognostic aid to predict CML responses to Imatinib.

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EFFICACY OF UPFRONT IMATINIB IN CML CHRONIC PHASE: INDIAN STUDY

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Background. 50 patients were enrolled starting from 2001. All patients were positive for either Philadelphia chromosome or bcr-abl gene tested by FISH/PCR. There were 35 males and 15 females. Median age of males was 42 yrs (6-72), and females was 51 yrs (21-68). The median TLC was 110 thous/µl (13-340). Blasts were seen in all patients with TLC>100 thous/µl (blasts ranging from 2-6%), at the time of diagnosis. *Design and Methods*. Patients were given Hydroxyurea to bring the counts in the normal range, after which, patients were given 400 mg of Imatinib daily (except 1 pediatric patient on 200 mg). Median follow-up of patients on Imatinib was 48 months. 49 out of 50 patients achieved complete hematological remission (CHR) (98%). Information of major molecular response (MMR) was available for 30 patients. *Results*. 20 out of these 30 patients achieved MMR at median follow-up of 36 months. 3 patients achieved complete molecular remission (CMR). Side effects included nausea/vomiting (20%), muscle pain (20%), depigmentation (95% pts), periorbital edema (80% pts), temporary neutropenia and thrombocytopenia (20% pts). The latter responded to dose reduction of Imatinib to 300 mg and use of G-CSF. 2 patients continue on 300 mg imatinib. Nausea/vomiting resolved within 2-3 months on its own. Lack of response was seen in 12 patients - 1 had primary resistance, 4 had only molecular relapse and 7 had hematological relapse. All 4 patients with molecular relapse responded to increased doses of imatinib. Of the 7 patients with hematological relapse, 2 underwent successful allogeneic BMT, 2 are on higher dose imatinib, 2 on second-generation TKI, and 1 died due to progressive disease (PD). 43 patients continue to take imatinib. Mutational analysis was done on 10 patients with relapse. Out of these, 3 patients were positive for mutations - 1 had M 351 T, and 2 had F 311 I mutations. Till date, 3 patients have died - 1 from PD, 1 during BMT (done for CML-CP) and 1 due to unrelated cause (myocardial Infarction).

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TREATMENT OF PHILADELPHIA - POSITIVE CHRONIC MYELOID LEUKEMIA WITH IMATINIB: ONE CENTER EXPERIENCE

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Background. Imatinib mesylate, a targeted inhibitor of the BCR-ABL tyrosine kinase, is the standard of care for chronic myeloid leukemia (CML). Imatinib has demonstrated a high level of efficacy and is associated with significant less toxicity than previously used therapies. Aims. To review the efficacy and safety of imatinib in patients with CML in chronic phase. *Design and Methods*. A retrospective review of patients in one department of hematology with a diagnosis of Ph/BCR-ABL positive CML and received imatinib. Criteria for treatment responses were according to the recommendations from the expert panel of European LeukemiaNet. *Results.* From 15.02.2002 to 15.02.2008, 48 patients in CML-CP received imatinib were introduced in the study. The median age at diagnosis was 34 years old (range 13 to 72) with 54% women. Sokal score was 1,30 (0,39-4,05) and Hasford score 1012 (58-2435). All patients had positive karyotiping for Philadelphia chromosome. Treatment and outcome. 16 (33%) patients received imatinib as upfront therapy, the others as second or third line treatment after hydroxiureea and/or interferon α, the mean time to initiate imatinib was 25 months (range 1-104). All the patients received a starting dose of 400 mg/day. Clinical assessment and laboratory investigations including cytogenetics studies were performed at regular intervals. The RT-PCR studies for bcr-abl were performed from 2007. Responses rate. After starting imatinib, a CHR was achieved at 3 months by 87,5% patients. The CyR achieved was major in 59,6% (an equal number of complete and partial responses), no CyR in 12 patients (25%). The molecular response was complete in 6 (12,5%) and major in 9 (18,8%) patients. Better cytogenetic responses were achieved by those who were treated with upfront imatinib (87,5 vs 45,2% *p* 0.006) and those who received imatinib up to 24 months from diagnosis (73,1% vs 42,9 p 0,043). The doses were increased in 5 patients but an improved response was achieved in only one. Three patients were switched to Dasatinib with an MjCyR in one patient at 12 months. Seven patients developed under imatinib additional cytogenetic anomalies : supplemental chromosome 8 (6), duplication of Ph1 (2), trisomy 17 and 19 (1). Survival data. The median of follow-up was 53 months (range 10-144) and under imatinib was 26 months (range 3-76). Seven patients died: five of blastic transformation (44-104 months after diagnosis) one of septic shock and one of caschexia (in CMR). The Sokal score was a better predictor than Hasford's. Safety profile. Adverse events were registered in 14 patients (29%). Ten experienced hematological toxicities but grade 3 or 4, only in four, without future consequences. One patient experienced a secondary MDS, and four an important edema. *Conclusions*. The age at diagnosis in CML patients was decreasing during the last decades. Imatinib remains effective and well tolerated treatment for most of the patients, but there are still a significant number of patients who did not achieve a CyR. The responses and survival were not influenced by the previous treatments but the earlier introduction of imatinib is better. The Sokal score seems to have a better prognostic role.

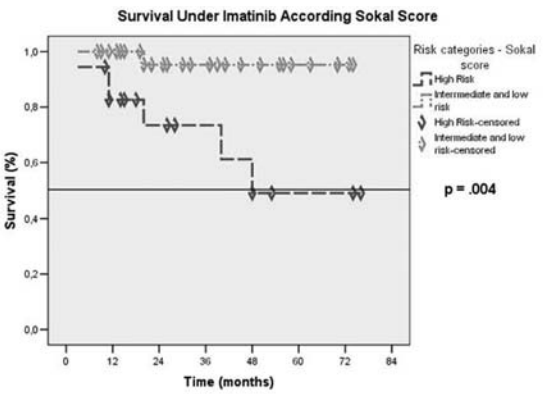

Figure 1. Survival of patients with CML after imatinib.

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B-LYMPHOID BLAST CRISIS OF P190 CHRONIC MYELOID LEUKEMIA RESPONSIVE TO DASATINIB: A CASE REPORT

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Background. Chronic myeloid leukemia (CML) is characterized by involvement of Philadelphia chromosome (Ph), which derived from a reciprocal translocation between chromosome 9 and 22. The breakpoint on the BCR gene occurs within the major breakpoint cluster region (M-BCR) and the BCR/ABL fusion gene codes for p210 protein. In two-third of patients with positive Ph-positive, acute lymphoblastic leukemia (ALL) and in rare case of CML, the breakpoint occurs within the minor breakpoint cluster region (m-BCR). In these cases the first BCR exon is fused to ABL exon 2 (e1a2) junction and a BCR/ABL protein of 190 kDA is formed (p190). Aim: We report a case of p190 CML in B-lymphoid blast crisis (B-Ly BC) responsive to dasatinib treatment. Case report: In March 2007, a 48 years old woman suffering from anemia, was referred to our centre. On the admission the patient had palpable liver but no splenomegaly. Hematological findings were: hemoglobin (Hb) 5.9 g/dl, platelets $104x10^9$/l and white blood cells (WBC) $25.2x10^9$/L (45% neutrophils, 5% metamyelocytes, 10% eosinophils, 1% basophils, 16%

lymphocytes, 10% monocytes and 10% blasts). The neutrophils alkaline phosphatase score was 3. A bone marrow (BM) aspirate showed marked hypercellularity, myeloid hyperplasia and 11% blasts. Cytogenetic analysis of BM showed 46 XX t(9;22) metaphases. Reverse transcriptase-polimerase chain reaction (RT-PCR) analysis showed a p190 BCR-ABL transcript (e1a2). A diagnosis of CML p190 was made and the patient was treated with imatinib 400 mg/day. The patient achieved complete hematological remission (CHR), but after 4 months she lose CHR. Main hematological findings were: WBC 40.2x10^9/l with 50% blasts and 40% blasts on BM smears. The blasts immunophenotype was CD10+, CD19+, CD22+, CD13+, CD33+, CD34+, DR+. Cytogenetic analysis of BM showed the presence of 46 XX t(9;22) methaphases and RT-PCR analysis showed a p190 BCR-ABL transcript. A diagnosis of B-Ly BC was made. The patient was treated with a polichemotherapy including vincristine, daunorubicin and prednisone and achieved CHR. Then she was treated with consolidation therapy with HAM protocol (ARA-C + mitoxantrone) and achieved complete molecular response (CMR). Therefore she started maintenance therapy with dasatinib 50 mg x 2/day until august 2008. CMR was costantly mantained. At this time, the patient received an allo-transplant from an HLA identical sibling donor using conditioning regimen with cyclophosphamide and Total Body Irradiation. Today, six months after the transplant, the patient is still in CMR with full donor chimerism, without therapy. *Conclusions*. To our knowledge, this is the first case of p190 CML B-Ly BC which lose CHR to imatinib and maintained with dasatinib the CMR obtained after polichemotherapy including antracyclines and cytosine arabinoside. This case comfirms the efficacy of dasatinib in patients with BC of CML also in the rare cases with p190 BCR-ABL transcript.

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PV AND ET PATIENTS PRESENT WITH ACQUIRED STORAGE POOL DISEASE LIKE PHENOTYPE

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Acquistion of Janus Kinase 2 (JAK2) V617F mutation heralds unregulated expansion of myeloid haematopoetic lineages which present as polycythaemia ruba vera (PV), essential thrombocythaemia (ET) or primary myleoifibrosis (PMF). These disorders are also associated with increased risk of thrombotic and hemorrhagic events, a major cause of morbidity and mortality. Therefore we studied the peripheral blood (PB) hemostatic status by measuring expression of platelet cell markers, pre and post stimuli to a number of agonists by flow cytometry (FCM), thereby assessed their function by measuring both P-selectin (CD62P) and CD63. In total 76 (F:40;M:36) patients (PV: 34; ET: 42) with median age 69.0 (range 30-94) were studied and 26 normal controls, Control Group (CG) (F:17;M:9); median age 28.3 (range 20-53) were included. With patients' informed written consent their asprin administration was withdrawn for 10 days prior to collection of blood. PB samples were collected into tri-sodium citrate and subsequently diluted 1:10 in physiological saline prior to incubation with the agnosists. The samples were then analysed by FCM within 1 hour of PB collection. Unstimulated patients´ platelets had higher basal level than the CG as measured by expression of CD62P. But, exposure to TRAP6 elicited a sub-optimum response from patients' platelets compared to the CG (see Table).

Table 1. Platelet cell marker expression data pre and post stimualtion with agonist.

	CD62P (%)	CD63 (%)	CD62P (%)	CD62P (%)		CD63 (%)		CD62P (%)	Peripheral blood counts at diagnosis	
	Basal levels	Basal levels	A.A 0.1mM	TRAP6 5μM	TRAP6 25μM	TRAP6 5μM	TRA6 25μM	ADP 5μM		
CG n=26	3.1 (1-8)	1.7 (0.8-3.9)	12.9 (4-79.6)	83 (10-99)	97 (88-99)	37 (2-89)	83.1 (46-97)	74 (50-93)	WBC (x10^9/l)	PLT (x10^3/l)
PV n=34	7.8 (1.6-36)	2.7 (0-22)	28.5 (4-77.5)	65 (5-93)	87 (49-98)	25.6 (3-76)	53.2 (15-96)	76.6 (38-88)	10.7 (4.2-24)	429 (454-1065)
ET n=42	5.1 (0-38)	2.6 (0-17.5)	19.9 (3-72)	64.8 (3-98)	91 (7-99)	22.2 (15-93)	63.5 (5-93)	70.8 (15-93)	9.0 (4.6-32)	821 (458-1697)

Median values are recorded with the range in parenthesis. CG: Control Group; PV Polycythaemia ruba vera; ET: essential thrombocythaemia. AA: Arachadonic acid. ADP: Adenosine diphosphate

Furthermore, PV and ET platelets response to AA was increased but in proportion to that seen in the CG. But no difference was seen in CD62P expression following stimulation with ADP. Together these data are reminiscent of acquired storage pool disease (ASPD). Therefore we tested the platelets for mepacrine uptake and release using FCM. We found 26 of 32 PV and 22 of 42 ET patients were negative, whilst all CG samples were positive. These observations mimic acquired storage pool disease (ASPD) phenotype and concur with the decreased CD63 expression post TRAP6 and normal response to ADP activation. However, we found no correlation between positivity for V617F mutation and the mepacrine test data. The increased basal expression of P-selectin and ASPD like phenotype imply dysregulated haemostatic function that probably contributes to the thrombohaemorrhagic events frequently observed in myeloproliferative disorders.

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THROMBOCYTE GLYCOPROTEINS MODIFIED STRUCTURE AND FUNCTIONAL STATUS IN CHRONIC MYELOPROLIFERATIVE DISORDERS - A MODEL OF ACQUIRED THROMBOPATHIESA

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Background. Chronic myeloproliferative disorders (CMPD) are highly associated with altered platelet homeostasis generating hemorrhage and thrombosis.Different laboratory methods are used for better understanding thrombocyte functional abnormalities. *Aims*. Combining methods to a complete investigation of platelet functional changes in CMPD - investigation of surface and intracellular expression of von Willebrand factor (vWF) receptor and αIIb-β3 integrin by flowcytometry, respectively western-blot method, and platelet functional status by aggregometry. *Design and Methods*.Whole blood flowcytometry for platelet surface glycoproteins-(Gp) (vWF receptor-GpIb-IX [CD42b,CD42a], fibrinogen receptor-GpIIb-IIIa [CD41,CD61], P-selectin [CD62P], granulophysin [CD63]) was performed in 42patients with Ph-negative CMPD and 13controls. CMPD group included 14patients with Myeloid Metaplasia, 12patients with Polycythemia Vera, 9patients with Essential Thrombocythemia and 7patients with Unclassified-CMPD. Platelet aggregometry after stimulation with collagen, ADP, ristocetine and epinephrine was completed in 41patients with CMPD. *Results*. Platelet immunophenotyping analysis revealed:1) statistically significant reduced expression of adhesion markers (CD42b, CD42a) in CMPD group vs. control (median of mean fluorescence intensity: 31% vs. 78.1%, $p<0.05$, respectively 20.22% vs. 80.04%, $p=0.003$);2)low expression of CD41 in CMPD vs. controls (median: 44% vs. 70.3%; $p=0.01$), without any differences in CD61 expression;3)no differences for the level of activation markers.[figure1a]. No platelet immunophenotyping differences were detected among specific CMPD subgroups. The aggregometry analysis revealed:1)CMPD cases vs. controls presented reduced amplitude (median 48% vs. 74%, $p= 0.003$) and slope at ADP stimulation (median: 73% vs. 99%, $p=0.027$) and also at epinephrine stimulation (median amplitude 3% vs. 68% and median slope 34% vs. 82%: $p<0.07$). We also noticed a drop in collagen slope in case groups (median 47% vs. 82%: $p<0.69$) without any significant differences in collagen amplitude.2)No statistically significant variations were noted in ristocetin response. [Figure1b]. Western-blot analysis for vWF Receptor revealed: subunit 42b presented reduced levels of expression in cases vs. controls; 42a and 42c components had no change; 42d unit was under the detection limit of the method. Examination of αIIbβ3-integrin showed a higher expression in cases vs. controls for αIIb subunit and no alteration for β3 subunit. [Figure1C]. *Conclusions*. Our data show that western-blot and flowcytometry are equally sensitive methods for investigating platelet glycoproteins expression. Ib-alfa subunit of vWF receptor had a reduced expression on cell surface and in platelet lysate, signifying either reduced production, or an inhibiting expression mutation, or transient suppression of synthesis. The decreased expression doesn't have functional implications when detected by aggregometry (normal response to ristocetin). Expression of GpIX(CD42a) and GpIIb(CD41) is reduced on cell surface, but it is normal/respectively over-expressed in platelet lysate, suggesting that these glycoproteins are initially present and then inter-

nalized, or deficiently expressed. Neither method showed any alteration in the expression of β3 subunit of the fibrinogen receptor. Analysis of activation markers (P-selectin,granulophysin) on platelet surface doesn't show any variation of platelet activation in patients vs controls.Aggregometry showed alterations suggesting acquired Glanzmann thrombasthenia, with noticeable decrease up to flatting of response to ADP and epinephrine. Finally, it appears that the functional role of GpIIb is much higher than it was believed-fibrinogen binds effectively to CD61 or activation doesn't induce conformational changes necessary to expose fibrinogen binding site.

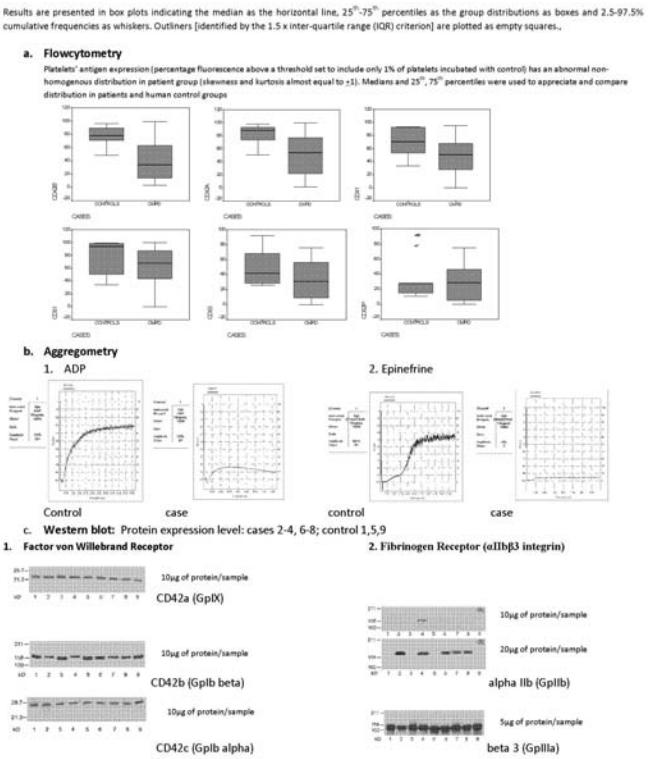

Figure 1. Multi method investigation of platelet behaviour in CMPD.

1494

QUALITITATIVE AND QUANTITATIVE ANALYSIS OF JAK2V617F MUTATIONS UNRAVELS HIGHER RESPONSIVENESS OF JAK2+ ET PATIENTS TREATED WITH HYDROXYUREA

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Background. JAK2V617F mutations are relevant genetic events that had been shown to be correlated to BCR-ABL negative myeloproliferative diseases. The large majority of PV patients, and 50-70% of Essential Trombocytemia (ET) patients, harbor the V617F allele. Recently, the allelic ratio (AR) of mutated to wild type JAK2 genes found in blood samples was suggested to be related to worst prognosis as the AR increases. Most of the AR determinations have been performed in cDNA samples, but this approach poses a real problem to laboratories that stored only DNA extracted from large granulocytic fractions collections. To approach the quantitative analysis of former stored DNA samples, we set a new methodology based on DNA and fluorescent-primer for a quantitative-based PCR approach. These extend our previously analyses with a qualitative JAK2 PCR and allowed the calculation of the AR ratio. Aims:To evaluate the JAK2 mutation status in PV and ET patients, it's impact in the response to Hydroxyurea and to correlate JAK2 allelic ratio to patient's response ratios. *Design and Methods*. PV (10 patients) and ET (27 patients) blood samples were separated in Ficoll gradient and the red cell/granulocitic fractions were submitted to hypotonic lysis and DNA isolation(DNAzol®). All patients included tested negative for BCR-ABL transcripts. Qualitative Allele-Specific PCR for JAK2 mutation was performed as described by Baxter *et al*. For Quantitative PCR, 6-FAM fluorescent-primers, adapted from Jones et al, were synthesized. A patient sample carrying high allelic rate mutation was selected and PCR fragments were cloned in TOPO-TA plasmid. Dilution curves of mutated and wild-type JAK2 templates were performed, PCR amplified and analyzed into a 377 DNA sequencer. *Results*. Using the qualitative JAK2 PCR essay, we found 90% of PV samples carrying the Val617Phe mutation (JAK2+). All PV patients were treated with Hydroxyurea (0,5-2,5g) before testing. Amongst JAK2+ patients, 72% had platelet counts above 450.000 at diagnosis and 50% of them reached platelet counts lower than 450.000 after treatment. ET patients tested JAK2+ for 55,6% of the samples. Of those, 82% were previously treated with Hydroxyurea (0,5-2,5g). Amongst the treated patients in JAK2- group, only 13,3% showed platelets counts <450.000. In contrast, 58,3% of JAK2+ treated ET patients showed platelets counts >450.000 (p=0.037, Fisher`s exact test). Median values for platelet counts at diagnosis were similar in both JAK2+ or negative groups with no statistic difference. Hydroxyurea doses used for the treatment of JAK2+ patients were lower (n=9, dose=0,78±0,26g) than the doses used for JAK2- patients (n=12, dose=1,21+/-0,54g, p=0,041 T test). Allelic sample calibration curves showed an excellent correlation coefficient (r2>0.98) and a sensibility of 2%, allowing the calculation of AR in every previously JAK2+ tested sample. *Conclusions*. The frequency of JAK2+ patients found in PV and TE groups were almost similar to those described in literature. In ET, JAK2- samples was correlated to pourer response to Hydroxyurea, requiring higher doses to achieve more discrete platelet count reductions. The allelic ratio data, obtained from DNA, allowed a more detailed analysis of patients. This could be relevant in future attempts to early predict good and bad responders to Hydroxyurea-therapy.

1495

ABSENCE OF REARRANGEMENTS OR ACTIVATING MUTATIONS IN THE IN THE RTK III AND IV FAMILY GENES IN BCR-ABL1 NEGATIVE AND JAK2V617F NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS (CMPNS)

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BCR-ABL1 negative chronic myeloproliferative neoplasms (CMPNs) are a heterogeneous group of clonal haematological malignancies. Over the last years, some genetic alterations have been described to cause these diseases, most of them activating tyrosine kinase (TK) genes. Tyrosine kinases (TK) have an important role in cell growth and oncogenesis, as gain-of-function mutations can lead to the constitutive activation of the signalling pathways in which they are involved. In this study, we have analysed all genes from the families III (PDGFRA, PDGFRB, CSF1R, KIT and FLT3) and IV (FGFR1, FGFR2, FGFR3 and FGFR4) of RTKs. All of them code for receptors with tyrosine kinase activity and some of them have been found mutated in CMPNs and in other tumor types. We have used FISH to detect cryptic rearrangements and dHPLC to detect sequence mutations on samples from 44 BCR-ABL1 negative and V617FJAK2 negative CMPN patients. Both analyses have shown that these genes are no frequently mutated in these diseases, implying that molecular events or cryptic rearrangements causing these diseases, if they exist, must be located in other genes.

1496

FISH AND MUTATIONAL SCREENING OF THE ABL, SYK AND JAK TYROSINE KINASE FAMILY GENES IN BCR-ABL1 NEGATIVE AND JAK2V617F NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS (CMPNS)

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Chronic myeloproliferative neoplasms (CMPNs) are clonal disorders of the hematopoietic stem cells, characterized by abnormal proliferation and survival of one or more cells of the myeloid lineage. BCR-ABL1 negative CMPNs are a heterogeneous group of diseases for which the molecular pathogenesis is not well understood. Over the last years some genetic alterations have been described, most of them activating some tyrosine kinase genes playing a role similar to ABL1 in CML. Tyrosine kinases (TK) have an important role in cell growth and oncogenesis. Deregulation of TK genes (mainly due to translocations, amplifications or point mutations) can result in constitutive activation of the signalling pathways in which they are involved, causing the abnormal proliferation and survival that characterize these pathologies. In this study, we have analysed all genes from the families Jak (JAK1, JAK2, JAK3 and

TYK2), Abl (ABL1 and ABL2) and Syk (SYK and ZAP70) of TKs. All of them code for cytoplasmic tyrosine kinase proteins and some of them have been found mutated in CMPNs and in other tumor types. We have used FISH to detect cryptic rearrangements and dHPLC to detect sequence mutations on samples from 44 BCR-ABL1 negative and V617FJAK2 negative CMPN patients. Both analyses have shown that these genes are no frequently mutated in these diseases, implying that lesions in other genes must be involved in the pathogenesis of these diseases.

1497

QUANTIFICATION OF PRV1 GENE USING ABL AND GAPDH AS REFERENCE GENES - COMPARISON OF RESULTS OBTAINED IN GROUP OF 21 PATIENTS WITH POLYCYTHEMIA VERA

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Primary polycythemia results from an intrinsic defect in hematopoietic stem cell, which results in increased production of erythrocytes. The most common form of primary polycythemia is polycythemia vera (PV) - one of Ph-negative chronic myeloproliferative disorders (Ph-negative cMPD). The aim of the study was to compare results of RQ-PCR obtained from 21 patients with PV performed using GAPDH and ABL as reference genes. GAPDH was used according to Klippel S. et al (Blood 2003, 15 (102); 3569-74). The method with use of ABL was established in our laboratory. A group of 21 patients with Ph-negative cMPD at the moment of diagnosis and 5 healthy volunteers were investigated. For PRV1 gene quantification only patients with negative result of multiplex-RT-PCR for BCR/ABL were taken. Briefly: from peripheral blood white cells preserved in TriPure RNA were isolated, then the reverse transcription from 1000ng of RNA (Superscript II, Invitrogen) was done. The next step was the RQ-PCR reaction based on TaqMan Gene Expression Assay Hs 00360669_mL (PRV1, Applied Biosystems). Two reference genes GAPDH (primers and probe (JOE, TAMRA) based on Klippel S. et al) and ABL (primers and probe (FAM, TAMRA) based on Europe Against Cancer program) were used. All reactions were performed in duplicates using 7500 Applied Biosystems platform. Our results were analysed with 3 different thresholds: 0,04 for PRV1/0,2 for GAPDH (*according to Klippel S. et al.*), and 0,1 for PRV1/0,1 for ABL (according to European Leukemia Net standards). The results were expressed as ratio: Ct PRV1/Ct GAPDH and Ct PRV1/Ct ABL. For statistic analysis t-student test was used. We did not find any differences between PRV1 expression obtained with use of two different reference genes. However three different thresholds were used. In the group of patients with PV analyzed by Klippel S. et al the mean ratio is 1,04 (range 0,85-1,17), and in healthy volunteers is 1,31 (range 1,20-1,52). The results obtained in our patients are shown in Table.1

Table 1.

	PRV1/ABL	PRV1/GAPDH
Mean ratio in PV group (range)	0,96 ± 0,12 (0,59 – 1,02)	0,94 ± 0,11 (0,64 – 1,06)
Mean ratio in healthy group (range)	1,05 ± 0,02 (1,02 – 1,07)	1,03 ± 0,05 (1,00 – 1,05)

The thresholds detected by Klippel *et al*. are lower than in our method. In many patients diagnosed as PV according to WHO criteria PRV1 expression in bone marrow was at the lower level than 0,85. The discrepancy like this could cause the difficulties with proper allocation of patients to PV or non-PV group. Therefore it would be suggested that each laboratory should establish individual thresholds in control group and patients diagnosed as PV according to WHO clinical criteria. It is possible to quantify the expression of PRV1 using GAPDH or ABL as reference gene

1498

DISAPPEARANCE OF JAK2 EXON 12 MUTATION BY HYDROXYUREA TREATMENT

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Background. Management of polycythemia has been dramatically changed with the discovery of Jak2 mutation. Actually, a negative search for Jak2 mutations at diagnosis excluded the diagnosis of polycythemia vera. However, this situation is more complex for patients already treated by cytoreductive drugs, as it was reported that interferon α and hydroxyurea can diminish the exon 14 Jak2 mutation allele burden or eventually can obtain molecular complete remission. In this setting, a negative search for Jak2 mutations is not sufficient to eliminate the diagnosis of polycythemia vera but necessitates to reconsider the diagnosis. *Aims*. We report the first case of disappearance of exon 12 Jak2 mutation on hydroxyurea treatment. *Methods*. A 42 year-old man was referred to our center for confirmation of polycythemia vera. He was diagnosed before the Jak2 area with polycythemia with 6 500 000 erythrocytes, 18,5 g/dL hemoglobin and 58% hematocrite confirmed by red cell mass evaluation. Leucocytes and platelets were normal. No cause of secondary polycythemia was retrieved. He was treated with hydroxyurea. After the discovery of exon 14 Jak2 mutation, the patient was screened for this mutation. Exon 14 Jak2 screening was negative. After the discovery of exon 12 Jak2 mutation, the patient was screened for this second mutation but was negative. Moreover, it was decided to stop hydroxyurea treatment to reconsider the diagnosis of polycythemia vera. Four months after the cessation of hydroxyurea, polycythemia occurs again with 0,60 of hematocrit. After the reported effect of interferon and then hydroxyurea on Jak2 allele burden, the patient was again tested for the two Jak2 mutations. Exon 14 Jak2 mutation was negative but exon 12 mutation was positive. The diagnosis of polycythema vera was confirmed and hydroxyurea treatment was restarted. Results - Negative screening for Jak2 mutations in a patient with polycythemia at diagnosis is a major criteria against the diagnosis for polycythemia vera. More recently, it was reported that treatment as interferon α or hydroxyurea can diminish Jak2 exon 14 mutation allele burden and even gives negative results. So, a negative result in a polycythemia vera patient treated can not exclude the diagnosis. To our knowledge, disappearance of exon 12 mutation on hydroxyurea treatment has never been reported. *Conclusions*. Hydroxyurea treatment and probably interferon α can modulate exon 12 mutation allele burden, as for exon 14 mutation. A negative result in a treated patient may be interpreted with caution.

1499

JAK2 V617F, C-MPL W515L AND W515K MUTATIONS IN ROMANIAN PATIENTS WITH POLYCYTHEMIA VERA, ESSENTIAL THROMBOCYTHEMIA AND PRIMARY MYELOFIBROSIS

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Background. Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are typical myeloproliferative disorders (MPDs). A major breakthrough in the ethiopathogenesis of MPDs has been made with the discovery of a somatic mutation, Janus kinase 2 (JAK2) V617F, which shortly afterwards became a major diagnosis criterion, in most of PV cases and around half of ET and PMF cases. Moreover, a small proportion of ET and PMF cases were shown to harbour somatic mutations in myeloproliferative leukemia virus oncogene (c-MPL) gene. *Aims*.The purpose of this study was to investigate JAK2 V617F and c-MPL W515L and W515K mutations in a group of Romanian patients with PV, ET and PMF. *Design and Methods*. A total number of 99 patients were included in the study, comprising 59 PV, 35 ET and 5 PMF patients. The JAK2 V617F mutation was studied by two different approaches, a nested PCR-RFLP (Polymerase Chain Reaction -

Restriction Fragments Length Polymorphism) and a tetra-primer PCR technique, respectively. c-MPL W515L and W515K mutations were screened by allele-specific PCR approaches in ET and PMF patients. *Results.* JAK2 V617F mutation could be demonstrated in 48 PV patients (81%), 21 ET patients (60%) and 4 PMF patients (80%). Overall, the expansion of the JAK2 mutant clone tended to be higher in JAK2 positive PV and PMF patients than in JAK2 positive ET patients. Both the techniques used for studying JAK2 V617F mutation, nested PCR-RFLP and tetra-primer PCR, respectively, gave concordant results in all the patients. As for c-MPL mutations, no ET or PMF patient was found to harbour neither the W515L mutation, nor the W515K mutation. *Conclusions*. The distribution of JAK2 V617F mutation in our group is concordant with data reported in literature. The absence of c-MPL W515L/K mutations in our group is not so surprising, taking into consideration their low frequency reported in ET and PMF patients. We cannot rule out the possibility of other JAK2 or c-MPL mutations in patients negative for JAK2 V617F and c-MPL W515L/K, which we were not yet able to analyze. The techniques used in this study for studying JAK2 V617F and c-MPL W515L/K mutations are quite inexpensive and they provide sufficient sensitivity for diagnostic purposes. As new molecular markers are discovered in hematological malignancies, their investigation become part of the investigations protocol, as diagnostic and sometimes as prognostic markers.

1500

COEXISTENCE OF POLYCITHEMYA VERA JAK2 V617F-POSITIVE AND MONOCLONAL GAMMOPATHY

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Background. Polycithemia vera (PV) is a clonal disease which originates from abnormal hematopoietic progenitor cells at the pluripotent stem cell level. The somatic mutation JAK2 V617F in exon 12 of the jak2 gene, with the constitutive activation of the gene, has been identified in about 81% (range, 65-97%) of patients with PV; in addition JAK2 V617F is not associated with Multiple Myeloma as recently showed. Concomitant cases of Monoclonal gammopathy (MG) with PV have been described in literature but gammopathies have been related to the precedent therapy for PV and JAK2 V617F was not performed. *Aims*. Here we report 2 cases of concomitant PV JAK2 V617F-positive and monoclonal gammopathy in which the MG has been diagnosed before or at the same time of PV. *Design and Methods*. The first one is about a 70-year old female who was referred to our institution because of elevated blood cell count and MG. Her blood test showed an increase in the number of white blood cells (WBC) and thrombocytosis of $12x10^9$/L and $0.7x10^{12}$/L, respectively, and hemoglobin of 178 g/L. Immunoelectophoresis showed 0.8 g/L of IgG/k monoclonal protein . Bence Jones (BJ) proteinuria was negative and renal function was normal. BM aspirates was consistent with PV and showed plasmacells (PC)<10% and eythroid iperplasia. Karyotype analysis revealed 46 XX. JAK2 V617F was identified in whole blood peripheral leucocytes with RT-PCR. PV was diagnosed according to WHO 2008 criteria. The second one is about a 67-year old male referred to our institution for MG. His blood test showed increased WBC, platelets and hemoglobin ($11x10^9$/L, $0.6x10^{12}$/L, 180 g/L respectively). He had 2 g/L of IgG/a monoclonal protein . BJ proteinuria was negative and renal function was normal. BM aspirates was consistent with PV and showed PC <10%. X-ray total body showed two osteolitic lesions in the skull. JAK2 V617F analyzed with RT-PCR from whole blood peripheral leucocytes was positive. Both patients were treated for PV with low dose aspirin and with periodic phlebotomy to reduce hematocrit to 47%. Laboratory blood test check for MG was performed every six months; a X-ray total body every year. *Results and conclusions*. Our case reports suggest that the coexistence of two different diseases from the same bone marrow milieu probably takes origin from different cell clones. In literature coexistence of PV and MG has been described but it has been associated to a precedent therapy for PV. Here the two diseases were diagnosed at the same time and the presence of JAK2 V617F mutation provides a molecular basis for the identification of PV-clone.

1501

JAK2V617F MUTATION IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA: INFLUENCE ON CLINICAL AND LABORATORY FEATURES AT DIAGNOSIS AND DURING FOLLOW UP

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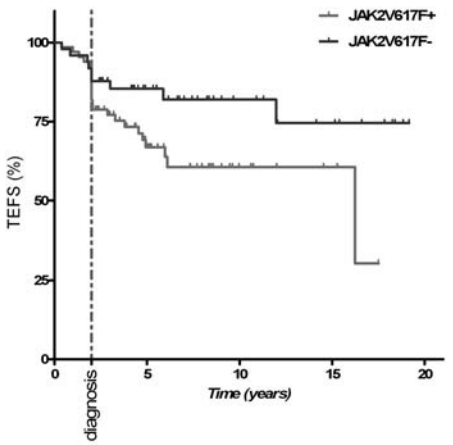
Background. Acquired mutation of JAK2V617F can be found in approximately 50-60% of Essential Thrombocytemia (ET). The impact of this mutation on its molecular pathogenesis and clinical phenotype is still debated AIMS: We investigated the frequency of JAK2 mutation and its possible impact and correlation with clinical and laboratory features at diagnosis and during follow-up in ET patients (pts) referred to our division between 1987 and 2008. *Design and Methods*. Allele-specific PCR for JAK2V617F mutational status was performed on genomic DNA from bone marrow cells or peripheral blood granulocytes in 115 ET (49 males, 66 females) pts. Clinical and laboratory features were evaluated at diagnosis and during follow-up, comparing mutated JAK2 ET pts versus pts harbouring wild-type (WT) gene. Data were processed using the Graph Pad PRISM 5 DEMO Software performing statistical non-parametric methods: univariate analysis was performed to evaluate differences in proportions by the chi-square and Fisher's exact tests for categorical nominal variables and the Mann-Whitney rank test for ordinal variables. Continuous variables have been categorized using median values. The log-rank (Mantel-Cox) test applied to Kaplan-Meyer method was employed to estimate thrombotic risk and thrombotic event-free-survival.



Thrombotic event free survival (TEFS) in JAK2V617F positive and negative patients

Figure 1.

Results. 66 ET pts (57,4%) were JAK2V617 positive. JAK2 mutated pts were older (median age 59 vs 48 years, p=0,0016) and presented at diagnosis significantly higher hemoglobin (Hb 14,3 g/dl vs 13,3 g/dl, p=0,0027), higher hematocrit (Ht 43% vs 39,8%, p<0,0001) and lower PLT count (PLT 725 vs 841 $x10^3$/L, p=0,005) respect to WT group. These PV-like features have also maintained during the course of disease, with statistical significance. No difference was observed in term of gender, white blood count, LDH, progression or entity of splenomegaly, and need of cytoreductive therapy between JAK2 mutated and WT pts. A highly significant increase of thrombotic complications was registered for JAK2 positive pts. Considering JAK2 WT ET as reference group, the relative risk (RR) of primary thrombotic event was 2,143 (95% CI: 1.053-4.360, p=0.035) for JAK2 mutated pts, with a TEFS lower than WT pts (as shown in Picture 1). Arterial events were more frequent than venous events without statistical difference between two ET groups. A trend of higher risk of recurrent thrombosis (p=0.056) was also shown for JAK2 mutated respect to WT pts. There was no difference about haemorragic complications, time and incidence of evolution in myelofibrosis between JAK2 mutated or WT pts. *Conclusions*. In our population, 57,4% ET pts displayed JAK2V617F mutation. This mutation divides pts into two distinct subtypes. JAK2 mutated pts present older age and laboratory features at diagnosis and during follow-up compatible with PV-like phenotype. Mutated pts also showed an increased risk of thrombosis, at diagnosis and during the course of disease, in terms of recurrent throm-

bosis, too. These observations suggest a more aggressive phenotype since the diagnosis in JAK2 mutated ET group. A possible biological continuum between JAK2 mutated ET and PV, or in general between JAK2 positive ET and JAK2 positive MPD may be supposed.

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EFFECT OF ANAGRELIDE ON PLATELET, COAGULANT AND ENDOTHELIAL FUNCTION IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

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Background. The large vessel thrombosis may complicate the essential thrombocyhemia (ET). Its pathogenesis is probably associated with platelet coagulant activation and this makes difficult its management. Anagrelide (ANA) is a platelet lowering drug that inhibits platelet aggregation. *Aims*. We investigated platelets and platelet factor 4 (PF4), prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complex (TAT), markers of platelet and coagulant activation, and tissue factor (TF) and von Willebrand factor (vWF), indicators of macrovascular damage in patients with ET. *Patients and Methods*. We recruited 19 patients with ET (12 males and 7 females, mean age 51 years) who fulfilled PVSG and WHO. All patients were on antiplatelets either aspirin (10 patients) or indobufen (7 patients) and ticlopidine (2 patients). Their mean duration of disease was 7 years. 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 $400x10^9$/L and with a average maintenance dosage of 2.1 mg/day. Platelets, PF4, F1+2, TAT, TF, vWF were measured before cytoreduction and to complete response defined as platelets < $400x10^9$/L. Platelets were measured by automated analyser. PF4, F1+2, TAT, TF and vWF were assayed by ELISA and immnunoturbidimetric assay, respectively. Results: Before ANA all patients had marked platelets ($1057\pm349x10^9$/L) and high PF4 (121±43 IU/mL vs 5.5±2.6 IU/mL) ($p<0.0001$), F1+2 (3.5±3.3 nmol/L vs 0.7±0.2 nmol/L) ($p<.0001$), TAT (28±32 µg/L vs 2.7±1 µg/L) ($p= 0.001$) and TF (247±184 pg/mL vs 4.8±2.5 pg/ml) ($p<0.0001$) and low vWF (22±8% vs 92±31%) ($p <.0001$). After ANA all patients had platelets <$400x10^9$/L (388 ± 66 x10^9/L) and normal PF4 (8.4±3.2 IU/mL) as well as F1+2 (1.1±0.8 nmol/L), TAT (2.4±1.1 µg/l), TF (8.3±0.4 pg/mL) and vWF (96±37%). A correlation was found between PF4 and F1+2 and TAT ($p<0.0001$ and $p=0.004$, respectively) and between PF4 and TF and vWF ($p<0.0001$ and $p=0.001$, respectively). Additionally, there was a correlation between F1+2 and TF and vWF ($p<0.0001$ and $p<.0001$, respectively) and TAT and vWF and TF ($p=0.001$ and $p= 0.023$, respectively). *Conclusions*. These findings suggest that the platelet coagulant activation is responsible for macrovascular damage and that ANA may repair the platelet coagulant endothelial injury and, hence, prevent the large vessel thrombosis.

1503

RAPID AND RELIABLE MUTATION SCREENING OF JAK2 EXONS 11-15 CODING SEQUENCE USING NON ISOTOPIC RNASE CLEAVAGE ASSAY (NIRCA)

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Background. The myeloproliferative disorders (MPD), polycythemia vera (PV), essential thrombocytosis (ET) and idiopathic myelofibrosis (IMF) exhibit gain-of-function V617 mutation of the Janus kinase (JAK) 2 gene (JAK2). Furthermore, 2 novel nucleotide exchanges in exon 14 and several exon 12 mutations of JAK2 have been identified in JAK2 V617F negative patients. In addition, there is low JAK2 V617F neutrophils allele burden in ET patents which can be omitted. *Aims*. In this study we report the development of a convenient and accurate method for screening of the coding region of the JAK2 gene which includes all potential mutations identified in exons 12 and 14 up to now and possible unidentified in the same regions. *Design and Methods*. Eight MPD patients (four PV, two ET and two IMF), who have been previously diagnosed to express JAK2 V617F mutation with allele specific PCR restriction enzyme analysis, four (two idiopathic erythrocytosis and two ET) negative in the same mutation and ten healthy individuals were cross-analyzed with NIRCA. RNA from peripheral granulocytes was isolated and PCR was performed using specific primers (containing a T7 promoter for upper primer and an SP6 one for the lower primer) spanning exons 11-15. These PCR products were analyzed using a NIRCA assay which is comprised by transcription, hybridization and digestion of these products. In transcription both strands of these products are transcribed along with wild type sample with the use of T7 and SP6 polymerases. These transcripts are hybridized in a fashion of sense and anti-sense patient strands mixed with complementary wild type strands. Wild type sense and anti-sense transcripts are hybridized in order to provide the control sample and the same procedure is repeated for the patient samples. Following hybridization, the products are digested using RNases in order to identify mutations by cleavage of mismatches. Then the digestion products are subject to agarose gel electrophoresis. *Results*. All eight patients positive to V617F mutation showed the same digestion pattern while digestion of all ten healthy individuals did not yield any fragments (results verified by direct sequencing). The same pattern of digestion was followed by a V617F negative ET patient, previously analyzed using restriction enzymes (direct sequencing verified low burden of V617F mutation). Different positive digestion pattern was also observed in two ET patients that were found negative in V617F mutation. Direct sequencing verified the presence of N542_E543del deletion in patient granulocytes. *Conclusions*. NIRCA is a rapid, highly sensitive and low cost technique that can be used in any laboratory. It has been proven to be accurate in the identification of even a low allele burden of JAK2 mutations in MPD patients and can be used for the simultaneous screening of the entire exon 11-15 JAK2 coding region with possible mutations while providing 100% sensitivity on negative results. As a result, its application could be used as a rapid screening test for the diagnosis of MPD patients since the digestion patterns are repeatable identifying particular mutations and direct sequencing can be avoided.

FIGURE 1. NIRCA digestion profiles in JAK-2 mutations.
Typical digestion profiles of A) healthy individuals, B) patients with V617F mutation, C) a patient with V617F mutation that showed false negative results with Baxter protocol and D) a patient with N542_E543del mutation. (ii) in B and D provides the corresponding sequencing for each digestion along with the wild type sequence (iii), while in C they are represented by (iii) and (iv) respectively. Lanes 1,2 and 3 in A(i), B(i), C(ii) and D(i) represent digestions, of the RNA duplex formed after hybridization of patient SP6 with wild type T7 opposable strands, with RNase 1, RNase T1 and a mix 1/1 of RNase 1 and RNase T1 respectively, while in C(i) the RNA duplex is formed after hybridization of patient T7 with patient SP6. Lane L corresponds to 100 base pair DNA marker (Fermentas). Digestion products are marked with arrows.

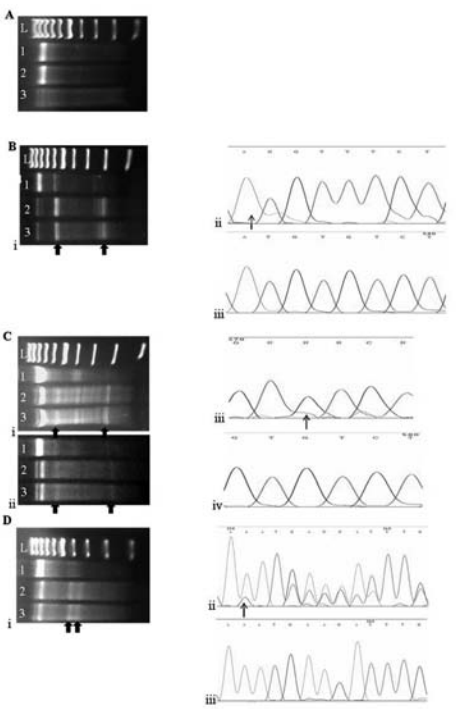

Figure 1.

1504

CLINICAL AND BIOLOGICAL PHENOTYPE IN ESSENTIAL THROMBOCYTHAEMIA PATIENTS WITH PLATELET COUNT BELOW 600X10^9/L

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Background. The WHO 2008 classification of Philadelfia negative chronic mieloproliferative neoplasms (MPN), by exploiting the crucial role of bone marrow biopsy and JAK2 V617F mutation, lowered the platelet (PLT) count cut-off for ET diagnosis from 600 to 450x10^9/L. *Aims*. To validate the diagnostic impact of the updated WHO PLT cut-off in a large series of ET patients, by evaluating the main parameters of the clinical and biological phenotype at diagnosis. *Design and Methods*. The patients, enrolled in the Registro Italiano Trombocitemia (RIT) after a written informed consent was reached, were divided on the basis of PLT count (x10^9/L) at diagnosis in three groups (451-600, 601-1000, >1000) and were compared by considering gender and other characteristics at diagnosis as age, symptoms, thrombosis, haemorrhage, splenomegaly, WBC count, Hgb level, bone marrow cellularity and fibrosis, and JAK2 pattern. The prescription of antiplatelet drugs was also considered. *Results*. The patients with a PLT count (x10^9/L) at diagnosis range >450 resulted to be 1844, being the PLT count 451-600 (group I) in 258 cases (14%), 601-1000 (group II) in 1219 cases (66.1%), and >1000 (group III) in the remaining 367 cases (19.9%). By moving from group I to group II and to group III, the female:male ratio increased (1.50, 1.58, 1.80), the median age was 60, 62, 58 years, the disease-related symptoms increased (36.8%, 37.3%, 45.5%, p 0.01), the thrombotic events decreased (27.2%,18.4%,12.9%, p 0.0001), the haemorrhagic events were 1.2%, 3.9%, 4.1%, a splenomegaly was 26.4%,25%, 26.4%. A rate of WBC count >8.7 x10^9/L increasd (34.5%, 48.9%, 62%, p 0.000), the median value of Hgb (g/dL) was 14.4, 14.3, and 13.7, the bone marrow biopsy documented an increase (p 0.000) of hypercellularity (36%, 50.4%, 59.5%) and of a grade 1 fibrosis (21.3%, 27,7%, 40,6%). In 665 evaluated cases, the JAK2 V617F mutation rate decreased (64.3%, 56.1%, 47.3%, p=0.04). The use of antiplatelet drugs was prescribed without significant differences in the three groups. (69.4%, 70.9%, 65.7%) *Conclusions*.In this RIT study the ET patients with a PLT count at diagnosis in the range 451-600x10^9/L showed a clinical and biological phenotype globally similar to that of patients with higher PLT count, althougt same characteristics resulted differently distributed. In fact, as expected they had a lower incidence of disease-related symptoms, haemorrhage, leukocytosis, bone marrow ipercellularity and grade 1 fibrosis, while surprisingly they had a higher incidence of thrombosis and Jak2 V617F mutation.

1505

PREVALENCE OF JAK2V617F MUTATION IN WOMEN WITH UNEXPLAINED RECURRENT MISCARRIAGE

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Background. The JAK2V617F mutation is associated with the group of Philadelphia-chromosome negative myeloproliferative disorders (MPD), e.g. polycythemia vera and essential thrombocytemia. The risk of a first trimester miscarriage is increased in pregnant women with MPD. Furthermore, an approximately five-fold increased risk of sporadic pregnancy loss (first trimester miscarriage, second trimester miscarriage and stillbirth) - was recently described in healthy women carrying the JAK2V617F mutation.[1] *Aims. W*e hypothesised that the JAK2V617F mutation plays an etiologic role in unexplained recurrent miscarriage and we assessed its prevalence in a well-defined group of healthy women with unexplained recurrent miscarriage. *Design and Methods.* The presence of the JAK2V617F mutation was determined in a cohort of 147 women diagnosed with unexplained recurrent miscarriage in an academic recurrent miscarriage clinic. Recurrent miscarriage was defined as two or more miscarriages with an upper gestational age of 20 weeks. Recurrent miscarriage was classified as unexplained when chromosome abnormalities, of both patient and her partner, uterine anomalies, antiphospholipid antibody syndrome (lupus anticoagulant and anticardiolipin IgM/ IgG) and hyperhomocysteinemia (fasting levels < 16 mmol/L) were ruled out. To test for the presence of the JAK2V617F mutation we used an allele-specific real-time quantitative TaqMan PCR. Results Of 147 women with unexplained recurrent miscarriage, 141 DNA samples could be tested for the presence of the JAK2V617F mutation. In none (0%, 95%CI 0.0-2.7%) of the women with unexplained recurrent miscarriage the JAK2V617F mutation was found. The significant association between JAK2V617F mutation and the risk of miscarriage could not be reproduced in our cohort. Conclusions The prevalence of JAK2V617F mutation in a group of women with unexplained recurrent miscarriage is negligible. The significant association between JAK2V617F mutation and the risk of miscarriage[1] could not be reproduced in our cohort. Our results do not support a significant etiologic role for JAK2V617F mutation in women with unexplained recurrent miscarriage. Currently there is no evidence to test for JAK2V617F mutation in the diagnostic work-up in women with recurrent miscarriage.

Reference

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1506

PHARMACOKINETICS AND SAFETY OF ANAGRELIDE HYDROCHLORIDE IN YOUNG AND ELDERLY PATIENTS WITH ESSENTIAL THROMBOCYTHAEMIA

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Background. Anagrelide hydrochloride is a megakaryocyte-selective agent, licensed in Europe for the reduction of elevated platelet counts in at-risk essential thrombocythaemia (ET) patients who are intolerant or resistant to their current therapy. *Aims*. The primary objective of this Phase II open-label, multicentre study was to assess the pharmacokinetic and pharmacodynamic profiles of anagrelide and its active metabolite, BCH24426, in young (18-50 years) and elderly (≥65 years) patients with ET. Assessment of the safety and tolerability of anagrelide were secondary objectives. *Design and Methods*. Patients who were receiving a stable dose of anagrelide (≥5 mg/day) for at least 4 weeks and provided informed consent were eligible for the study. For 3 days prior to pharmacokinetic evaluation, each patient evenly divided their standard total daily dose of anagrelide into a twice daily (BID) regimen. Serial blood samples were obtained for pharmacokinetic and pharmacodynamic analysis over a 12-hour dosing interval. Drug plasma concentrations were normalised to a common 1 mg BID regimen to account for dosing differences between subjects. Patients were routinely monitored for platelet count, blood pressure, heart rate and adverse events (AEs). Results: Twelve patients aged 18-50 years and 12 patients aged ≥65 years were enrolled in the study. Mean (± standard deviation) total daily doses were 2.09 (± 0.74 mg) and 1.42 (± 0.67 mg) in young and elderly patients, respectively. Compared with younger patients, the geometric mean dose-normalised Cmax (maximum observed plasma concentration) and AUCtau (area under the concentration-time curve over one dosing interval) in elderly patients were higher for anagrelide (Cmax: 3.63 vs 2.66 ng/mL; p=0.09, AUCtau: 10.3 vs 6.4 ng-h/mL; p=0.01) and lower for BCH24426 (Cmax: 4.19 vs 7.26 ng/mL; p=0.02, AUCtau: 17.4 vs 27.6 ng-h/mL; p=0.03). No significant difference was observed in geometric mean terminal half life (T1/2) of anagrelide in elderly and young patients (1.4 vs 1.3 hours, respectively; p=0.38). The geometric mean T1/2 of BCH24426 was significantly longer in elderly patients than young patients (3.5 vs 2.7 hours, respectively; p=0.01). There were no substantial changes in platelet count, blood pressure or heart rate between age groups. Six AEs were reported in three younger patients, all of which were mild-to-moderate in intensity. Headache was the most common AE. There were no deaths, serious AEs or AEs that led to withdrawal from the study, and no clinically significant abnormalities in electrocardiogram or cardiovascular function. Conclusions: Anagrelide and BCH24426 have equipotent anti-megakaryocyte activity. Therefore, despite different exposures to anagrelide and BCH24426 in elderly and younger patients, anti-megakaryocyte activity is expected to be similar. Safety findings were as expected and consistent with the pharmacological profile of anagrelide and underlying disease. The results of this study do not suggest a different anagrelide dosing paradigm (starting regimen or dose-titration steps) should be implemented to achieve individual, patient-optimised platelet control in young or elderly patients with ET, thus simplifying the therapeutic approach for the clinician.

1507

THE RISK OF RECURRENT THROMBOSIS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA CARRYING HOMOZYGOUS OR HETEROZYGOUS JAK2 V617F MUTATION

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Background. Some evidence suggests that the JAK2 V617F mutation is associated with an increased risk of first thrombosis in patients with essential thrombocythemia (ET). Whether is a risk factor for recurrent thrombosis too is currently unknown. *Aims*. In order to investigate the impact of the JAK2 V617F mutation on the risk of recurrent thrombosis in patients with ET, we carried out a multicenter retrospective cohort study. *Design and Methods*. We recruited 143 patients with ET, with previous arterial (64.4%) or venous major thrombosis (34.8%) or both (0.8%). The total observation time after thrombosis was 922 pt-years (median 5.5). All patients had to be tested for the presence of the JAK2 V617F mutation in granulocytes by allele-specific polymerase chain reaction; 98 of them (68.5%) carried the mutation. Information about the patient's mutant allele burden was recorded in 47 mutated patients. Heterozygous (n=42) or homozygous (n=5) status was defined as a mutant allele burden < 50% or > 50%, respectively. The relative risk of recurrence was estimated as a hazard ratio (HR) using a Cox proportional hazards regression model. The HR was adjusted using recurrence as the dependent variable and selecting as covariates gender, age at the time of the initial thrombosis (>60 or <60 years), presence of one or more vascular risk factors, history of remote thromboses, type of first thrombosis (arterial or venous), presence of the JAK2 mutation, and type of treatment following thrombosis. Results: Thrombosis recurred in 43 patients (30%); after adjustment for sex, age, presence of vascular risk factors, and treatment after the first thrombosis, the presence of the JAK2 mutation did not predict recurrence (multivariable HR, 0.88, 95%CI 0.46-1.68). This finding was substantially unchanged after stratification of the patients according to the age at the time of the first thrombosis (<60 years or >60 years), the type of first thrombosis (arterial or venous), and administration of cytoreduction after the first thrombosis. Indeed, homozygotes for JAK2 V617F had an increased risk of recurrence in comparison with wild-type patients (HR 6.15, 95%CI 1.51-24.92); in respect to the heterozygous patients, the risk was increased but without reaching the statistical significance (HR 2.94, 95%CI 0.73-11.78). The heterozygous patients had a risk of recurrence quite similar to that of the wild-type patients (HR 0.99, 95%CI 0.45-2.14) Conclusion: The JAK2 V617F mutation is not a risk factor for recurrent thrombosis in patients with ET, unless the patient is homozygous for the gene.

1508

COMBINED USE OF THE PLATELET FUNCTION ANALYZER (PFA)-100 AND PLATELET AGGREGOMETRY IN THE DIFFERENTIATION OF ESSENTIAL THROMBOCYTHEMIA FROM REACTIVE THROMBOCYTOSIS

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Background. The most crucial component of all diagnostic criteria for essential thrombocythemia (ET) has been the exclusion of reactive thrombocytosis (RT). *Aims*. Our goal was to evaluate the diagnostic performance of the PFA-100 collagen-epinephrine (CEPI) cartridge test and epinephrine-induced aggregometry individually, but mainly combined, in the differentiation of ET from RT. *Design and Methods*. Twenty-six patients with ET and 25 with RT were studied. Platelet function was analyzed by the PFA-100 using CEPI and collagen-ADP (CADP) cartridges, and by light transmission aggregometry with epinephrine and ADP. Haematological parameters, plasma von Willebrand factor antigen and activity levels were also assessed. *Results*. The sensitivity (Se), specificity (Sp), positive predictive value (PPV), and the negative predictive value (NPV) of PFA-100 CEPI vs epinephrine-induced aggregometry in the differentiation of ET from RT were estimated as follows: Se (%): 78.9 vs 84.6, Sp (%): 92.0 vs 96.0, PPV (%): 88.2 vs 95.7, NPV (%): 85.2 vs 85.7, respectively. When both of these methods were combined, a lower sensitivity of 68.4%, but a specificity of 100% was attained. The PPV observed with this double abnormal combination was 100% and the NPV 80.6%. Lastly, when we assessed the abnormality for either CEPI CT or epinephrine-induced aggregometry, the sensitivity was 100%, the specificity 88.0%, PPV 86.4% and NPV 100%. *Conclusions*. An abnormal combination was strongly suggestive of ET, while normal results with both methods excluded ET. If our results are replicated by further studies, these two methods could be used very effectively as adjunct markers in the differentiation between ET and RT.

1509

PLATELETS AND WHITE BLOOD CELLS (WBC) DO NOT INCREASE IN JAK2-WILD TYPE (WT) POLYCYTHEMIA VERA (PV) AFTER OBTAINING A NORMAL HEMATOCRIT (HT) WITH PHLEBOTOMIES

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Background. Diagnostic tools in cases presenting with isolated erythrocytosis have been recently improved by the mutations of the janus-kinase 2 (JAK2), that identify more than 90% of patients with PV. This observation induced the recent modification of WHO diagnostic criteria for PV and now the presence of JAK2 mutation is a major diagnostic data for the disease. Nevertheless, PV represents a small proportion of patients with isolated erythrocytosis and other disorders display the same clinical phenotype. Other absolute erythrocytosis, both congenital or acquired, do not carry the JAK2 mutations. In the presence of an acquired absolute erythrocytosis, after the exclusion of secondary and familial causes, if no JAK2 mutation is found, the diagnosis may be of PV or idiopathic erythrocytosis (IE). The former is characterized by an increase of red blood cells without an identified cause and the patients usually undergo phlebotomies to avoid the thrombotic risk due to hyperviscosity.

Table 1.

	Group 1	Group 2	p
N° of patients	24	16	
Sex (M/F)	21 / 3	12 / 4	NS
Mean age at diagnosis (years)	66 ± 10	62 ±16	NS
Median follow-up (years)	5.85	4.41	NS
Platelets x 10^9/L	320 ± 123	281±129	NS
	552 ±178	316 ±154	0.001
WBC x 10^9/L	8,04 ±2,25	8,54 ±2,44	NS
	14,55 ±5,48	8,40 ±2,32	0.001

Material, methods and patients. We retrospectively evaluated 201 patients with PV diagnosed in agreement with the criteria in use at the first diagnosis and treated with phlebotomies to maintain Ht <48% in males and <45% in females. The present study comprehends 40 (19.9%) of these patients who had platelets and WBC within normal limits (respectively 150-450x10^9/L and 4.4-11x10^9/L) at the time of diagnosis. The patients were divided on the basis of platelets and/or WBC increase over the 25° percentile after Ht goal achievement. Group 1 is formed by 12 patients with both platelets and WBC, 10 patients with platelet and 2 with WBC increase. Patients of group 2 did not develop platelets or WBC modification with phlebotomies. We searched JAK2V617F mutation in DNA from granulocytes by allele specific PCR assay and exon 12 JAK2 mutations with sequencing. Our patients were thereafter stratified on the basis of presence of JAK2 mutations. Chi square and Student's t tests were used for statistical analysis. *Results*. 17 patients of group 1

and 4 of group 2 (p=0.01) were found to carry JAK2V617F mutation while no patient had exon 12 mutations. In respectively 18 and 12 patients with JAK2 mutation and in 6 and 2 JAK2-WT, platelet or WBC increased significantly (respectively p=0.001 and p=0.005) at Ht achievement. *Conclusions*. Our results suggest that phlebotomies do not increase platelet and WBC counts in PV patients that do not carry JAK2V617F mutation. Because dubious exist in considering JAK2-WT patients with PV as "true" PV, we can surmise that no increase in platelets and WBC occurs in IE rather than PV. Larger and prospectic studies are needed to confirm our observation.

1510

PREVALENCE OF ESSENTIAL THROMBOCYTHEMIA IN THE JAK2V617F ERA: A POPULATION BASED STUDY APPROACH IN A SPANISH COMMUNITARY HOSPITAL

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Background. Essential thrombocythemia (ET) is an infrequent myeloproliferative disease lacking population based epidemiological data particularly after JAK2V617F mutation was described. Essential thrombocythemia diagnosis is still challenging and shares different features with other myeloproliferative diseases which make difficult population based studies. Prevalence data from Spain has not been published yet despite undergoing surveys. *Aims*. To calculate the prevalence of ET in an Spanish mixed urban-rural area of 189.877 inhabitants (Departamento 3, Agencia Valenciana de Salud, Spain). *Design and Methods*. From January 2007 until December 2008, a Laboratory Information System (Modulab Gold®, Izasa, Barcelona) was used to detect thrombocytosis ($750000x10^9$) in blood counts centralized in our laboratory. Thrombocytosis had to be confirmed after 15 days. Confirmed thrombocytosis patients were electronically notified to be reviewed at Hematology Dept. Thrombocytosis were classified as reactive if patients had a previous history of cancer, chronic inflammatory diseases, iron deficiency or splenectomy. A myeloproliferative disease was suspected in remaining patients and WHO diagnostic criteria were applied to proper diagnosis and classification of patients. In order to confirm ET diagnosis all patients underwent a bone marrow biopsy and PCR for JAK2V617F . ET prevalence was calculated from raw number of confirmed diagnosis in two years and expressed as N per 100,000. Results 540 patients had thrombocytosis. Non-reactive thrombocytosis was detected in 63 patients. 18 patients did not meet criteria for diagnosis of myeloproliferative disease. 4 patients were diagnosed of CML. 1 patient was diagnosed of PMF. 25 patients were diagnosed of PV and 26 patients were diagnosed of ET. JAK2V617F mutation was detected in 25% of myeloproliferative disease cases and 47% of ET cases. For ET we estimate a prevalence of 6,8 cases per 100,000. *Conclusions*. To our knowledge this is the first study to assess the prevalence of ET in a large Spanish population and it´s in agreement with previous data from other industrialized countries. Centralized Laboratory Information Systems are an interesting tool to develop population based epidemiological studies.

1511

MOLECULAR REMISSION ON LOW-DOSE NILOTINIB AFTER IMATINIB INTOLERANCE IN A PATIENT WITH FIP1L1-PDGFRA POSITIVE CHRONIC EOSINOPHILIC LEUKEMIA

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The FIP1L1-PDGFRA fusion gene results from a cytogenetically invisible interstitial deletion on chromosome 4q12. It is the most frequent genetic aberration in chronic eosinophilic leukaemia (CEL) and the vast majority of patients achieve complete molecular remissions on low-dose imatinib (50-100 mg/day). Adverse effects are rare, and therefore the optimal management of patients with unexpected toxicity or intolerance to imatinib are yet unknown. We here report on a 58-years old male patient with FIP1L1-PDGFRA-positive CEL with severe hepatotoxicity after rapid complete haematologic remission on imatinib 100 mg. The patient was switched to low-dose nilotinib and achieved rapid complete haematological and complete molecular remission. Initially, the patient presented with an eosinophil count between 5 to $7x10^9$/L. Allergic disease or parasitic infection were excluded. The bone marrow showed a 30% infiltration of eosinophils. The karyotype was normal, FISH revealed a deletion of CHIC2 in only 0.3% of cells. A FIP1L1-PDGFRA fusion gene was identified by nested RT-PCR. Complete haematological remission was achieved after one week on imatinib 100mg. After four weeks, hepatotoxicity occurred, accompanied by fever, abdominal discomfort and limb pain. When toxicity resolved, imatinib was restarted at 50 mg/day with rapid complete haematological response. Reappearance of hepatotoxicity led to definite discontinuation of imatinib treatment. No data exist on the clinical use of nilotinib or dasatinib in FIP1L1-PDGFRA-positive CEL. *in vitro* data have shown that both components effectively inhibit cell lines transformed by FIP1L1-PDGFRA. Dasatinib inhibits proliferation of EOL-1 cells with an IC_{50} of 0.5 to 1 nM, which is about the same range compared to imatinib. Nilotinib inhibits proliferation of Ba/F3 cells transformed by FIP1L1-PDGFRA with an IC_{50} of 23 nM, the corresponding IC50 for imatinib only being 3 nM. Because of the similar chemical structure, cross intolerance is expected with nilotinib rather than dasatinib and was shown in one out of four patients with CML who were switched from imatinib to nilotinib due to hepatotoxicity. Comparing the two drugs, the higher risk of cross intolerance for nilotinib was rated lower than the possible side effects related to the greater off-target activity of dasatinib. Although IC_{50} of nilotinib might suggest the use of even higher doses than in CML patients, a reduced dose of 200 mg b.i.d. was chosen due to history of hepatotoxicity. After start of nilotinib, eosinophil count normalized within one week. Although no adverse effects occurred, the patient requested a further dose reduction to nilotinib 200 mg/day. After eight months on nilotinib, the patient remains in ongoing complete haematological and complete molecular remission. We conclude that nilotinib is a well tolerated alternative treatment option in imatinib-intolerant FIP1L1-PDGFRA positive CEL and may potentially achieve similar rates of haematological and molecular remissions. This case demonstrates that responses can be achieved at lower doses than expected from *in vitro* data.

1512

DEPENDENCE OF CLINICOHISTOLOGICAL PARAMETERS FROM THE PRESENCE OF JAK2V617F MUTATION IN PATIENTS WITH PRIMARY MYELOFIBROSIS

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Background. Among Philadelphia-negative chronic myeloproloferative disorders (CMPD): essential thrombocythemia, polycythemia vera and primary myelofibrosis - mutation of Jak2V617F tyrosine kinase occurs only in half of patients with primary myelofibrosis (PMF). Phenotypic similarity in the group of patients with PMF does not correlate with the presence of Jak2V617F mutation and require identification of main features for making diagnosis of PMF. *Aims*. To determine the frequency of Jak2V617F mutation in patients with PMF and impact of this mutation on clinical and histological characteristics in such patients. *Design and Methods*. The Jak2V617F mutation was detected by polymerase chain reaction. Morphological assessment of bone marrow biopsy was performed in 37 patients with Ph-negative CMPD (29 patients with PMF, 8 patients with post-PV myelofibrosis). *Results*. Patient's groups were formed according to the presence or absence of Jak2V617F mutation: 25 (68%) patients were Jak2V617F-positive and 12 (32%) patients were Jak2V617F-negative. The median age was 45-48 years in both groups. There were no differences in main clinical and hematological features in both groups. Group of patients with Jak2V617F-positive mutation had higher level of hemoglobin (137g/L versus 119 g/L), red blood cells (5, $1x10^{12}$/L versus 3, $4x10^{12}$/L), lactate dehydrogenase (984 versus 687 E/L). However, presence of Jak2V617F mutation predisposed to more frequent vascular complications, then in Jak2V617F-negative group of patients (48% versus 0%, p<0,001). Comparative analysis of histological parameters of bone marrow in this groups showed, that fibrosis is present in both groups and is more evident in Jak-negative group (67% versus 44%, p< 0, 02). Significant proliferation of the erythroid lineage was observed in Jak2-positive patients (44% versus 8% of patients). There were seen distinction in morphology of megakaryocytes, prevalence of enlarged forms occurred in Jak-positive group (36% versus 8%, p<0,001), and pleomorphic forms of megakaryocytes dominated in Jak-negative group (75% versus 40%, p<0, 01). The majority of Jak2V617F-positive patients with PMF had the same histological features of bone marrow like patients with polycythemia vera. Patients with PMF who had Jak2V617F mutation differed from patients without Jak2V617F

mutation and Jak2V617F-positive patients characterized by prognostic unfavorable bone marrow histology. *Conclusions*. We consider that presence of Jak2V617F mutation can be prognostic unfavorable factor for vascular complications and may have an effect on prognosis and clinical course. Histological differences in these two groups of patients can be explained by presence of other molecular markers or/and by definite stage of the disease.

1513

IRON STATUS PARAMETERS IN POLYCYTHEMIA VERA

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Background. Polycythemia vera (PV) is classified as one of the myeloproliferative neoplasms. Several studies have shown that iron deficiency is common complication in PV patients. However still little is known about iron status in PV. There is no data in the last decade documenting iron metabolism in polycythemia vera despite the progress in laboratory techniques measuring iron status parameters. *Aims*. The objective of this study was to estimate concentrations of prohepcidin and other iron metabolism parameters in PV patients. *Design and Methods*. The study was performed in 56 PV patients (F/M 23/33) aged 38-84 years (65,6±10,5). The control group consisted of 20 healthy persons age and sex matched. The following parameters were determined in blood serum samples: prohepcidin, iron, unsaturated iron binding capacity (UIBC), transferrin saturation and soluble transferrin receptor (sTfR). Results: All patients with polycythemia vera showed significantly lower levels of prohepcidin (93,85 ng/mL [80,64; 108,00] vs 293,67 ng/mL [172,81; 353,25]; p<0,0000001) and higher levels of sTfR (2,14 µg/mL [1,31; 4,19] vs 1,48 µg/mL [1,07; 2,01]; p=0,02) compared to healthy controls. Almost 36% of the PV patients showed concentrations of ferritin below the normal range and significantly lower levels of serum iron (35 µg/dl [24; 90,5] vs 100,5 µg/dl [68,5; 121,5]; p=0,0001), transferrin saturation (8,22% [5,86; 23,25] vs 34,04% [25,74; 47,59]; p=0,000002) and significantly higher levels of sTfR (4,39 µg/mL [2,74; 6,11] vs 1,77 µg/mL [1,04; 2,27]; p=0,00003) and UIBC (353,70±73,91 µg/dl vs 177,36±65,92 µg/dl; p<0,0000001) in comparison with PV patients with normal ferritin values. In this group of patients prohepcidin concentrations (87,54 ng/mL [76,20; 98,05] vs 99,39 ng/mL [85,85; 113,05]; p=0,045) were significantly lower than in other patients. *Conclusions*. The results of our study confirm that many patients with polycythemia vera suffer on iron metabolism disorders. The lower serum levels of prohepcidin in patients with polycythemia vera may be the consequence of iron deficiency. We indicate that the lower levels of prohepcidin in patients with ferritin concentrations below the normal range are the result of organism compensation which increase iron absorption in the intestine.

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1514

A NOVEL JAK2 MUTATION INVOLVING 14TH INTRON SPLICING REGION IN A PATIENT WITH ESSENTIAL THROMBOCYTHEMIA

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Background. The discovery of the V617F point mutation of JAK2 has revolutionized the understanding of the pathogenesis of BCR-ABL-negative myeloproliferative neoplasms (MPNs) Further discoveries of JAK2 exon 12-15 and MPL mutations have extended the scope of the research in this field. However, a very small proportion of patients with polycythemia vera (PV) and 50% of patients with essential thrombocythemia (ET), as well as primary myelofibrosis, are negative for JAK2 or MPL mutations. This indicates that other forms of the mutations may exist. Recently, we analysed a patient with ET by routine methods for exon 14 and 12 detection. Aims To evaluate the optimal protocol in molecular diagnosis in patients with MPNs. *Design and Methods* The genomic DNA of mononuclear cells from heparin-treated bone marrow as well as he gastric tissue from paraffin embedding samples obtained from the patient was extracted using a DNA extraction kit, according to the manufacturer's instruction (Promega, Madison, WI, USA), and stored at -20'. JAK2 mutation with genomic DNA from this patient was studied by RFLP, AS-PCR, RT-PCR and direct sequencing. Results No JAK2 V617F mutations were detected by RFLP, AS-PCR, or RT-PCR analyses. A C/T mutation was detected by sequencing in intron 14 at the position 14 downstream of the exon 14 (see Figure 1) with bone marrow cells. Informatively, this mutation was not found in genomic DNA of the gastric tissue (Figure 2). *Conclusions*. The C-to-T mutation found in this study is localized in intron 14 with no JAK2 V617F mutation or mutations in exon 12 of JAK2. The mutation has not been reported. The change in intron 14 restricted to hematopoietic tissue demonstrates that this nucleotide change is not due to gene polymorphornisms, but might be a mutation that can cause disease. We hypotheses that mutation at the intron splicing region could cause the changes of the exon ligation, which infects the mature mRNA structure and function. Thus the normal inhibition effect of JH2 domain on the JH1 tyrosine kinase domain would be attenuated. Further examination of the effect of this nucleotide change in mutated JAK2 cell lines as well as animal models would be interesting.

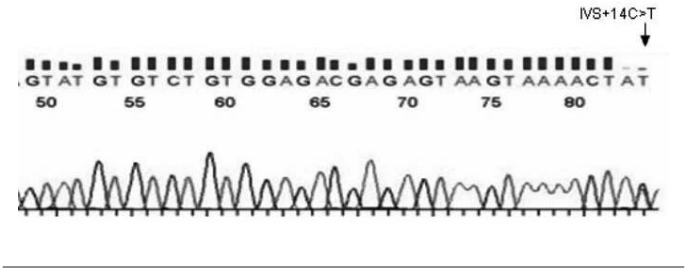

Figure 1.

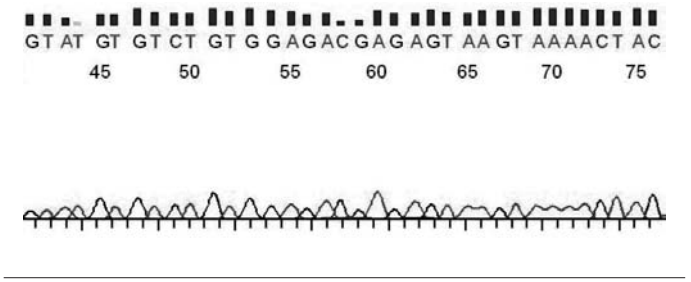

Figure 2.

1515

ERADICATION OF MPL W515A MUTATION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION IN A PATIENT WITH POST ESSENTIAL THROMBOCYTHEMIA MYELOFIBROSIS

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Background. Myelofibrosis (MF) may occur as disease evolution in essential thrombocythemia (post-ET MF). Allogeneic hemopoietic stem cell transplantation (ASCT) is currently the only curative option for these patients, although associated with high rate of transplant-related mortality. Patients with post-ET MF may carry the JAK2 V617F mutation or more rarely mutations within the MPL gene. *Aims*. To evaluate the clinical course and minimal residual disease (MRD) in a patient with MPL-positive post-ET MF who received ASCT. *Design and Methods*. Diagnosis of post-ET MF was according to International Working Group on Myelofibrosis Research and Treatment (IWG-MRT) criteria. MRD was assessed by chimerism analysis using microsatellites evaluation and by MPL mutation analysis using high resolution melting (HRM) and sequencing.*Results*. The patient was a 49 year old male when he received diagnosis of post-ET MF. One year later, when he was referred to our Division, he showed post-ET MF with transfusion-dependent anemia (hemoglobin 7.1 g/dL), leukopenia (white blood cell 3.4x10^9/L) and splenomegaly. Circulating CD34$^+$ cells were 49x10^6/L, and lactate dehydrogenase was 1085 mU/mL. Bone marrow biopsy showed hypercellularity (90%), megakaryocytic hyperplasia with atypia, grade 2 myelofibrosis (EUNMET criteria), and 3% CD34$^+$ blasts. HRM and sequencing analyses on circulating granulocytes showed MPL W515A mutation. After failure of conventional treatments, he received ASCT from

matched-unrelated-donor with fully myeloablative conditioning (total body irradiation, cyclophosphamide and anti-thymocyte globulin). Peripheral CD34+ cells (8.9x10⁶/kg) were infused. Engraftment was on day +16 after transplantation. During the post-transplant period he experienced CMV reactivation, treated with gancyclovir. At day +100, a complete remission was documented according to IWG criteria: resolution of splenomegaly, normal peripheral blood counts and differential, normal bone marrow histology without fibrosis. Circulating CD34+ cells returned to normal (3.1x10⁶/L). Full donor chimerism was achieved. Eradication of MPL W515A mutation was demonstrated with HRM and sequencing analyses, defining a status of molecular remission (Figure). *Conclusions*. This is the first report of a patient with MPL-positive post-ET myelofibrosis who obtained the eradication of the MPL mutation with allogeneic hemopoietic stem cell transplantation.

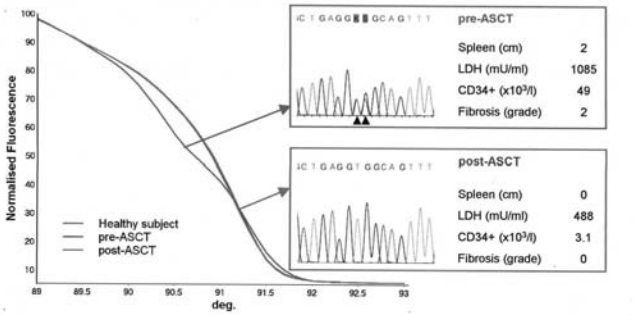

Figure 1.

1516

ANALYSIS OF CLINICAL COMPLICATIONS IN ESSENTIAL THROMBOCYTEMIA: A RETROSPECTIVE STUDY OF 74 PATIENTS

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Background. Essential thrombocytemia (ET) is one of "myeloproliferative neoplasms" (MPN), characterized by an indolent clinical course. ET does not generally shorten life expectancy. Still, medical supervision of individuals with ET is important to prevent or treat complications, as the propensity to develop thrombohemorrhagic complications, myelofibrosis or secondary malignancies. *Design and Methods*. In this study, we retrospectively investigated long-term development of haematological and non-haematological second malignancies and thrombosis in 74 patients (male 26/ female 48) with ET. Our group followed 74 patients with diagnosis of ET from 1987 to 2008, with median follow-up of 72 months (range 1-144 months); median age was 70 years (range 23-90),. RESULTS In our series, we considered risk factors the preesistent presence of arterial hypertension, diabetes, smoking, and hypercholesterolemia. Of the 74 patients observed, we found the one cardiovascular risk factor in 24 pts (32%), two risk factors in 7 pts (9%) and three risk factors in 1 pts (1,3%). At the diagnosis we found 7 cases (9%) of primary malignancies: 3 breast (4%), 1 prostate (1.3%), 1 skin cancer (1.3%), 1 bladder (1.3%) and 1 endometrial (1.3%). After a median follow-up of 36 months from the start of treatment (range 0-144 months), 20 patients (27%) developed a clinical complication. In 11 of 20 patients (55%) a second non-haematological malignancy was documented (2 colon, 1 prostatic, 1 pancreas, 3 endometrial, 2 bladder, 1 breast and 1 large B-cell NHL. Nine patients (45%) developed vascular complications including: ischemic stroke (n=1, 11.1%), acute myocardial infarction (n=2, 22, 2%), deep vein thrombosis of the of the lowr extremities (n=5, 55, 5%). No clinical progression to myelofibrosis or leukaemia occurred. Of the 62 pts (83, 7%) who were treated with chemotherapy, 45 (72, 5%) received only hydroxyurea (HU), 3 pts (0, 48%) only anagrelide (ANA), 2 pts (0, 32%) only interferon (IF), 6 pts (0, 96%) HU followed by ANA, 6 pts (0, 96%) other combination chemotherapy. According to the type of complications, 8/11 pts (72%) with second malignancies were treated with only HU, 3/11 pts (27%) with more than one citotoxic agent; of the 9 patiens with vascular complications 5/9 pts (62%) received only HU, 3/9 more than 1 line of therapy and 1 no treatment. *Conclusions*. Our retrospective study shows that ET is a real chronic disease which affected patients having a long survival exceeding 144 months. In our group all patients who developed second malignancy or cardiovascular complication were in treatment with HU; no patients in clinical observation developed any complication.

1517

SAFETY AND EFFICACY OF PEGYLATED INTERFERON THERAPY FOR ESSENTIAL THROMBOCYTHEMIA DURING PREGNANCY

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Background. Management of essential thrombocythemia during pregnancy remain a difficult challenge for both patients and doctors. Treatment may allow reducing the fetal complications without side effects for the fetus. Few data have been published on the safety of treatment for essential thrombocythemia during pregnancy. Classical Interferon α is considered the drug of choice in this context. *Aims*. We report the case of a woman with essential thrombocythemia who was treated with pegylated interferon during pregnancy. *Design and Methods*. A 25 year old woman was diagnosed with essential thrombocythemia. She was treated with hydroxyurea because of high platelet count. At 35 year-old, the patient wants to become pregnant and so hydroxyurea was stopped and aspirin was continued. Two months after hydroxyurea cessation, a rebound thrombocytosis was observed with 2 000 000 of platelets and biological acquired von Willebrand disease. So it was decided to introduce treatment with pegylated interferon α 2a at the dose of 90 µg/ week. Five months after the cessation of hydroxyurea and three months after beginning of pegylated interferon α 2a, the patient becomes pregnant. The patient has been under joint care of an obstretician experienced in the care of patients with high risk pregnancies. During treatment, the intrauterine development was closely monitored without abnormalities. The pregnancy was managed according to the European recommendations for pregnancy during essential thrombocythemia. Platelet count was maintained with pegylated interferon α below 600 000 during all the pregnancy. She delivered three weeks before term a healthy child. The child is now 1 year old and has a completely normal growth. *Results*. Little is know about the safety and efficacy of pegylated interferon α2a during pregnancy. Classical Interferon α is considered the drug of choice during pregnancy because it is not expected to cross the placenta barrier. However it must be administered generally three times a week and so it may be an inconvenient schedule during pregnancy. Pegylated interferon α2a is formulated by attaching polymers of ethylene glycol of large molecular weight to the native interferon α2a molecule and so a once-a-week schedule is sufficient. The passage of the placenta barrier has not been studied for pegylated interferon α but molecular weight is greater than classical interferon. *Conclusions*. Pegylated interferon α2a seems safe and might be a more convenient treatment option than classical interferon for pregnant women with essential thrombocythemia. More cases are necessary to confirm this data.

1518

LEUKEMIC TRANSFORMATION OF PHILADELPHIA CHROMOSOME-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASE - ANALYSIS OF 10 CASES

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Introduction. Chronic myeloproliferative disease (CMPD) is a clonal proliferation arising from a hematopoietic progenitor of stem cell. Recently, the discovery of mutations on the genes for the Janus kinasa 2 (JAK2) and the trombopoietin (TPO) receptor and MPL gene in Philadelphia chromosome (Ph1)- negative MPD lead to the reassesment of classification of CMPD and provided new tools for diagnosis and treatment. On the other side, leukemic transformation of Ph1 negative CMPD, mainly polycythemia rubra vera (PRV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are unfrequent events with incidence about 2-20%. A little is knowen about mechanisms and factors that cause leukemic transformation of chronic MPD. *Aims*. To analize the course, therapy and survival of 10 patients with leukemic transformation of Ph1 negative CMPD. *Patients and methods*. We studied 10 patients (pts) median age of 60 years (range 50-73 years). Seven pts were diagnosed as PMF, one as ET and two as PRV. Cytogenetic analysis were performed on all pts in the chronic phase of disease and at the time of leukemic transformation. Flow cytometry or immunohystochemical analysis were used to confirm type of accutisation. We coment on therapy, response and survival of patients. *Results*. The median duration of chronic phase of MPD was 92 (range 32- 288 months). All pts developed transformation of MPD to acute leukemia: 8 pts to AML, FAB

M-2; and the last two to AML, FAB M-4. At the time of acutisation, mean values of blood counts were: hemoglobin 100g/l (range 78- 134g/l); WBC 14,5x10^9/l (range 2,9- 35,3x10^9/L) and platelets 199,2x10^9/L (range 20- 478x10^9/l/l). Evolution of cytogenetic abnormalities in kariotype were detected in four pts: 48,XY,+8, +16 in one patient; del (20q) in two pts and del (13q) in one. Normal kariotype was confirmed in last 6 pts. Four pts (two with PMF, one with ET and one with PRV) were treated with chemotherapy according to the standard protocol "3+7' (Doxorubycin and ARA-C). Complete remission was achived in 3 pts; and a partial remission in one patient. All patients with complete or partal remission hade normal kariotype at the time of accutisation. Palliative therapy was applied in 4 pts with PMF. Supportive therapy was applied in 2 pts due to advance age and high ECOG score. At the commencement of the study 6/10 pts were still alive, 3 pts in complete remission, lasting 24, 14 and 35 months, one in partial remission lasting 48 months, and two patients with stabile disease lasting 10 and 32 months. Four remaining pts died 2, 4, 5 and 6 months after acute transformation. *Conclusions*. Blastic transformation of Ph1-negative CMPD requires further studies in both pathogenetic and therapeutic sense. Our modest experience with a small number of patients confirms that complete hematological response is obtainable by conventional chemotherapy. This gives hope that pts within this spectrum could be successfully treated.

1519

THROMBOEMBOLIC AND HEMORRHAGIC COMPLICATIONS AND JAK2 V617F MUTATION POSITIVITY IN PHILADELPHIA NEGATIVE MYELOPROLIFERATIVE DISEASES

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Background. Thromboembolic and hemorrhagic complications are the leading cause for morbidity and mortality in myeloproliferative diseases (MPD) representing the initial presentation for 12-39% of patients subsequently diagnosed with polycyhemia vera (PV) and 11-25% for those given a diagnosis of essential thrombocythemia (ET). In recent years, there was relationships between JAK2 V617F mutation and the diagnosis and complications of MPD. *Aims*. To investigate the prevalance of thromboembolic and hemorrhagic complications and the correlation between these complications and JAK2 V617F mutation in MPD. *Design and Methods*. According to criteria, Philadelphia (Ph) negative MPD was diagnosed to one hundred-seven patients (51 males, mean age 64±14 years). There were ET in 60 (56%), PV in 25 (23%), primary myelofibrosis (PM) 19 (18%), and unclassified MPD in 3 (3%) patients. Thromboembolic and hemorrhagic complications were recorded. Survival duration was followed up. JAK2 V617F mutation was examined using by PCR. Ph chromose was investigated using by PCR and sitogenetic examinations. Results: Thromboembolic complications was detected in 45 (42%) patients with MPD. The most common complications were cerebrovascular events (19%) and myocardial infarction (12%). Hemorrhagic complications were found in 54 (51%) patients with MPD. The most common complications were skin (30%) and gastrointestinal (22%) bleeding. There was no difference for thromboembolic and hemorrhagic complications between subgroups of MPD (p>0.05). While leukemic transformation (1%) was seen in only one patient, no secondary myelofibrosis was detected. JAK2 V617F mutation was examined 68 (64%) of 107 patients. The prevalance of JAK2 V617F mutation was 70% in all patients. The prevalence of this mutation was 61% in ET, 80% in PM, 83% in PV, and 50% in unclassified MPD. However there was no difference for JAK2 V617F mutation positivity between subgroups of MPD (p>0.05). Mean and median survival durations of all the patients was 48±46 months (range 2-262 months) and 36 months, respectively. There was no difference for mean survival duration between subgroups of MPD (p>0.05). 24 (23%) patients died. The most common cause of death were thromboembolic events (42%). *Conclusions*. Thromboembolic and hemorrhagic complications were the most common causes of mortality in MPD. Although the prevalance of JAK2 V617F mutation in PV wass higher than other MPD, this prevalence was not statistically different. Moreover there was no correlation between JAK2 V617F mutation and these complications.

1520

COMPLICATIONS OF MYELOPROLIFERATIVE DISEASES

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Myeloproliferative diseases (MPD) belong to the group of clonic malignant diseases of parent cell hematopoiesis, characterized by abnormal increase of one or several blood lines with normal or nearly normal maturing of those cells, both in bone marrow and in extramedullary hemotopoietic organs.The objectuve of the paper is to confirm which are the most frequent diseases that occur as the complication of MPD. The investigation included 219 subjects of both sexes, between 17 and 83 years of age with the diagnosis of MPD. The patients were divided into five groups: A. Chronic myeloid leukemia (CML)-group; B. Polycythaemia vera (PV)-group; C. Idiopathic myelofibrosis (IMF)- group; D. Essential thrombocythaemia (ET)-group; E. Myeloproliferative disease that cannot be classified (MPS)-group. Among possible complications, we followed hypertension, presence of ulcus disease, coronary disease, thrombotic complications and neurological disorders. The methods of clinical examinations, endoscopies, exosonographies and computer-assisted tomographies have been used. Hypertension was described with the third of the subjects with MPD. It was the mostly expressive with the patients with PV (p<0,01). Presence of thrombotic complications was recorded in almost 20% of all subjects. In the group with ET, there were statistically remarkably more subjects with thrombotic complications than in other groups (p<0,05,p<0,001). Presence of coronary diseases was established with slightly more than 20% of patients. The most subjects were within the group with ET (p<0,001). Presence of ulcus disease was noticed with slightly less than the fifth of subjects. In groups with PV, ET and MPS, between 30 and 40% of subjects had ulcus disease (p<0,001). Neurological disorders were represented in nearly 20% of patients. The highest percentage of neurological disorders was established within the group with ET (52%), which is statistically remarkably more frequent in comparison with the group with PV (p<0,05) and IMF (p<0,01), and especially in comparison with CML (p<0,001). With our patients with PV we noticed significantly less frequency of neurological disorders than we could find in literary data. We proved that examined complications often follow myeloproliferative diseases and they are mostly represented with patients with ET, and then with patients with PV and MPS, but it is remarkably rare manifestation with patients with CML and IMF.

1521

SKIN CHANGES AFTER LONG-TERM TREATMENT WITH HYDROXIUREA

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Background. Hydroxyurea is a potent inhibitor of DNA synthesis and recently has been recognized to enhance carcinogenesis and various skin changes (ulcers, skin cancers, alopecia, lichenoid eruptions, nail pigmentation etc). *Aim*. To present our experience with hydroxyurea-related cutaneous changes. *Design and Methods*. We followed up patients with myeloproliferative disorders who were treated with hydroxiurea in Clinical Hospital Center of Rijeka, Croatia prospectively during five year period. *Results*. During five year 97 persons were treated for myeloproliferative disease with hydroxyurea . Five of them had unusual skin changes which we could not explained by any reason but long term treatment with hydroxiurea (range 6 months to 15 years). Most of them had multiple skin changes (i.e. multiple skin carcinomas, hand ulcers, melanonychia with skin necrosis in the same patient). Clinical findings, dermatologic evaluation , patohystology analysis, cytogenetics (bcr/abl, JAK2, fragile site for 3p14, p53 mutation), PDGF and PCR for retroviruses were evaluated. *Conclusions*. We emphasize that long term treatment with hydroxiurea could be associated with mucocutaneous side effects. Close dermatological follow up should be imperative during and even after many years after discontinuation of hydroxyurea therapy.

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CAN ESSENTIAL THROMBOCYTHEMIA BE CURED BY AUTOLOGOUS TRANSPLANT?

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Background. Essential thrombocythemia is the most begnin myeloproliferative disorder but follow-up can be complicated by thrombotic complications or haematological transformations as myelofibrosis or acute leukaemia. Actual management of essential thrombocythemia diminish thrombotic complications but no treatment can cure the disease. The discovery of Jak2 mutation has led to the development of Jak2 tyrosine kinase inhibitors which may affect positively the disease and benefit many patients. Allografting is reserved for patients with haematological evolution as myelofibrosis. Autologous transplant is not a standard procedure in myeloproliferative disorders, apart for few cases reported in the palliative treatment of myelofibrosis. Aims - We report the case of a woman with essential thrombocythemia who was treated for concomittant plasmocytoma with intensive chemotherapy and autologous transplant. *Design and Methods*. A 60 year-old woman was diagnosed with essential thrombocythemia after the observation of elevated platelet count. She was treated with hydroxyurea and aspirin. Thereafter, a diagnosis of costal plasmocytoma was made on a biopsy and treated chirurgically. Plasmocytomas recidive in costal and vertebrae. A treatment with bortezomib and dexamathasone was introduced with good results. Hydroxyurea treatment was stopped. Consolidation therapy consists of intensive chemotherapy with Melphalan 200 mg/m2 rescued by autologous transplant. This treatment led us to monitor evolution of platelet count and Jak2 mutation allelle burden after autologous transplant. Jak2 mutation was positive before transplant at 45-50%. Three months after autologous transplant, platelet count was normal. Jak2 mutation diminish to 15-30%. Six months after autologous transplant, platelet count was 550 000 and Jak2 mutation was in augmentation at 30-50%. *Results*. No treatment can cure essential thrombocythemia at that time. However, recent reports have showed that pegylated interferon α and at a lesser extent hydroxyurea could modulate Jak2 mutation allelle burden. Moreover, complete molecular remission with indectable Jak2 mutation detection could be obtained in some patients with interferon α or allografting. In contradiction, intensive chemotherapy with autologous transplant can not diminish Jak2 mutation allelle burden for long time in our patient. *Conclusions*. This exceptional observation of coexistence of plasmocytoma and essential thrombocythemia has led us to monitor Jak2 mutation allele burden after intensive chemotherapy and autologous transplant. Studies on cytapheresis cells are ongoing in order to detect Jak2 mutation on CD34 positive cells.

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^{18}F -FLT PET IN MYELOFIBROSIS. REPORT OF TWO CASES AND REVIEW LITERATURE

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Actually few diagnostic procedures are available to determine the quantity of bone marrow cellularity and the numbers of cycling cells in patients with bone marrow disorders. Non invasive imaging of the bone marrow compartment may be helpful. The PET tracer 3'-fluoro-3'-deoxy-L-thymidine (18F-FLT) has been developed in recent times. 18F-FLT uptake is linked to the rate of DNA synthesis and increases with higher proliferation rates in many types of tumour. We have had the opportunity to perform the PET investigation in two patients with idiopathic hypecellulary myelofibrosis before medical treatment. Case report: Two patients, respectively 40 years old male and 68 years old woman were hospitalized for checks on splenomegaly, night sweats, bone pain and alteration of peripheral blood. The physical examination revealed splenomegaly in both without abdominal masses or lymphadenopathy. The remainder of the examination was normal. Laboratory studies showed the following: in woman patient lactic dehydrogenase 1477 IU/L, β2 microglobulin 2719 ng/ml, Ferritin 126.20 ng/ml, WBC 5.5x10^3/ul, HGB 10.8 g/dl, RBC 3.82x10^3/ul; PLT 283. X10^3/ul. The other laboratory results were normal. In male patient : WBC 7.14 x 10^ 3/ul, HGB 13.2 g/dl, PLT 508x10^3/ul, Lactic dehydrogenase 1408 IU/L, β 2 microglobulin 2034 ng/ml, γglobulin 0.76 g/dl. The other laboratory results were normal. A computed tomography scan of the abdomen disclosed splenomegaly in both ; no other abdominal masses or adenopathy were showed. A Positron Emission Tomography scan demonstrate markedly increased uptake throughout the bone marrow, without uptake of the spleen. The bone marrow biopsy made it possible to diagnose in both idiopathic myelofibrosis. Man patient was treated with anti-platelet drugs for thrombocytosis and woman patient with small doses of prednisolone and. vit. D3 with a good recovery of hemoglobin from 10 to 12.5 g/dl. *Discussion*. Idiopathic myelofibrosis is a chronic myeloproliferative disorder with neoplastic proliferation of myeloid stem cells, primarily in the spleen. As the disease progresses the marrow becomes hypocellular and fibrotic. Many patients develop osteosclerosis of the cortical bone. Marked extramedullary hematopoiesis results yielding massive enlargement of the spleen and moderate enlargement of the liver. Background uptake of 18F-FLT in bone marrow is common. ^{18}F-FLT PET might, for that reason, visualize the high cycling activity of hematopoietic cells in the bone marrow compartment. The feasibility of visualization and quantification of the activity of the bone marrow compartment with ^{18}F-FLT PET is so very important to discriminate diverse hematologic disorders. Reported in the literature are few work on the topic; an important increase in ^{18}F-FLT uptake was observed in all of the studied patients with myeloproliferative disorders. The findings on the F-18 FDG PET scan reflect the increased metabolism associated with the extramedullary hematopoiesis in the markedly enlarged liver and spleen, as well as peripheral bone marrow expansion. *Conclusions*. ^{18}F-FLT PET can be used to visualize the proliferative activity of the bone marrow compartment and may be helpful to distinguish separate hematologic disorders.

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JAK2VAL PHE 617 MUTATON IN A PEDIATRIC CASE WITH POLYCTHEMIA VERA

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Background. Polycthemia vera (PV) is a clonal myeloperative disorder charactherized by abnormal increase of precursors in predominatly erythroid, myeloid and megacaryocytes series. It is very rare in childhood and account for only 0.1% of total PV patients. V617F mutation is very rare in children although constitute 90% of mutations in adult patients. Aim:We present clinical properties of youngest PV patients with V617F mutation in our clinic. *Case Report*. Ten year old female patients admitted to our clinic with complaints of pain on the lower extremities lasting a few days. She was referred to hematology clinic for his high level of blood counts. She was pleotoric, and on examination cervical lymphadenopathy 1X1cm and massive splenomegaly (4 cm below costal margine) were detected. Complete blood analysis were as follows Hb:18g/dl, Hematocrit: 55%, WBC: 14x10^9/L, RBC:5,7x10^{12}/L, Plt: 687 x10^9/L, blood smear was normochormic and normocytic and 79% PNL, 4% eosinophilia, 12% lymphocyte reported. Bone marrow aspiration was performed and increased megacaryocytes detected with a M/E rate 3/1. Cytogenetic analysis was 46XX. Ultrasonography confirmed splenomegaly and also showed increased renal echogenicity. Her O2 saturation was 96%. HbA2 was 2.9% and HbF: 0,6%. Erytrocyte mass index was 51,7 ml/kg (36 ml/kg) with Cr51 analysis. Leukocyte alkalen phosphatase level was normal and erytropoetin level was low (2 Mu/ml). Clinical and hematological findings met the criteria for the diagnosis of polycythemia vera. She was diagnosed as PV and low dose aspirin treatment was started. Phlebetomy was done 4 times for controlling hematocrit, red cell volume and progressed asympthomatic till 17 years old. She was readmitted to our clinic with abdominal pain and abdominal swelling. She has broken her routine controls and did not take aspirin treatment for one year. Her complete blood counts were as follows ; Hb: 17 g/dl; Hematocrit: 53%, WBC: 11x10^9/L, RBC: 6x10^{12}/L , and Plt: 634 x10^9/L. Abdominal computerized tomography was consistent with Budd-Chiari disease. Hydoxyurea and anticoagulant treatment were given. Peripheral blood analysis detected JAK2 exon 12 (n1849G>T; Val 617Phe) mutation. She is now asymptomatic and treated with aspirin, hydroxyurea and cumadin for 3 months. Majority of previously reported patients were 10-13 years at time of diagnosis. Our case was diagnosed at the age of 10 years old and she was well being until the age of 17 on which the complication Budd-Chiari occured. *Conclusions*. As pediatric cases with PV in the literature is very rare and diagnostic criteria is not well established as adult patients, JAK2 mutation can give a clue about the diagnosis for pediatric patients.

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APRIL SERUM LEVELS PREDICT TIME TO TREATMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. In chronic lymphocityc leukemia (B-CLL) B lymphocytes gradually accumulate and survive due to apoptosis resistance. Since B-CLL lymphocytes rapidly die *in vitro*, it is postulated that molecular and cellular interactions occurring in tissue microenvironment support their survival *in vivo*. APRIL (a proliferation-inducing ligand), a TNF superfamily member involved in normal B cells survival and differentiation, is known to play an important role in protecting B-CLL cells against spontaneous apoptosis through paracrine and autocrine stimulation of their receptors. In B-CLL APRIL may be released both by microenvironment cells and by malignant lymphocytes themselves. Several studies suggest that APRIL is involved in other B cell derived malignancies, in autoimmune diseases and in cancer. *Aim.* to examine APRIL serum levels in B-CLL patients compared to healthy donors and to evaluate the correlation of soluble APRIL with clinical-biological parameters and survival. *Methods*. we retrospectively evaluated by ELISA serum samples from 83 B-CLL patients referred to our Institution from 1993 to 2008. Sera were collected at diagnosis and before any treatment upon patients consent. Sera from 25 age and sex matched healthy donors were used as control. The following clinical characteristics were considered: gender (51 male; 32 female); age (47 <65 years; 36 ≥65 years); classification according to Rai (73 stage 0-2; 10 stage 3-4) and Binet (56 stage A; 27 stage B-C); lymphocyte count (41<13000/m^3; 42 13000/m^3); development of autoimmune cytopenia (AIC) (34 with AIC, 49 without AIC). We also evaluated: ZAP-70 (24 negative; 50 positive) and CD38 expression (28 negative; 37 positive); IgV(H) mutation status (12 mutated; 26 unmutated); sCD23 (37<60 U/L; 24 ≥60 U/L), Thymidine Kinase (30<9.14 U/L; 31 9.14 U/L) and B2 microglobulin levels (25<2 mg/L; 47 ≥2 mg/L); cytogenetic analysis (23 with normal or favourable karyotype, 9 with unfavourable karyotype). *Results*. We found significantly higher levels of APRIL in the sera of B-CLL patients as compared to normal donors (17.37±3.773 vs 4.186±0,683 ng/ml; Mann-Whitney test, p<0.0001). The mean APRIL serum level was increased in patients with unfavorable karyotype as compared to those with favourable karyotype (17.22±7.629 vs 5.68±0.623 ng/ml, p=0.032). No other significant associations were found between APRIL levels and the other clinical and biological markers evaluated. To better evaluate the clinical relevance of APRIL, we divided patients according to median APRIL serum level (<7.08 ng/mL vs >7.08 ng/ml). Interestingly, patients with APRIL serum level below the median showed a significantly higher time to treatment as compared to patients with APRIL level above this value (34.40 months±5.45 vs 10.8 months±4.279) (p=0.021). On the other hand, the two groups of patients did not significantly differ regarding overall survival and other clinico-biological parameters. *Conclusions*. although based on a limited number of patients, our results suggest that APRIL is increased in B-CLL patients as compared to healthy donors. Furthermore APRIL levels above the median value significantly correlate with a shorter time to treatment.

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ZAP-70 EXPRESSION ASSESSED BY FLOW CYTOMETRY BECOMES A RELIABLE, REPRODUCIBLE AND PREDICTIVE MARKER FOR IGVH MUTATION STATUS IN CLL PATIENTS IF THE RATIO OF B- TO T-CELL FLUORESCENCE INTENSITY ('RATIOMETRIC METHOD') IS TAKEN INTO ACCOUNT

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Background. ZAP-70 expression in B-CLL lymphocytes is a powerful prognostic marker and is strongly correlated to IgVH mutation status (IgVHms) as shown by gene expression profiling yielding concordance in 93%. However, methods assessing ZAP-70 expression by flow cytometry vary and results may differ considerably. Recently, a ratiometric analysis method based on comparison of ZAP-70 expression in B- and T-cells was described to circumvent intra-/interlaboratory variation, but cut-off values are not well established. *Aims*. Assessment of the performance of an in-house ZAP-70 analysis method by flow cytometry (FC) incorporating a ratiometric approach. *Design and Methods*. On previously acquired data, we compared retrospectively our 'standard' (healthy donor as an external control; cut-off >20% for positivity according to Rassenti *et al.*) with a 'ratiometric' (mean fluorescence intensity of B-cells / T-cells x 100 [%]) analysis method. If available, results were compared to IgVHms. ZAP-70 analysis was done by separation of mononuclear cells from EDTA anticoagulated blood by Ficoll density gradient, permeabilization by incubation within 80% ice ethanol for at least 24 hours, 3 color FC assay with ZAP-70-PE (Clone 1E7.2; Caltag Labortories), CD3-PerCP (BD) and CD19-APC (BD) and corresponding isotype controls on patient and control sample, respectively. Results: ZAP-70 measurements in the peripheral blood of 104 pts. with SLL/CLL were available. The Mean + 3 SD of ZAP-70 expression of 84 normal controls was 21% (B-cells) and 19.9% (B-/T-cells) with the standard and ratiometric method, respectively. 37 pt. samples were ZAP-70 pos., 64 ZAP-70 neg. and 3 yielded borderline results. In 75 pts. IgVHms was available: 28 pts. had unmutated IgVH and 47 mutated IgVH. 80% of the ZAP-70 results were concordant to IgVHms (8 false pos., 8 false neg.) with the standard, and 76% (5 false pos., 13 false neg.) with the ratiometric method using the same cut-off (20%). However, ROC curve analysis was better for the ratiometric than for the standard method (AUC 0.84 with a cut-off of 13.2% and 0.77 with a cut-off of 22%, respectively). Combining both methods (cut-off >20% for ZAP-70 positivity using standard or ratiometric method, cut-off ≤20% or ≤13.2% for ZAP-70 negativity using standard and ratiometric method, respectively, ratio = 1st priority) the concordance was 86% (4 false pos., 7 false neg.). 4 pts. had undergone ZAP-70 analysis at different time points with all results being concordant with the combined but not the standard method (2 pts. discordant). 11 samples were remeasured after a median of 161 (78-238) days and showed concordant results (Spearment's coefficient with standard and ratiometric method 0.74 (p=0.02) and 0.94 (p=0.003), respectively). *Conclusions*. The performance of ZAP-70 expression analysis by flow cytometry in CLL patients can be improved by combination of the 'standard method' with the ratio of the B- and T-cell expression ("ratiometric method"). This may be due to methodology and/or by concomitant but variably abnormal ZAP-70 expression in the T-cells of CLL patients with mutated IgVH compared to unmutated IgVH.

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EXPRESSION OF TLRs IN B-CLL: RELATIONSHIP WITH DISEASE ACTIVITY

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Background. Toll-like receptors (TLRs) are co-stimulatory molecules expressed on cell of the immune system and represent major agents of innate immunity and initiators of adaptive immunity. Main function of TLRs is to directly activate the immune system recognizing viral and microbial agents; moreover, TLRs are involved in the self-antigen recognition and could play a role in autoimmune phenomena. It is well established that B-chronic lymphocytic leukemia (B-CLL) is characterized by a variable clinical course, and by an increased incidence of autoimmune phenomena and immunodeficiency, which can greatly influence the disease outcome. Aims. To study the gene expression of TLRs in 25 patients with B-CLL (mean age+/-SD 72+/-12 years, range 42-90, 10 female and 15 male) and to relate it with the clinical course of the disease and the expression of prognostic factors (mutational status of Ig VH region, CD38 and ZAP70 expression, and cytogenetic alterations). *Design and Methods*. The gene expression of TLR4, TLR9, and TLR10 was evaluated in B cell from B-CLL patients and controls. Total RNA was extracted from B-CLL patients and controls (pool of 10 healthy donors), cDNA was synthesized, and real-time PCR was performed. For each real-time PCR reaction 50 ng of cDNA were mixed with 1.25 microl of TaqMan primer/probe set and 10.25 microl of TaqMan Universal Master Mix. Real-time PCR was performed with a model 7300 real-time PCR system (Applied Biosystems). The TLR4, TLR9, and TLR10 expression was normalized according to GAPDH as an internal control gene and it was expressed as percentage of control. *Results.* We found that TLR4 gene expression was lower in B-CLL patients compared to controls (23%±4.5%) while TLR9, and TLR10 gene expression was higher (4467%±773% and 2705% +/- 387% for TLR9 and TLR10 respectively). TLR4 gene expression was significantly lower in "progressive" B-CLL patients (n=15) compared with "indolent" B-CLL ones (n=10): 9.5%±2.7% vs 38.8%±7.9% (p<0.001). The 2 patients with autoimmune diseases (hemolytic anemia and Evan's syndrome) showed no characteristic TLRs gene expression pattern. At present no clear relationship was found between TLRs expression and CD38/ZAP70 levels. *Conclusions*. The reduced expression of TLR4, which is even more pronounced in "progressive" B-CLL patients, is consistent with the reduced ability to a proper immune response to Gram-negative bacterial lipopolysaccarides; the clinical significance (number, severity and outcome of infectious episodes and autoimmune phenomena) as well as the relationship with standard prognostic markers is under investigation in a larger number of patients.

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METHYLATION OF VON HIPPEL-LINDAU GENE IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background. The Von Hippel-Lindau (VHL) is a tumor suppressor gene and its protein product, pVHL, is part of an E3 ubiquitin ligase complex that targets HIFα subunits for destruction in the presense of oxygen. Accordingly, pVHL defective tumor cells overproduce a variety of HIF-induced target genes, which have been implicated in metabolism, mitogenesis and angiogenesis. Aim. To investigate whether the inactivation of the VHL gene, through its promoter methylation, is present in CLL and if there is any correlation with clinical parameters and patient survival. *Design and Methods*. 41 CLL patients (36 male, 5 female) were studied. Genomic DNA was isolated from patient sera in 38 cases and from parrafin-embedded biopsies in 37. All samples were taken at diagnosis. The methylation status of the VHL promoter region was determined using methylation -specific polymerase chain reaction (MSP) with primers for methylated and unmethylated alleles for VHL. All samples previously subjected to bisulphite-modification, according to pre-established protocols. DNA from 10 individuals without malignancies served as negative controls. Human male genomic DNA universally methylated for all genes was used as positive control for methylated alleles. Results. Methylation of the VHL promoter was found in 7 of the 38 serum DNA samples (18%) and in 12 of 37 bone marrow DNA samples (32%). The presence of methylated DNA for VHL either in bone marrow or in serum was correlated significantly with advanced Rai stage (2 p=0.036) and high serum LDH (χ^2 p=0.022). At a median follow up of 61 months, 12/31 patients with serun unmethylated VHL died (mortality 39%) versus 4/7 of those with methylated VHL present in serum (mortality 58%). In contrast, 10/25 patients with bone marrow unmethylated VHL died (mortality 40%) versus 5/12 of those with bone marrow methylated VHL present (mortality 42%). The median survival of patients with serum unmethylated VHL was 80 months (95% CI 59-103) whereas that of patients with serum methylated VHL was 67 months (log-rank p=0.38). *Conclusions*. Methylation of the VHL promoter occurs in a fifth to a third of CLL patients and is associated with adverse clinicopathologic characteristics and inferior outcome. The promoter methylation status of the VHL gene in serum carries potential prognostic significance, probably due to predominant derivation of serum DNA from the neoplastic clone. Validation in larger cohorts is warranted.

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TNFRSF13B GENE EXPRESSION AND MOLECULAR ANALYSIS IN B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS

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Background. Mutations in the TNFRSF13B gene, encoding TACI, a tumor necrosis factor (TNF) superfamily member, have recently been implicated in the pathogenesis of common variable immunodeficiency. Expression of this gene, yet of unknown significance, has been detected in malignant lymphocytes of patients with chronic lymphoproliferative disorders (LPDs). *Aims*. Since certain genotypes of other TNF superfamily members have been implicated as contributing to the enhanced survival potential of malignant lymphocytes, this study was scheduled to investigate in LPDs the mutational status of the TNFRSF13B gene and its expression, in relation to the disease phenotype. Materials and *Design and Methods*: One-hundred and forty-five patients (M/F: 78/67, mean age: 67.9 years, range: 41-87) with LPDs (108 with B-cell chronic lymphocytic leukemia, B-CLL; 29 with low-grade non-Hodgkin lymphomas (NHL); 3 with diffuse large B-cell lymphoma, DLBCL; 5 with hairy cell leukemia, HCL) were enrolled in the study. Diagnoses were made by standard criteria. The expression levels of TACI were estimated by flow cytometry using an anti-CD267 monoclonal antibody (Abcam, clone: 1a1) in 102 LPD patients (81 with B-CLL and 21 with low-grade NHL). The two most common polymorphisms of TNFRSF13B (V220A and P251L) were detected by PCR-RFLP, using DNA extracted from peripheral blood or bone marrow. Thirty-seven individuals (M/F:19/18, mean age: 64.8 years, range: 47-87), free of disease, were also analyzed for TACI expression and TNFRSF13B polymorphisms and were considered as normal controls. Statistical analysis was performed using the SPSS statistical software. Results: Patients with B-CLL displayed significantly lower expression levels of TACI (mean±STDEV: 16.2±21.3%) compared to both patients with low-grade NHL (43.2±27.6%) and normal controls (40.6±20.6%) (p<0.001 and p<0.001, respectively). Moreover, the presence of TNFRSF13B-P251L was associated with a 2.8-fold increased probability for LPD development (allele frequencies 17.2% vs 6.8%, 95%CI: 1.05-7.87, p=0.039), while the allele frequency of TNFRSF13B-V220A did not significantly differ between patients and controls (3.1% vs 5.4%, p=0.338). Finally, no correlation of TACI expression or the presence of TNFRSF13B polymorphisms with autoimmune manifestations, the presence of hypogammaglobulinemia, or monoclonal gammapathy was observed. *Conclusions*. A positive association of TACI expression and TNFRSF13B-P251L polymorphism with LPD phenotype was observed and considering the development of new anti-TACI treatments, our findings might have significant therapeutic consequences.

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WITHDRAWN

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INHERITED POLYMORPHISMS IN THE LIGAND OF CD38, THE CD31/PECAM-1 PROTEIN, ARE ASSOCIATED WITH PREDISPOSITION TO B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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The platelet endothelial adhesion molecule-1 (CD31/PECAM-1) is the ligand of CD38 signaling receptor, an important prognostic marker in B-cell chronic lymphocytic leukemia (B-CLL). B-CLL cells co-express variable levels of CD38 and CD31/PECAM-1, thus interactions within B-CLL clone and bone marrow microenvironment may influence natural history of B-CLL. Interestingly, the CD31/PECAM-1 gene is known to have functional single nucleotide polymorphisms (SNPs) that alter the protein structure,though little is known on the significance of these genetic variants in B-CLL. The objective of this hospital-based case control study was to compare allelic frequencies of three non-synonymous CD31/PECAM1 SNPs in 127 Caucasian B-CLL patients and 207 healthy individuals, and to analyze potential impact of these polymorphisms on clinical outcome of B-CLL. The genotyping of the 373C>G (Leu125Val), 1688G>A (Ser563Asn) and 2008A>G (Arg670Gly) SNPs was performed using PCR-based assays. We found that some CD31/PECAM-1 genotypes were significantly associated with the risk of B-CLL. The logistic regression analysis revealed an association between B-CLL and genotypes: 373 CG [odds ratio (OR) = 2.22; 95% confidence interval (95%CI) = 1.3-3.8, p=0.005], 373 GG (OR = 8.56; 95%CI = 4.3 - 17.0, p<0.0001) and 1688 AA (OR = 4.93, 95%CI = 2.6 - 9.4, p<0.0001). In contrast, none of the studied CD31/PECAM1 SNPs was found to influence survival of the B-CLL patients. In conclusion, our data suggest that inherited variants of CD31/PECAM1 protein may be associated with susceptibility to B-CLL in Caucasians. The mechanism of this association and its potential link with CD38 signaling need to be further elucidated.

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MLPA ASSAY FOR SIMULTANEOUS DETECTION OF GENOMIC REARRANGEMENTS IN B-CELL CHRONIC LYMPHOCITIC LEUKEMIA

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Background. B-Chronic lymphocytic leukemia (B-CLL) is the commonest leukemia of adults populations in Western countries, characterized by highly heterogeneity at the clinical as well as at the molecular and cellular level. Chromosomal changes have been detected in the majority of B-CLL samples by use of interphase fluorescence *in situ* hybridization (I-FISH) and an association has been found between chromosomal abnormalities and clinical prognosis or disease progression. In clinical practice, FISH is performed, at diagnosis, for the most frequent chromosomal abnormalities such as 13q14, 11q22-q23, 17p13 deletions and trisomy 12. However, a new method has been described for the measurement of gene copy number: Multiplex Ligation-dependent Probe Amplification (MLPA). *Aims.* The purpose of this study was to performed MLPA assay in a panel of 33 B-CLL patients in early stage disease (Binet stage A) and to compare the results with FISH data. *Design and Methods*. The MLPA assay was performed for all samples in two independent reactions, one for each probe mix (SALSA Probe-Mix P037 and P038) according to the manufacturer's recommendations (MRC-Holland). This mixes contains 55 target sequences specific for different chromosome regions: 17p13 (8 probes), 13q14 (12 probes), ATM 11q23 (7 probes), 10q23 (2 probes), 2p24 (3 probes), 8q24 (3 probes), 6q25-26 (3 probes), 9p21 (2 probes) chr. 12 (10 probes) and chr. 19 (5 probes). Data were analyzed with Coffalyser Software (MRC-Holland) using DNA from ten healthy donors as controls. FISH study was performed on a panel of highly purified (>90%) peripheral mononuclear CD19+ cells. A set of commercially available probes (Vysis, Downers Grove, IL, USA) was used, as follows: del(11)(q22-23), ATM and centromeric probe for chromosome 11; +12, centromeric probe for chromosome 12; del(13)(q14), D13S325 and subtelomeric probe 13q34; del(17)(p13), p53 and centromeric probe for chromosome 17. A minimum of 100 interphase nuclei were evaluated per probe for each patient. *Results*. MLPA and FISH approaches detected 13q14 deletion in twenty (60%) patients, 4 of which showing biallelic deletion. Same results were obtained for four (12%) cases with trisomy 12. Deletions in TP53 were detected by MLPA in two of the three cases detected by FISH (3/33, 9%). Deletions in ATM were found by MLPA in four of the five B-CLLs detected using the FISH approach (5/33, 15%). In these two cases, detected only by FISH, the low percentage of cells carrying the alterations (< 30%) may hamper MLPA detection. Finally, in one additional case, FISH assay was not able to detect deletion at 11q22-23, instead identified by MLPA since a deletion could be too small for the FISH size probe. *Conclusions*. Our study showed a good correlation between the MLPA and FISH results (91%), as most of the alterations were detected by both techniques. MLPA and FISH showed a comparable sensitivity. However, MLPA unlike FISH has proven to be rapid, cost effective, relatively easy to perform and enabled the simultaneous analysis of many samples with automated data processing, so representing a useful technique for the analysis of genomic alterations in B-CLL.

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EPIGENETIC ALTERATION IN DLK1/MEG3 IMRPINTING GENES IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background. DLK1 and MEG3 are reciprocally imprinted genes on human chromosome 14q32 . DLK1 is a paternally expressed gene and encodes a transmembrane protein that is involved in the Notch signaling pathway. The maternally expressed MEG3 (also known as GTL2) allele encodes non-conding RNA. DLK1 and MEG3 are coexpressed and respond in a reciprocal manner to loss of DNA methylation. Regulation of imprinted expression of DLK1 and MEG3 involves a differentially methylated region (DMR) that encopasses the MEG3 promoter. Imprinting status and epigenetic alterations of DLK1/MEG3 have been studied in Wilms, pituitary and brain tumors and lymphomas. *Aims*. To study the methylation status of DMR of MEG3 promoter in patients with CLL in order to detect epigenetic alterations of DLK1/MEG3 gene and investigate possible prognostic significance of these alterations. *Design and Methods*. 41 CLL patients (36 male, 5 female) were studied. Genomic DNA was isolated from patient sera in 38 cases and from paraffin-embedded biopsies in 37 cases. All samples were taken at diagnosis. DNA methylation pattern was determined by methylation-specific PCR of samples previously subjected to bisulphite-modification, according to standard protocols. The normal pattern consists of 2 bands, one corresponding to the methylated paternal allele, a 160bp PCR product and an unmethylated maternal allele, a 120bp PCR product. DNA from 10 individuals without malignancies served as negative controls. *Results*. Abnormal methylation pattern of the MEG3 DMR was observed in 8 of the 38 serum DNA samples (21%) and was significantly correlated with early Rai stage (0-I), nodular/interstitial bone marrow infiltration, late initiation of therapy (2 $p<0.05$). Abnormal methylation pattern of the MEG3 DMR was observed in 13 of 37 DNA bone marrow samples (35%) and was correlated significantly with late initiation of therapy (χ^2 $p<0.05$). At a median follow up of 61 months, 17/41 died. 14 out of 30 patients who had normal serum DNA methylation pattern died (47% mortality) in contrast to 2/8 patients with abnormal methylation pattern (25% mortality). The group with normal pattern of serum DNA methylation had a median survival of 67 months (95% CI 45-89) whereas the median survival of the group with abnormal pattern was not reached yet (log-rank $p=0.47$). 11 out of 24 patients who had normal bone marrow DNA methylation pattern died (46% mortality) in contrast to 4/13 patients with abnormal methylation pattern (31% mortality). The former group had a median survival of 83 months (95% CI 40-126) whereas the median survival of the latter was 80 months (95% CI 44-116) (log-rank $p=0.36$). *Conclusions*. Serum and bone marrow DNA hypermethylation of the promoter of the tumor suppressor gene MEG3 was seen in 21 and 35% of our CLL patients respectively and was associated with favourable clinicopathologic characteristics and outcome. This contradictory result, if validated, may be due to epigenetic inactivation of DLK1 or to unknown molecular cellular pathways that need further study.

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DIAGNOSTIC VALUE OF CD19 COUNT FOR B-CLL IN PATIENTS WTH ABNORMAL WBC'S IN A PRIMARY HEALTH CARE SETTING

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Background. B-cell chronic lymphocytic leukaemia (B-CLL) is often diagnosed by coincidence in patients with an increased absolute lymphocyte count (ALC). Immunophenotyping is advised for all patients with an increased ALC of unknown aetiology, however, this will result in high costs and unnecessary complete immunophenotypings, since a considerable number of patients with increased ALC has no B-CLL or other malignancy. On the other hand, early haematological consultation at diagnosis is becoming more important, since initiation of treatment of B-CLL is changing from a symptom-based strategy towards a strategy based on biological markers. In this context, an additional CD19 count might be a valuable laboratory test to reduce unnecessary complete immunophenotypings and to facilitate early haematological consultation of B-CLL patients. *Aims*. To investigate the value of a CD19 count in patients with an abnormal WBC to detect and exclude B-CLL in a primary care laboratory. *Design and Methods*. In the period from March 2001 until October 2006, a CD19 count was performed in all patients, living in a previously defined geographic area, aged ≥40 years with an abnormal White Blood Cell count (WBC), defined as ALC ≥6.0x10^9 cells/L, relative lymphocyte count (RLC) ≥60% or atypical lymphocytes (AL) ≥2+. Patients with positive EBV or CMV serology were excluded. CD19 counts were classified as normal (<1.0x10^9/L) or abnormal (≥1.0x10^9/L). In case of an abnormal test, referral to a haematologist was advised. A geographic area in which the contingency areas of the laboratory and the referral hospitals overlapped was based on postal codes in order to link patients in whom a CD19 count was performed to newly diagnosed B-CLL patients in the hospitals. In October 2007, all cases of B-CLL in this area were identified by a stepwise search-strategy with a survey among general practitioners, including two postal questionnaires and a telephone reminder and by consultation of three different hospital-based databases. All data were collected anonymously and CD19 count cases and B-CLL cases were cross-referenced by postal code and date of birth. Results: A CD19 count was performed in 543 patients of 199,108 (0.3%) patients in whom a WBC was performed; 229 (42%) were abnormal and 314 (58%) were normal. In total, 120 patients with a B-CLL were identified; 119 in the abnormal group en 1 in the normal group. This resulted in a sensitivity and Positive Predictive Value (PPV) of 99%(95%CI 97.9-99.7) and 52%(95%CI 47.6-56.2) respectively and specificity and Negative Predictive Value (NPV) of 74%(95%CI 70.1-77.7) and 100%(95%CI 98.7-100) respectively. This resulted in a Number Needed to Screen (NNS) of 1.9. The AUC and PPV for CD19 combined with ALC, 0.92(95% CI 0.90-0.94) and 52% respectively, were significantly higher than the AUC and PPV for ALC; 0.85(95% CI 0.81-0.88) and 34% respectively. In retrospect, 37% of patients with an increased ALC of ≥5.0x10^9/L could be avoided from further complete immunophenotyping due to a normal CD19 count. *Conclusions*. CD19 is a valuable laboratory test in the detection and exclusion of B-CLL. It facilitates the diagnostic follow-up of an abnormal WBC and reduces unnecessary referrals for complete immunophenotyping.

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LENALIDOMIDE TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS HAS DIVERSE EFFECTS ON B-CELLS, BUT CONSISTENTLY CHANGES THE ACTIVATION STATE OF CD8+ T-CELLS, REDUCES REGULATORY T-CELLS AND INDUCES TH17 T-HELPER CELLS

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Background. Lenalidomide shows promising activity in patients with refractory chronic lymphocytic leukemia (CLL). However, lenalidomide has side effects, and the mechanism(s) of action is unclear, and the drug is likely affecting several cell populations and a variety of cellular pathways. Understanding the effects of lenalidomide in CLL in-vivo is important as severe side effects have been reported that are specific to CLL, and markers that would allow identification of patients prone to develop these side effects are required. In addition, understanding how lenalidomide exerts its function would also allow logical combination with other drugs effective in CLL. *Design and Methods*. In order to shed light on the in-vivo mode of action of lenalidomide, we collected samples from patients during the course of treatment with lenalidomide. In order to characterize its cellular and molecular effects in CLL in-vivo, sequential samples from patients undergoing therapy with lenalidomide were analyzed with FACS analysis. We performed an in-depth analysis not only of the activation of CLL cells, but also of changes in composition and activation of T-cell, NK cell and regulatory T-cell populations. *Results*. In contrast to previous findings, a tumor flare reaction was not correlated with an *in vivo* activation of CLL cells. Interestingly, the numbers of immunosuppressive regulatory T-cells consistently decreased upon treatment. This is in keeping with an immunostimulatory effect of lenalidomide, and consequently numbers of NK cells, $CD4^+$- and $CD8^+$ T-cells were increased in a subset of patients. Intriguingly, the population of pro-inflammatory $CD3^+CD4^+IL17^+$ Th17-cells expanded at the same time as CLL cell counts decreased. *Conclusions*. These findings support an immunostimulatory and pro-inflammatory mode of action of lenalidomide in CLL *in vivo* via suppression of regulatory T-cells and an induction of Th17-cells.

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IMPAIRED EXPRESSION OF CD3 γ, Δ, ε AND ζ GENES IN PERIPHERAL BLOOD T CELLS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. In leukemia patients, T-cell function has been suppressed with disease progress. This immune dysfunction may be due to the disorder of recent thymic output function, the abnormal expression of T cell receptor repertoire and, may in part, due to abnormal signal transduction, possibly through altered expression of CD3 genes. Recently, quantitative CD3 ζ chain associated protein kinase 70 kDa (Zap-70) is considered as a marker of IGHV mutational status and a powerful prognostic factor in B-cell chronic lymphocytic leukemia (B-CLL). *Aims*. The expression level of TCR signaling transduction factor CD3γ, δ, ε and ζ genes in T cells from patients with B-CLL were investigated. *Design and Methods*: Real-time RT-PCR with SYBR Green technique was used for detecting the expression level of CD3 γ, δ, ε and ζ genes in peripheral blood mononuclear cells (PBMCs) from 17 patients with B-CLL, and 17 healthy individuals served as control. The β2-microglobulin gene (β2M) was used as an endogenous reference. Relative mRNA expression level of genes was analyzed by using the 2Δ -Ct-100% method. *Results*. All of four CD3 genes could be detected in samples from healthy individuals, however, CD3 γ, ε and ζ genes were not detected in 2, 3 and 1 samples from B-CLL respectively. The expression level of CD3 γ, ε and ζ genes were significant decreased compared with healthy controls (p=0.002, p<0.001 and p=0.014, respectively). Although the expression level of CD3 delta gene was 1.18 times higher in B-CLL patients than that from controls, there was not significant different (p=0.054). The expression pattern of these four CD3 genes was compared, there was not significant different in the expression level in B-CLL patients, however, the expression level of CD3 delta in healthy controls was significant different compared with the other CD3 genes (p<0.0001). Additional, the expression level of all four CD3 genes was not significant different associated with age in B-CLL patients as well as in healthy controls. *Conclusions*. Decreased expression of CD3 genes might associate with the immune dysfunction in the patients, which was thought to be impaired

in activation and results in an increased susceptibility to apoptosis of T cells. The study might contribute to better understand the cellular immune features in B-CLL patients.

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APOPTOSIS INDUCED BY VERBENA OFFICINALIS OIL AND CITRAL IN PERIPHERAL BLOOD LYMPHOCYTES OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Isoprenoids, a broad class of mevalonate-derived phytochemicals which are ubiquitous in the plant Kingdom, may suppress the proliferation of tumour cells as breast adenocarcinoma (MCF7), human leukemia (HL-60), and human colon adenocarcinoma (CaCo2). *Verbena officinalis L.* (verbenaceae), commonly known as vervain, is a medicinal plant which grows wild everywhere. Despite its pharmacological mechanisms of action is still unclear, V. Officinalis essential oil has several traditional medicinal uses and is almost completely constituted of isoprenoids compounds. *Aims*. To verify the CLL cells ability of udergoing apoptosis in the presence of the isoprenoid compounds deriving from Verbena Officinalis (Vervain) and their main component Citral. *Design and Methods*. We obtained the essential oil *V. Officinalis* from plants grown in the Garden of Medicinal Plants in the Campus of the State University of Salerno, Italy. The chemical composition of the volatile oil was achieved by GC and GC-MS methods. The essential oil and citral, its main constituent, were tested on 13 untreated patients (8 M, 5 F, median age 68 yrs) with B-cell chronic lymphocytic leukemia (CLL) and from 10 healthy donors (5 M, 5 F, median age 55 yrs). Mononuclear cells were isolated from peripheral blood samples by centrifugation of a Ficoll/Hypaque gradient and cultured at a cell concentration of $2,5x10^6$/mL in RPMI 1640 culture medium in a humidified incubator 5% CO2 at 37°C. Cells were incubated for up to 24 hours with vervain essential oil or citral at different concentrations. The proapoptotic effect of the compound was evaluated after three different times of incubation (4, 8 and 12 hours in dark conditions) by adding to cells annexin-V 5 microL, and propidium iodide 5 microL (Annexin V-FITC Apoptosis Detection kit 1, BD Pharmingen). FACSCanto flow cytometer (Becton Dickinson) was used to acquire and analyze samples. *Results*. Both vervain essential oil and pure citral induced a significant apoptosis in CLL samples with respect to controls ($p<0.0001$). Necrosis was also found to increase with time, reaching the maximum after 24 hours. The presence of del 17p13, was associated to a reduced ability to undergo apoptosis compared with other rearrangements or nomal karyotype. *Conclusions*. Our data agree with the literature in indicating natural compounds as possible precursors of new therapeutic agents.

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PROGNOSTIC VALUE OF DYNAMICS OF EXPRESSION OF COSTIMULATORY MOLECULES CD 80+, CD86+ AND THE LEVEL OF INTERLEUKIN-2 IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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51 patients with B-CLL were studied with median age of 56.7±3.1. Among them 19 patients were on stage II-III according to Rai et al. and 32 of them - in remission phase. Treatment was given according to FC scheme. The expression of costimulatory molecules were measured with FACScan flow cytometer (BD) and the level of Interleukin-2 - with immuno-enzyme method. In patients with clinical expression phase of B-CLL we observed the decline of following molecules' amount: CD19+CD80+ tumor lymphocytes - 4.1 times, CD19+CD86+ - 3.2 times ($p<0.05$), CD4+ lymphocytes - 6.0 times ($p<0.01$), CD8+ - 4.7 times ($p<0.01$), CD3+ lymphocytes - 9.2 times ($p<0.001$), CD3+CD28+ - 7.8 times ($p<0.001$). During the remission phase we observed increased expression of CD19+ and CD3+ by lymphocytes of B7 family costimulatory molecules (CD80, CD86) and CD 28 ligand respectively. Interaction of these molecules leads to CD4+ synthesis by interleukin-2, which activates killer functions of CD8+ antigen-specific lymphocytes. Data analysis of expression of these molecular structures and their comparison to average value in the groups showed, that in patients with remission phase of B-CLL the amount of CD19+CD80+ were decreased by 32.4%, CD19+CD86+ B lymphocytes - by 2.2% ($p<0.05$). CD19+CD86+ lymphocytes were decreased by 1.6 times in 18.9% of patients ($p<0.05$). CD3+CD28+ were decreased by 1.8 times in 8.1% of patients ($p<0.05$). The dynamics of immunologic data showed that revealed changes in patients with B-CLL in remission phase were early predictors of disease recurrence, which clinically manifest 2-4 months later.

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IGVH MUTATIONAL STATUS AND GENE SEGMENTS USAGE IN A SPANISH GROUP OF 224 PATIENTS DIAGNOSED WITH B-CLL

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Background. The mutational status of IgVH gene identifies patients with different outcome in B-CLL. Thus, IgVH unmutated patients have poor prognosis. However, the information related with different IgVH gene segments usage and the patients characteristics and prognosis is controversial and the series are limited. *Aims*. i) To assess the frequency of the IgVH gene segments usage and the mutational status in a Spanish large cohort of patients with B-CLL. ii) To correlate the results with CD38 expression and cytogenetics. iii) To analyze the survival and the time to first therapy of the patients according to the IgVH gene segments usage and mutational status. *Patients and Methods*.Mutational status was performed in 224 patients diagnosed with B-CLL (152 male; ratio: 2.1). Median age was 63 years (range: 29-90). Most of cases (77%) were in Binet A stage while 46% showed CD38 expression. A total of 150 patients (67%) displayed cytogenetic aberrations analysed by FISH: 13q deletion was present in 47.5%, +12 in 14.3%, 11q- in 9.4%, IGH rearrangement in 5.8%, and 17p- in 4.5%. IgVH gene usage and mutational status were investigated by PCR and sequencing (Ghia P, et al. Leukemia 2007; 21:1-3). Unmutated cases were considered when a VH homology ≥98% was present. *Results*. A total of 125 patients (55.8%) presented mutated IgVH. The most frequent VH gene segments used were VH3 (49%), VH1 (24%), and VH4 (18%), while VH5 and VH7 were used in only 4% and <1%, respectively. We did not observe expression of VH2 or VH6 gene segments. VH1 family showed a predominance of non-mutated cases (68.5%), being VH1-69 the most frequent usage and strongly associated to non-mutated IgVH (86%, $p<0.0001$) with male preference. Most of patients of VH3 family were mutated cases ($p = 0.004$), mainly VH3-23 and VH3-74. By contrast, all VH3-11 ($n = 5$) were non-mutated cases ($p = 0.0016$). VH3-30 ($n = 22$) and VH3-23 ($n = 19$) were the VH-3 segments more used. It should be noted that VH3-21 only supposed 3.1% of patients in our study with 6/7 mutated cases. VH4 family was more frequent in females with more mutated cases ($p = 0.022$). All but one of cases with VH5 usage affected VH5-51. Interestingly, all VH5 patients were non-mutated ($p = 0.023$) and all of them were male. Mutated patients showed correlation with CD38- ($p<0.001$) and 13q- ($p=0.011$), while non-mutated cases correlated with 11q- ($p<0.0001$), 17p- ($p<0.0001$) and +12 ($p=0.004$). Three out of eight cases of VH5 segment showed 11q deletion ($p=0.025$), while all five cases of VH4-04 had 13q deletion ($p=0.023$). The median overall survival was longer in mutated patients (215 months vs 117 months, $p=0.027$). However, no differences in the overall survival were observed when the analysis was performed according to the IgVH usages. Furthermore, the median time to first therapy was also longer in mutated patients (227 months vs 29 months, $p<0.001$). Of note only 29 out of 107 VH3 patients required therapy ($p=0.016$) while six out of seven VH5 patients were treated during follow-up ($p=0.01$). A trend of VH3-21 patients to require early therapy was also observed ($p=0.07$). *Conclusions*. In patients diagnosed with B-CLL, the identification of IgVH gene segments could provide additional information related to the outcome. VH3-21 usage had a low incidence and VH5-51 identified a group of patients with a bad prognosis. Overall survival and therapy free interval were significative related with IgVH mutational status.

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CYTOCHROME C LEVELS MEASURED BY ELISA IN SERUM OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS AND IN LYSATE OF THEIR LYMPHOCYTES

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Background. It has been reported that the levels of cytochrome c in serum can indicate the presence of *in vivo* apoptosis and can serve as a prognostic factor during cancer chemotherapy. This finding can help to determine the efficiency of chemotherapy in it beginnings. It has also been shown that cytochrome c levels in different hematological malignancies can correlate with aggressive disease or may be a prognostic marker. *Aims*. To determinate the levels of cytochrome c and bcl-2 in lymphoma patient's serum using ELISA method before and after initiating chemotherapy. To examine the levels of cytochrome c and bcl-2 in the serum and in lymphocyte's lysates taken from chronic lymphocytic leukemia (CLL) patients. To try to find a correlation between the level of those apoptotic factors and clinical and laboratory parameters and known prognostic factors. *Design and Methods*. This study included 7 non-Hodgkin's lymphoma (NHL) patients that received RCHOP chemotherapy and 51 untreated CLL patients (65% stage A, 21% stage B, 13% stage C, median follow-up time 79 months, mean age 84 years, 69% males). Informed consent was obtained from all patients. In NHL patients, blood was taken before and after therapy at different timepoints. We examined serums levels of cytochrome c and bcl-2 using ELISA method with kit from ''Bender Med systems'' company, Burlingame, CA, USA. In CLL patients we checked cytochrome c and bcl-2 levels in serum and in lymphocyte's lysates by the same method. In CLL patients these levels were compared with known prognostic factors. Results: In NHL patients we could not detect levels of cytochrome c, and bcl-2 by this method in the serum before or after treatment. In CLL patients we could not detect levels of cytochrome c and bcl-2 in serum, nor bcl-2 levels in lysates of CLL cells. The results of cytochrome c in lymphocyte's lysates were positive. Figure 1 shows the distribution of results. We have found a negative correlation between cytochrome-c levels in lymphocyte lysates and serum hemoglobin level (p=0.035) and a positive correlation between cytochrome c levels in lymphocyte lysates and WBC (p=0.014) and lymphocytes count (p=0.005). We did not find a correlation between cytochrome c levels in lymphocyte's and other clinical and laboratory parameters of the CLL patients.

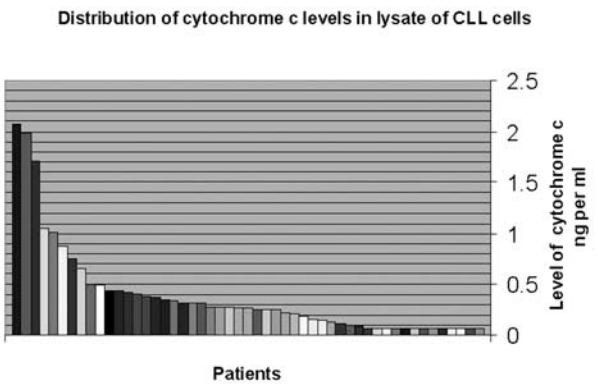

Figure.

Conclusions. Using this ELISA kit cytochrome c and bcl-2 could not be detected in serum of all patients including NHL and CLL. Therefore it was impossible to compare the levels before and after treatment and the study continued on lymphocyte's lysats from CLL patients. We find a negative correlation between cytochrome c levels in lymphocyte's lysates and hemoglobin levels and a positive correlation with WBC and lymphocytes count. We did not find a correlation between cytochrome c levels in lymphocyte's lysates and parameters that are known as prognostic factors in CLL patients, such as stage, β2 microglobulin and LDH.

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THE VALUE OF ULTRASTRUCTURAL CHARACTERIZATION IN DIFERENTIAL DIAGNOSIS OF HAIRY CELL LEUKEMIA (HCL), HAIRY CELL LEUKEMIA VARIANT (HCL-V), SPLENIC MARGINAL ZONE LYMPHOMA (SMZL) AND PROLINFOCYTIC LEUKEMIA (PL)

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Background. In the chronic lymphoproliferative disorders, although the information derived from immunophenotyping, is essential to define the type of the lymphoid population and it´s helpful to distinguish between the various conditions: the clinical, morphologic, cytochemical and histopathological features of these diseases are also important in order to establish a more precise diagnostic. Despite the HCL is a B proliferation, a peroxydase activity was found in the hairy cells. *Aims*. Analyze the more typical morphological and cytochemical ultrastructural characteristics of HCL, HCL-V, SMZL and PL to establish the ultrastructural criteria useful for diagnosis of uncertain cases. *Design and Methods.* Blood cells were obtained from patients with typical or suspicious HCL, 33 cases, 6 HCL-V, 31 SMZL, and 5 B-PL. The Roels and Breton-Gorius methods were applied for the peroxydase reaction. *Results*. With the Roels method the 75-94% of hairy cells showed peroxydase activity in the perinuclear envelope, ER cisternae and the ER surrounding the ribosome-lamellar complex (RLC). With the Breton-Gorius method, the hairy cells were negative or only slightly positive. In the HC variant cells, the peroxydase reaction was positive in all cases, in more than 71% of cells. Highlight that the intensity of the reaction was always slightly. In the cells of SMZL and PL only the mitochondria were reactive by the cytochrome. Morphological features: The most typical morphological features of HCL were: a) Markedly variable shape of nucleus and large cytoplasm with multiples organelles and the numerous, short and long projections, frequents "lake like" and pseudopods that showed the HC membranes in all the cases b) The RLCs were observed in 30/33 of the cases. They were found in 6-75% of hairy cell and some cellular fragments. RLCs are cylindrical cytoplasm structures constituted by spirals with rows of ribosomes spaced among the lamellae. HCL-V, an infrequent entity, could be identified not only for the peroxydase reaction present in all six cases but also for her typical central nucleus (2/3 cases), round or oval nucleus shape (85%) with medium-sized nucleoli and their short and long cytoplasm projections. Moreover 3/6 patients cells show a variable frequency of RLCs including a case with 60% RCLs. In the SMZL, 62% of cells were small size, normal or low nucleus/cytoplasm ratio, central or excentric round or oval nucleus, single nuclear pockets (43,5%), small nucleoli (29%), large cytoplasm with scarce organelles, RER circular (11/31), absent RLCs and short projections or wide base around of all plasmatic membrane or pseudopods (12/31). For the PL, the lymphoid cells showed medium size, high N/C ratio, round nucleus with scantily condensed chromatin, median-large sized, round nucleoli (63-83% cells) and small cytoplasm with scanty organelles, without RLC or projections. *Conclusions*. The peroxidase unfixed cells and morphological ultrastructural study have been useful to confirm uncertain HCL or HCL-V cases and to demonstrate that RLC is not exclusive of classic Hairy cell leukemia. For SMZL, a combination of ultrastructural methods and immunophenotyping combination allow to exclude HCL and to confirm the SMZL diagnosis. For PL, the ultrastructural study was characteristic.

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FAMILIAL AGGREGATION OF MALIGNANT HEMOPATHY AND AUTOIMMUNE DISEASES

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We selected from the last 20 years case record a number of 34 subjects with malignant hemopathy and autoimmune diseases who were constituted in 15 familial groups. In four cases the 1st generation was represented by father and the 2nd generation by daughters. The diagnosed pathology was: 1. father: IgA λ Multiple Myeloma (MM) stage III, acute renal failure secondary to the hypercalcemia syndrome, and daughter: RAEB t complicated with Sweet syndrome; 2. father: DLBC Lymphoma, CD 20 + stage III B, and daughter: Follicular lymphoma grades 2 - CD20+; 3. father, son and daughter Chronic Lymphocytic Leukemia

(CLL); 4. father and daughter: Mixed cellularity Hodgkin's disease (MC - HD). One group where the 1st generation was represented by the mother with MC-HD, the 2nd generation represented by the daughter with micromolecular λ Multiple Myeloma and the 3rd generation by the niece with immunodeficiency IgA. One group where the 1st generation was represented by mother with diagnosis Polycythemia Vera and the 2nd generation was a son with diagnosis non-Hodgkin lymphoma. One familial group where the wife had Nodular Sclerosis Hodgkin's Disease (NS - HD) diagnosed in 2002 and husband was diagnosed in 2008 with MC - HD. One group where the 1st generation, the father, had IgA lambda MM, severe hyperviscosity syndrome, and the 2nd generation, daughter had Takayasu disease and the 3rd generation, grand daughter had SLE. The other 14 cases are grouped in 7 familial clusters with SLE, Systemic sclerosis, Cryoglobulinemia with vasculitis, Polymyositis, Juvenile rheumatoid arthritis. The group with hemopathy of the first generation the average diagnosis age was 69 (62-79) and the average age of the second generation was 41 (29-53) The analysis of this sample showed from a clinical point of view: a predominance of the father daughter sequence; an increase of suffering in the 2nd generation, meaning a maximal severity of the condition in the second generation and early onset, approximately three decades before the onset in parents. we also point out other observations: the result of the HLA evaluation class I and class II at brothers diagnosed with Chronic Lymphatic Leukemia, descendents of parents with CLL: DE sister, born in 1948, has blood group A, Rh+ and HLA class I: A2, 29; B44, 62; CW3 ;BW4; BW6 HLA class I: DR7, 16; DR51; DR53; DQ1,2, MG brother, born 1950, has blood group A, Rh+ and HLA class I: A2,2 ; B44,62; CW3; BW; BW6. HLA class II: DR7,16; DR51; DR53; DQ1,2. We highlight the identity HLA class I and II. Flow Cytometric Immunophenotyping shows: at the sister (DE): 80% of Lymphocyte population: [CD19 +, low; CD 20 neg.; CD 45 +; CD 23 +, low] at the brother (MG): 80% of Lymphocyte population: [CD 20+; CD 23+; CD5 +low, CD45+ high] We also observed: HLA B5 common in the group HD father-daughter; HLA A1, B8 common in the SLE familial group; HLA B8 common in the IgA MM - Takayatsy disease group; HLA DR3 common in the SLE/ Polymyositis family group. In the husband-wife group with HD, we only observed serology IgG + for EBV (negative for EBV- IgM, CMV, HTLV - I, VHB, VHC, Toxoplasmosis, HIV) *Conclusions*. an increase of suffering in the second generation meaning an earlier onset by approximately 25 years, predominant father-daughter transmission , genetic susceptibility suggested by the identity HLA class I and II in brothers with CLL without identical phenotype of lymphocyte population, we suggest as useful the deepening of the analysis of these type of subjects in a medical center with better technological endowment.

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GENISTEIN-INDUCED APOPTOSIS ON MEC1 CELL LINE

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Apoptosis is a physiological cell suicide program, critical for the development and maintenance of healthy tissues, therefore aberration of this process can de detrimental. B-cell chronic lymphocytic leukemia (B-CLL) is a neoplasic disorder characterized by defective apoptosis. In many cases the classical therapy failed and we must find new therapeutical strategies. One of this strategies being the use of biological active agents as genistein. Genistein is a nontoxic isoflavone with chemopreventive activity present in soy. During the past years soy consumption has been associated with lower cancer incidence. The aim of this study was to investigate the apoptosis-induced (at mitochondrial and caspase level) by genistein on MEC1 (cell line derived from B-chronic lymphocytic leukaemia). The genistein activity was putted in evidence by flowcytometry and western blot technique and our results indicate that MEC1 leukemia cell line is sensitive to the apoptotic effect of genistein in a dose-and time-dependent manner as well as a caspase dependent way. Genistein may prove to be a safe and effective anti-cancer therapeutic agent, either as a single agent and/or in combination with other therapies, therefore antileukemic activity of genistein merits further investigation.

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NEW DIAGNOSTIC MARKERS IN CHRONIC LYMPHOID LYMPHOCYTIC LEUKEMIA

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Introduction. Chronic lymphocytic leukaemia (CLL) is a B-cell chronic lymphocytic leukemic condition occurring in a premature stage of maturation, when immunoglobuline types IgM and/or IgD on surface are scarcely expressed. Several epidemiological factors may contribute to its occurrence, including familiar predisposition, level of industrialization (higher in Western Countries), age (50-70 years), and gender (M/W 2: 1). For the study (of CLL) , clinical and laboratory parameters are used: LDH, b2 microglobulin, clinical stage, bone marrow infiltration type, lymphocytic doubling time as well as others biochemical parameters such as IgVH gene somatic mutations, chromosomal alterations (i.e. deletions on chromosomes 13, 11, and trisomy 12), and CD38 expression. Molecular markers include ZAP-70. CLLU1, the first gene-specific marker in Chronic lymphocytic leukaemia with elevating pattern of expression, which represents a predictive index of CLL risk. Additionally, MUM1 is a recent factor whose expression levels that prognosticates the form of CLLU1 with a favorable clinical course and long survival. Aims of the study: i) to characterize the molecular alterations, mutational states and gene rearrangements, associated with this disease; ii) to find biological markers of specific stages of the disease. These prognostics parameters should allow a more correct therapy and a better estimation of the prognosis for patients in the high risk category (Binet stage B-C). *Materials and methods*. 23 cases with a diagnosis of CLL were analyzed. The study was performed between 2006 and 2008 at the "A. Businco" Cancer Hospital in Cagliari, Sardinia, (Italy). Case age, 42-88 years (W/M 39% /61%, ratio M/F 2:1). DNA and total RNA from mononucleated cells obtained from bone marrow and peripheral blood were extracted. serum surveying, measurement of prognostic indicators by real time Polimerase Chain reaction, immunohistochemical and immunocytometrical analysis, and study of trascripts by sequence analisys were performed. *Results*. 30% of the patients showed an aggressive clinical course, while 70% of them had a good clinical course. The CD79b immunologic level resulted immeasurable or very weak in all cases (91%), except in two (9%) where it was high. CD38 was positive in 43% of the cases, while it remained negative in 57%. Mutational status of immunoglobuline IgVH and CD38 correlation: 5% were ZAP-70 +/ IgVH unmutated, 69% were ZAP-70 -/ IgVH mutated, with absence of correlation in 26% of cases. Histopathological analysis was performed using standard markers (CD5, CD79a, CD20, CD23) in order to differentiate between classic CLL and its variants. The mutation status of IgVH was performed by RT-PCR using 3 sets of primers specific for VHL, VHFs, and VHD families. IgVH extension molecular analysis show: 89% mutated, and 11% not mutated. For the IgVH carrying somatic mutations, we studied also the specific interested families: 19% VH1, 52% VH3, 29% VH4. VH3-21 family was absent (unique index negative prognosticates in IgVH with somatic mutation). ZAP-70 and CLLU1 has been performed through RT-PCR by ABI-PRISM 7000 and relative quantification expressed as (2Δ-CT). From real time evaluation : Positive ZAP-70: 26%, Negatives ZAP-70: 74%: CLLU1 and IgVH not any correlation Expression of MUM1 was high in all ZAP-70 negative cases . *Conclusions*. In the IgVH analysis we found discordant data with respect to the European guidelines, in particular the absence of VH3-21 expression family. The ZAP-70 values performed by molecular biology techniques remain the more important index of the IgVH mutational status. The high expression of MUM1 may be indicative of patients with favorable clinical course and longer survival expectancy. CLLU1, CD38, and ZAP-70 correlation have characterized groups of patients with a better or a worse prognosis. Because of the limited number of cases (23 patients diagnosed) analyzed, our results should in future be extended to investigate the prognostic factors, disease clinical stage study on mutational state of the IgVH, expression of the polymorphism of IL1B and IL6 genes, doubling time of the lymphocyte study CD38 and ZAP-70 quantitation expression.

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HIGH PREVALENCE OF CLONAL B-CELL DISEASE IN PATIENTS WITH LGL-LEUKEMIA

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Background. When we recently reported on 52 patients with LGL-leukaemia, we serendipitously found a high prevalence of monoclonal gammopathy. This was in line with a recent report by Viny et al showing high prevalence B-cell dyscrasias in LGL-leukaemia. *Aims*. This prompted us to systematically address the issue of concomitant clonal B-cell disease including monoclonal gammopathy in an extended group of patients with LGL-leukaemia diagnosed at our hospital. *Design and Methods*. Between October 2001 and December 2008, 65 patients were diagnosed with LGL-leukaemia at Rikshospitalet, Oslo University Hospital. The median age was 59 years (range 26-86), and the cohort consisted of 30 men and 35 women. The medical records were retrospectively scrutinized to identify patients with monoclonal gammopathy and/or lymphoproliferative disease. *Results*. Results of protein electrophoresis of serum and/or urine were available in 59 patients. Monoclonal gammopathy was detected in 12 patients (20% of those assessed by electrophoresis). In six patients, the monoclonal gammopathy was diagnosed as monoclonal gammopathy of undetermined significance (MGUS). In the other six patients with monoclonal gammopathy, a lymphoproliferative disease was disclosed in the bone marrow by immunhistochemistry and/or flow cytometry (smoldering myeloma in two patients, indolent lymphomas in four patients). Three patients were diagnosed with chronic lymphoprolifetrative disease with no signs of monoclonal gammoapthy; CLL in two patients and follicular lymphoma in one patient. *Summary*. All together, in our cohort of 66 patients with LGL-leukaemia 15 patients (23%) were diagnosed with concomitant indolent clonal B-cell disease, which is considerably higher than expected. This is in line with data recently reported by Viny and coworkers. This finding may have some relevance to the pathogenesis of LGL-leukaemia.

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LUMILIXIMAB TREATMENT RESULTS IN INCREASES IN SERUM CD23 BUT NO MODULATION OF MEMBRANE CD23 IN RELAPSED CLL PATIENTS

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Background. Lumiliximab (IDEC-152) is an anti-CD23 monoclonal antibody that binds to CD23, the low-affinity IgE receptor. CD23 is a trimeric molecule with both membrane-bound and serum forms. Membrane CD23 is a 45 kD glycoprotein expressed on B cells, monocytes /macrophages, eosinophils, platelets, and dendritic cells, with expression being increased on chronic lymphocytic leukemia (CLL) cells. Serum CD23 is produced via the cleavage of membrane CD23 into fragments by endogenous proteases, with the 25 kD fragment occurring at ng/mL concentrations in normal human serum. Increased levels of serum CD23 are found in patients with several disease states, including CLL. *Aims*. We report two trials that assessed the effects of lumiliximab treatment, alone (Ph I) or in combination (Ph I/II) with FCR (Fludarabine, Cyclophosphamide, and rituximab), on membrane and serum CD23 levels in relapsed CLL patients. Receptor occupancy levels in patients were also evaluated following weekly (monotherapy) or monthly (+ FCR) treatment with lumiliximab. In addition, potential correlations between the level of membrane CD23 expression and absolute lymphocyte count (ALC) decrease or clinical response were studied in both trials. *Design and Methods*. Serum CD23 levels were measured using a validated enzyme-linked immunosorbant assay. Two non-competitive CD23 antibodies (anti-CD23 PE and lumiliximab-FITC) were utilized in a flow cytometry assay to assess the receptor occupancy of lumiliximab on the surface of B-CLL cells, as well as membrane CD23 levels. Lymphocytes were gated on CD45 and SSC followed by gating on CD5+CD19+ cells to identify B-CLL cells. CLL cells were assessed for membrane CD23 by gating on CD23+CLL cells. The receptor occupancy (fraction of lumiliximab-bound CLL cells) was reported as the ratio of CD23+CLL cells divided by lumiliximab+CD23+CLL cells. Results Membrane CD23 levels remain relatively consistent following treatment with lumiliximab, both in a monotherapy and combination therapy setting. However, serum CD23 levels increased within several hours of dosing with either lumiliximab alone or in combination with FCR. The intensity of the serum CD23 increase varied from patient to patient, and was dose-dependant. Following initial increases in serum CD23, subsequent kinetics are dose and schedule dependant. CD23 receptor saturation with lumiliximab was maintained, and clinical activity was observed, despite the presence of high serum CD23 levels in both trials. Membrane CD23 levels did not appear to correlate to ALC decreases and clinical response. *Conclusions*. These analyses show that treatment of relapsed CLL patients with lumiliximab results in increases in serum CD23 levels in a dose- and schedule-dependant manner. These studies also found that lumiliximab treatment did not result in a reduction in membrane CD23 levels. As CD23 receptor saturation and a favorable response profile occurred in the presence of high circulating CD23 levels, these studies suggest that increased levels of serum CD23 do not negatively impact on clinical activity and do not appear to behave as an antigen sink. Furthermore, the level of membrane CD23 expression did not correlate with ALC decreases or clinical response to lumiliximab. These findings will be further evaluated in ongoing clinical trials.

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ERUFOSINE, A NOVEL ALKYLPHOSPHOCHOLINE, IN CHRONIC LYMPHOCYTIC LEUKEMIA: IN VITRO COMPARISON WITH BENDAMUSTINE AND FLUDARABINE

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Background. Alkylphosphocholines represent a new class of cytostatic drugs with a novel mode of action. Erucylphospho-N,N,N-trimethylpropylammonium (erufosine - ErPC3), the first compound of this class that can be administered intravenously, has recently been shown to be active against human tumor and leukemic cell lines and is currently undergoing clinical trials in leukemia patients. Erufosine has high activity against leukemic cells without affecting the normal hematopoiesis. The aim of the study was to evaluate *in vitro* the antileukemic potential of ErPC3 in chronic lymphocytic leukemia (CLL) compared to fludarabine and bendamustine, that are routinely used drugs for the treatment of CLL. *Design and Methods*. The cytoreductive activity of ErPC3, fludarabine, and bendamustine was evaluated in primary cell cultures of mononuclear cells isolated from peripheral blood from newly diagnosed patients with CLL using the MTT-dye reduction assay. The levels of bcl-2 and NF-kB proteins were measured by Western blot. *Results*. Erufosine showed clear concentration-dependent efficacy against CLL cells that exceeded in some cases the efficacy of the reference drugs fludarabine and bendamustine (IC50 values ranging from 12 to 30 μM). Subsets of cell cultures showed greater sensitivity towards fludarabine or bendamustine as estimated by the corresponding IC50 values. Immunoblot analyses revealed about 90% Bcl-2 and about 70% NF-κB positive samples. Bcl-2 protein expression showed a decrease after cytoreductive treatment. *Conclusions*. Erufosine is a promising agent for the treatment of lymphoproliferative disorders such as CLL because of its remarkable antitumor activity *in vitro* and its ability to intensify normal hematopoiesis.

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FLUDARABINE AND CYCLOPHOSPHAMIDE (FC) VERSUS CYCLOPHOSPHAMIDE, VINCRISTINE AND PREDNISONE(CVP) AS FIRST LINE THERAPY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The introduction of Fludarabine into the treatment of chronic lymphocytic leukemia (CLL) has improved the complete remission rate (CR), overall response rate (OR) and progression free-survival (PFS) compared with alkilator based regimens. Its synergistic action with cyclophosphamide has demonstrated semnificative advances as front line therapy in untreated CLL patients. *Aims*. To evaluate the response rate time, the disease progression and survival of the patients of FC (Arm A) vs CVP (Arm B) as first line CLL treatment. *Design and Methods*. Starting from I 2004 until I 2008, 87 untreated patients with CLL were enroled, diagnosed and treated in the Hematology Department from the western part of the country. The group was formed by 56 males and 31 females who were randomised into the two treatment arms approxi-

matelly 43 in each group. The diagnosis of CLL was established according to the criteria of International Workshop on CLL (IWCLL) 1989. The median age was 66,3 years, range (37-78), ECOG performance status 0-II with high risk category (RAI stage III-IV or RAI stage I-II if they have at least one of the followings: one or more of the disease related symptoms, progressive marrow failure, massive splenomegally or lymphadenopathy or progressive lymphocytosis). Arm A received: cyclophosphamide 250 mg/m^2 i.v. D1 to D3 and Fludarabine 25 mg/m^2 D1 to D3. Arm B received Cyclophosphamide 400 mg/m^2 i.v. D1 to D3, Vincristine 1,4 mg/m2 D1 and Prednisone 100 mg/m^2 D1 to D5. Cycles to be repeated every 21 days. Hematological toxicity was recorded according to NCI-WC for diagnosis and treatment and evaluation of response was done according to the NCI-WC response criteria. Were excluded from the study with stable or progressive disease after 3rd cycle. While PR and CR cases continued to 6 cycles of the same treatment. To confirm the repsonse to treatment, were performed Bone marrow biopsy and immunophenotyping. Results: 28 patients had stage IV, 34 patients had stage III and 25 patients had stage II. The median WBC was 93x10^9/L, the median lymphocyte count was 75x10^9/L, the median hemoglobin level was 8,3 gr/dl, the median platelet count was 110x10^9/L. Bone marrow biopsy showed difuse pattern in 84% and the median lymphocyte in the bone marrow was 90,5%. Complete clinical remission was reported in 29/44 patients in arm A (66%) compared to 9/43 in arm B (21%), p=0,15. Confirmed CR by bone marrow biopsy was reported in 14 patients in arm A (31,8%) and in only 4 cases in arm B (9,3%). Partial respons with nodules was reported in 10 patients (22,7%) in arm A and 5 cases (11,6%) in arm B. Median time to progression was 28 months in Arm A and 8 months in Arm B (p=0,03). In therms of hematological toxicity in Arm A 9 patients developed grade IV neutropenia and received G-CSF treatment while 2 patients developed severe anemia (grade III and IV) that required red blood cell transfusion. Two patients developed transient febrile neutropenia of unknown origin, which required hospitalization. Mild extra-hematological toxicity consisting of nausea and vomiting occured in 10 patients during the treatment in both Arm A and B. Treatment related mortality occured in 2,0% in FC Arm and 1,5% in CVP Arm. *Conclusions.* The overall response rate (ORR) was significantly higher in FC Arm in comparison with CVP Arm. Also the complete response rate of FC Arm was high compared with CVP Arm. The combination of FC is able to induce higher response rate at the level of bone marrow biopsy. The hematological end extrahematological side effect were mild and manageable. There was a statistically significant difference in time to disease progression in favor of FC regimen.

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PREDICTORS OF DEATH IN CRITICALLY ILL CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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For chronic lymphocytic leukemia (CLL), prognostic factors which are the most used by haematologists are the clinico-biological staging of Rai/Binet, along with newly identified cytogenetic and molecular markers. In that retrospective monocentric study, we sought to identify risk factors for in-hospital mortality in critically ill CLL patients. We studied 62 consecutive patients with CLL admitted to the medical intensive care unit (ICU) at Saint-Louis hospital, Paris, France, between 1991 and 2008. Binet staging was as follows: stage A, 21%, stage B, 16% and stage C, 58%. Five percent of the patients had a Richter disease. Before ICU admission, 15% of the patients had never received any treatment for CLL, 60% had already received at least one course of Fludarabine, 36% had received Rituximab and 35,5% had received prolonged steroid treatment. Thirteen percent of the patients were receiving anti-pneumococcal disease prophylaxis, 26% anti-herpes and varicella-zoster viruses prophylaxis and 30% anti-pneumocystis prophylaxis. Haematopoietic stem cell transplantation (autologous or allogeneic) had been performed in 8% of the patients. During the course of CLL, 36 patients (58%) had presented an infectious episode, including 9 patients with low immunoglobulin levels. The two major reasons for ICU admission were acute respiratory failure (75,8%) and sepsis (61,3%). Twenty-nine percent of the patients presented with shock. Median LOD (Logistic Organ Dysfunction) scores at day 1 and 3 were 5 (25%-75%, 0-20) and 4 (idem), respectively. Noninvasive mechanical ventilation was needed in 27,4% of the patients and 50% needed invasive mechanical ventilation or vasopressors throughout the ICU stay. ICU, in-hospital and 90-day mortalities were 35,4%, 45% and 58%, respectively. Univariate and multivariate logistic regression analyses were performed. By multivariate logistical regression analysis, we identified 3 variables independently associated with in-hospital mortality: (i) previous infection during CLL course, (ii) admission to the ICU for shock and (iii) low oxygen saturation at ICU admission. The risk for in-hospital mortality increased with an increasing number of these risk factors. In this study of CLL patients, ICU was necessary mainly for acute respiratory failure, primarily from infectious origin. Prognosis relied on severity of lung injury and on the presence of septic shock, rather than on the characteristics of the underlying malignancy. The high mortality in sepsis-related ICU admission in CLL patients suggests that infection prevention may translate into reduced ICU admission and associated mortality.

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WHAT DO WE KNOW ABOUT THE REAL INCIDENCE OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AND WHERE AND HOW ARE THE PATIENTS WITH CLL TREATED?

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Background. Currently, pathogenesis, modern prognostic factors, or modern therapy in CLL are frequently discussed, however, up-to-date data concerning the incidence and the management of CLL in "real life" are still missing. *Aims*. The aim of our study was to find out the actual epidemiological situation of CLL, and the diagnostic and therapeutic preferences of hematologists in the preselect area - the South Moravian Region. *Design and Methods*. We asked 18 specialized hematological departments to fill in our questionnaire in order to get exact information about epidemiology and treatment of CLL patients. All hematologists filled the questionnaires out. Acquired data were merged with our database. According to the Czech Bureau of Statistics, 1,127,718 inhabitants live in the South Moravian Region. *Results.* The total number of 540 patients (median age at the time of diagnosis: 65 years, range: 33-92; sex: 306 males, 234 females) who were followed up in the year 2008 were included in the analysis. Therefore, the incidence of CLL was 6.2 per 100,000, the prevalence 48 per 100,000. The Rai stage at diagnosis was as follows: 0 (n=274, 50%), I (n=173, 32%), II (n=36, 6.7%), III (n=25, 4.6%), IV (n=25, 4.6%). In 7 patients (1.3%) the Rai stage wasn't determined. Flowcytometry was carried out in 525 patients (97%), the others were diagnosed by a histological examination either of bone marrow or a lymph-node. Two hundred eighty-seven patients (53%) were followed up at the local hematological ambulances, 253 (47%) were treated at one main hematological center. The median follow-up was 56 months (range: 5-1262). Two hundred ninety-one patients (54%) were cytogenetically examined (del 17p, n=5; del 11q, n=18; +12, n=29; del 13q, n=111; normal karyotype, n=94, combined cytogenetic abnormalities, n=34). IgVH mutational status was examined in 305 (57%) patients (155 mutated, 134 unmutated, 16 polyclonal). ZAP-70 was examined in 191 (35%) patients (77 positive, 114 negative). The treatment of CLL was indicated in 194 patients (36%), 93 (17%) of them also underwent the second line treatment. As the first line treatment, 64 patients (33%) were given fludarabine-based regimen, 40 (20.6%) of them achieved complete remission (CR), 74 (38%) received chlorambucil with 12 (6%) CRs, 28 (14%) CHOP-like regimen with 8 (4%) CRs, 10 (5%) alemtuzumab with 6 CRs, 18 (9%) corticosteroids with 1 CR. Ninety-three patients (17%) underwent the second line treatment: fludarabine-based regimen (n=39, 42%) with19 CRs, chlorambucil (n=19, 20%) with 1 CR, CHOP-like regimen (n=8, 9%) with 1 CR, alemtuzumab (n=10, 10%) with 4 CRs, and corticoid-based treatment (n=17, 18%) with no CR. Thirty patients were treated within clinical trials. *Conclusions*. According to our analysis, fewer patients with CLL were surprisingly indicated to therapy than we expected. Although the treatment was indicated in the minority of patients, the modern prognostic factors were examined in almost all patients. It remains questionable if in *real life* the examination of the modern prognostic factors is necessary in all CLL patients.

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FLUDARABINE REFRACTORY AND VERY ADVANCED CLL: VALUE OF RITUXIMAB MONOTHERAPY

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Background. In chronic lymphocytic leukaemia (CLL) therapeutic

options are limited for heavily pretreated patients, or patients who have failed or relapsed shortly after treatment with fludarabine (F-refractory). Many patients with advanced stage CLL have compromised bone marrow capacity, and many are elderly with significant co-morbidities further limiting choice of therapy. Thus, there is a need for mild 'salvage' regimens that combine efficacy with reduced hematotoxicity. Rituximab has been widely explored as a single agent in CLL and has considerable advantages due to its manageable, predictable safety profile. Rituximab monotherapy is evaluated here in the context of heavily pretreated and F-refractory CLL. *Aims*. The objective of this literature survey was to conduct a comprehensive analysis of existing clinical data to evaluate reported efficacy of Rituximab in heavily pretreated and F-refractory CLL. A secondary objective was to evaluate reported efficacy of Rituximab in combination with novel, non-chemotherapy combinations. Methods A search of all electronic information including Medline databases, conference proceedings (ASH, EHA, ASCO) and trial registers was conducted. Extracted data included prior therapies, treatment regimen, dosing schedule, response rate, median follow-up time, progression free survival (PFS) and time to progression (TTP). *Results*. The total number of publications exploring utility of single agent Rituximab in heavily pretreated or F-refractory CLL was 21 and involved 292 patients. When administered at a dose of 375mg/m2 weekly for 4 weeks, Rituximab gave variable efficacy (ORR ranging from 0-38%) and limited durability of response (TTP ~ 4 months). Eight studies employed alternative Rituximab dosing regimens, including high dose (up to 2250 mg/m^2), dose dense (administered 3x/week) and prolonged dosing regimens. These modified dosing schedules were associated with improved ORR (ranging from 33 to 85%) and considerable improvements in durability compared to the standard (375 mg/m^2 weekly x 4) dosing regimen (TTP ranging from 6 to over 25 months). Where individual data on F-refractory patient sub-sets was reported, clinical responses were observed in 41-50% of patients. Three separate case studies report successful use of Rituximab in F-refractory CLL. Rituximab-non-chemotherapy combinations offer the potential for improved efficacy with acceptable toxicity. An emerging body of evidence supports utility of Rituximab in combination with high dose glucocorticoids, with five recent studies reporting ORRs of 78-93% in F- refractory CLL, and TTP ranging from 12-15 months. *Conclusions*. Rituximab monotherapy has utility in CLL patients for whom alternative therapies have been exhausted or are not advisable due to hematotoxicity. Intensified Rituximab monotherapy regimens have resulted in response rates of up to 85% in CLL patients, with ORRs of 41-50% in F-refractory subsets. Rituximab-non-chemotherapy combinations offer the potential for improved efficacy with acceptable toxicity and a combination that is demonstrating exciting synergistic efficacy as salvage for refractory patients is the Rituximab-glucocorticoid combination, with ORRs greater than 90% reported in F-refractory CLL. Thus, Rituximab as monotherapy or in combination with high dose glucocorticoids is an efficacious and well tolerated treatment option for patients with very advanced or F-refractory CLL. Together, these studies support utility of Rituximab in CLL patients that are unsuitable candidates for more cytotoxic regimens.

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SERUM LEVEL OF BFGF AND HIS CORRELATION WITH BIOLOGICAL PARAMETERS IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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A lot of study indicate that angiogenesis may play important role in the pathogenesis of hematological malignancies, including B-cell chronic lymphocytic leukaemia (B-CLL). Angiogenesis is regulated by many substances with pro- and anti-angiogenic activity. Basic fibroblast growth factor (bFGF) is a pleiotropic cytokine that plays an important role in angiogenesis. bFGF can also act as a hematopoietic cytokine. It is found in megakariocytes and cells of the granulocyte lineage. Elevated levels of bFGF have been previously reported in cells derived from renal cell carcinoma, malignant melanoma. Some investigators have shown that clonal B cells from B-chronic lymphocytic leukemia are able to synthesize bFGF and a correlation between increased levels of bFGF and impaired prognosis. We investigated whether is an association between the concentration of bFGF and other biological parameters in untreated CLL patients. Two hundred sixty one patients (110 women, 151 men) with Rai stage 0-4 were studied. They were newly diagnosed according NCI criteria. Analysis of bFGF serum concentration, Zap-70, CD38 expression and detection of genomic aberrations (del 17p, del 11q, del 13q, trisomy 12) was performed. Serum bFGF levels were measured in 261 CLL patients with range from 2.49 to 556.09 pg/mL with a mean of 88.9703 (SD=132,92) pg/mL. The serum concentration of bFGF was higher in the CLL group (mean 90.1960 pg/mL, SD=134,15) when compared to the control group (mean 1.3837 pg/mL, SD=1.38) and that difference found to be significant (p<0.003). The mean bFGF concentration in ZAP-70 positive patients was 79.32 (SD=120.23) pg/mL and ZAP-70 negative patients was 103.72 (SD=152.43)pg/mL. Among CD38 positive patients mean concentration of bFGF was 102.03 (SD=160,48) pg/mL, and among CD38 negative patients was 87.40 (SD=126,16) pg/mL. However, differences between bFGF concentration in group of ZAP- 70 positive versus ZAP-70 negative patients, and CD38 positive patients versus CD38 negative patients were no statistically significant. There were no statistically significant differences in concentration of bFGF between gender groups and between four clinical stage groups. No statistically significant correlation was found between bFGF serum concentration level and patients sex, time to treatment, overall survival and genetics aberrations. Lack of relationship between CD38, ZAP-70, genomic aberrations and another clinical parameters show, that further investigation of the exact role of bFGF in CLL is warranted.

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AN ANALYSIS OF EXPRESSION OF MAJOR LYMPHOID ANTIGENS IN B-CLL IN PATIENTS WITH DIFFERENT STAGES OF DISEASE

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The application of the immunophenotyping method for diagnosing chronic lymphoproliferative diseases allows not only to reveal the expression of major antigens but to assess the number of cues expressing these antigens. Currently there is no common opinion of a prognostic role of degree of «ANCHOR» antigens expression. The purpose of the study was the assessment of major lymphoid antigens (CD5, CD19, CD23) expression in patients with an established diagnosis of B-linear chronic lymphocytic leukemia (B-CLL) to reveal phenotypically heterogeneous groups of patients. Samples of bone marrow (BM) aspirate from 71 patients with B-CLL were studied. The diagnosis was made on the basis of standard clinical and laboratory criteria. The standard panel of monoclonal antibodies for CLL typing was utilized for immunophenotyping. Assessment of the results was made by flowing cytometer FACS Caliber (BD). B-linear belonging was determined by the presence of major B-antigens (CD19, CD20, CD22, CD23, HLA-DR). Belonging of cells to B-CLL was confirmed by the presence of specific co-expression CD5+\CD19+, CD5+\CD20+, the presence of antigen CD23, by clonality according to one of light chains (**K** or **λ**) and a negative reaction with FMC7 and CD10 antigens. Thus it was established that the number of cells with monoclonal CD5+ accumulation in B cells (CD19+) is different in patients with a clinically and morphologically established diagnosis B-CLL. We analyzed the degree of expression of antigens depending on the stage (according to Rai classification) of disease. We found the level of CD5+ and CD19+ cells is the highest (median 71%) for stage 1 and gradually lowers to stage III (median 48%), and at stage IV increases again (median 70%). The greatest number of cells with co-expression CD5+\CD19+ was observed in the subgroup with stage IV of disease (median 63%). While studying the expression of membrane antigen CD23 which is carried by lymphocytes in B-CLL, we also observed high values of the given antigen expression (max = 98%), but noted a decrease in CD23+ level (up to 6%) in some cases. Therefore, the greatest number of cells positive to CD23 antigen was in the subgroup with stage 1 (median 79%) and the least - with stage IV (median 51%). The given antigen was expressed by the same cells which were CD19+\CD5+. The cells with high values according to one antigen also expressed a considerable amount of other antigen molecules, indicating direct dependence. The obtained results give evidence that heterogeneity regarding the level of expression of *key* antigens is observed in group B-linear CLL. First stage disease patients demonstrated the highest amount of lymphocytes carrying major antigens B-CLL on the superficial membrane. The given values gradually decrease to stage III. At stage IV the amount of cells with co-expression CD5+/CD T9+ increases, and CD23 level continues to decrease. The study of phenotype in patients with different stages of B-CLL and follow-up of the patients with revealed differences in the phenotype will be continued. The aim of continuing the investigation is to determine the correlation of antigen changes in a clinicomorphologic picture.

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CAUSE OF DEATH IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL): EFFECT OF AGE AND LYMPHOCYTE COUNT AT DIAGNOSIS

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Background. Limited data is available on the cause of death in patients with CLL. CLL has been reported as the underlying or contributory cause of death in 52-69% of patients, secondary malignancies in 16-34% and non malignant causes in 32%.[1-3] This retrospective study analysed the causes of death in patients diagnosed with CLL in Wellington Hospital from 1992-2007. *Aims*. To see if CLL was the primary cause of death in our population and if age and lymphocyte count were independent prognostic factors for dying of CLL related causes.

Table.

Exposure variable	Odds Ratio	95% confidence limits Lower	Upper	*P* value
Sex (female:male)	0.615	0.246	1.538	0.299
Age at diagnosis (≥65 relative to <65)	0.210	0.078	0.571	0.002
Log2 of lymphocyte count (modelled as linear predictor)	1.47	1.05	2.47	0.026

Design and Methods. Patients with CLL were identified from laboratory diagnostic records. Information on age, gender, date of diagnosis, lymphocyte count was collected from clinical records. Date and cause of death was established from the New Zealand Health Information Service. All results reported are given for CLL-related relative to non-CLL related cause of death. Exposure variables included in the model were patient gender, patient age (under 65 years or 65 and older) and a log-transformed lymphocyte count (obtained at diagnosis). Cause of death was modelled using logistic regression. All analyses were performed using PROC LOGISTIC in SAS 9.1 (SAS Institute Inc, NC, USA). *Results*. 392 patients with CLL were identified and of these, 153 patients had died. Data on cause of death were available for 119 of the deceased patients with a lymphocyte count at diagnosis recorded in 108. CLL was the leading cause of death in 47% of patients, with non-malignant causes in 30% and secondary malignancy in 22%. Of the 108 patients included in the analysis, a total of 50 patients died of CLL-related causes (CLL or Richters Transformation) and 58 died of non-CLL related causes. From the 11 patients missing lymphocyte counts, 6 died from CLL-related causes. Table 1 reports the odds ratios associated with each of the variables in the model. As can be seen, patient gender did not significantly influence the likelihood of dying from CLL-related causes. Patients aged 65 or over at the time of diagnosis were less likely to have died from CLL-related causes than those patients aged less than 65 years. Furthermore, lymphocyte count at diagnosis was significantly related to the likelihood of dying from CLL -related causes: for each two fold increase in lymphocyte count, the odds of dying from a CLL related cause increased by a factor of 1.47 (95% confidence interval 1.05, 2.07). These risk factors were independent of each other. *Conclusions*. CLL accounted for nearly half of all deaths in our population. The risk of dying of CLL or related causes increased with younger age at presentation and increasing lymphocyte count at diagnosis.

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IMMUNOHISTOCHEMICAL DETERMINATION OF ZAP-70 PROTEIN IN BONE MARROW BIOPSIES IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS

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Background. CLL is the most common type of leukemia in the western world, with a variable clinical course. The biology of CLL partly depends on the mutational status of the variable region of immunoglobulin heavy chain genes (IgVH), which defines two subgroups of tumors: mutated and unmutated. The expression of ZAP-70 protein is a surrogate marker of more aggressive unmutated cases. *Aims*. Our study aimed to assess the ZAP-70 immunohistochemical protein expression on bone marrow biopsies (BMBs) and examine possible associations with clinicopathologic characteristics and patient survival. *Design and Methods*. 37 BMBs of typical CLL patients were studied (32 male, 5 female) at diagnosis. 21 patients were stage Binet A, 12 stage B and 4 stage C. Immunohistochemistry (IHC) was performed in paraffin sections usinig streptavidin-biotin method. The applied antidody was mouse monoclonal 2F3.2 (Cell Marque). IHC profile also included CD3, CD20, CD5, CD23. A threshold of 25% of CLL cells expressing the ZAP70 protein was used as cut-off for classification of samples as negative (<25% cells positive) or positive (>25% of cells positive). We also evaluated percentage of positive cells along with intensity of staining (0+ absent, 1+ weak, 2+ moderate, 3+ strong staining) in a composite semi-quantitative ZAP70 score (range 0-300). To grade the staining intensity, T-cells were used as strongly positive controls. *Results*. 16/37 cases were negative (43%) and 21/37 (57%) were positive. All positive cases exhibited >50% staining cells . ZAP70 protein expression was associated with advanced age (χ^2 p=0.017), need for therapy (χ^2 p=0.002) and early initiation of therapy (χ^2 p=0.012). At a median follow up of 61 months, 4/16 patients with ZAP70 negative subtype died (mortality 25%), whereas 10/21 among those with ZAP70 expressing tumor died (mortality 48%). The median survival of patients with ZAP70 negative CLL was 90 months while that of patients with ZAP70 positive CLL was 80 months (log-rank p=0.07). When cell number and staining intensity were evaluated by means of the ZAP70 score, the median value of 150 was used to classify patients to ZAP70 negative or ZAP70 positive CLL subgroup. The difference in overall survival favouring the former group reached statistical significance (log-rank p=0.05). *Conclusions*. Immunohistochemistry for ZAP70 protein expression in bone marrow leukemic cells from paraffin-embedded biopsies is a convenient and reliable surrogate marker for aggressive biology of CLL and inferior patient outcome. Its association with adverse clinicopathological characteristics and molecular links to proven cellular pathways of cell kinetic and therapy resistance should be further studied.

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CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS WITH V3-21 GENE REARRANGEMENT HAVE A HIGHLY VARIABLE CLINICAL OUTCOME DEPENDING ON MUTATIONAL STATUS AND CYTOGENETIC FEATURES

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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). Growing evidence suggests that patients with rare rearrangement of a specific variable region (V3-21) gene have a uniformly unfavourable clinical outcome irrespective of the IgVH mutational status. *Aim.* To analyze the haematological features, clinical behaviour and outcome of patients with V3-21 gene rearrangement. *Method*. IgVH mutational status was analyzed in 352 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 cut-

off of 98% was used to define the IgVH mutational status. The V3-21 gene was clonally rearranged in 22/352 (6.25%) cases and mutated in 12/22 (55%) patients. FISH analysis was performed in 19/22 patients (86%); high-risk genetic abnormality (17p or 11q deletion) was detected in 8/19 (42%) cases. The median age at diagnosis was 66 years (36-76). Binet clinical stages were as follows: A 32%, B 36% and C 32%. Treatment was initiated in 18/22 patients (82%), with first-line therapy comprising chlorambucil (6/22), fludarabine-based regimen (7/22) or C(H)OP-like regimen (9/22). After a median follow-up of 54 months, 9/22 (41%) patients died. *Results*. Overall survival at 7 years (7y OS) reached 61% (95% CI 0.35-0.87); treatment-free survival at 2 years (2y TFS) was 39% (95% CI 0.16-0.61). Prolonged TFS was strongly associated with unmutated IgVH (log rank 0.004) and with the absence of high-risk cytogenetics (log rank 0.02). Patients without 17p or 11q deletion had longer survival (3y OS 90% vs 57%, log rank 0.02). Similarly, those with mutated IgVH showed a trend for favourable OS (7y OS 72% vs 40%, log rank 0.09). No correlation was found between 17p or 11q deletion and IgVH mutational status (χ^2 0.6). There was a trend for treatment initiation in patients with mutated IgVH and unfavourable cytogenetics (χ^2 0.1). *Summary*. Patients with V3-21 gene rearrangement do not share the same clinical outcome. Survival and treatment initiation are influenced by both unfavourable cytogenetics and unmutated IgVH status. Most patients with mutated IgVH are long- term survivors. Currently used standard cytogenetic and molecular cytogenetic methods do not lose their predictive role in the population of B-CLL patients with V3-21 gene rearrangement.

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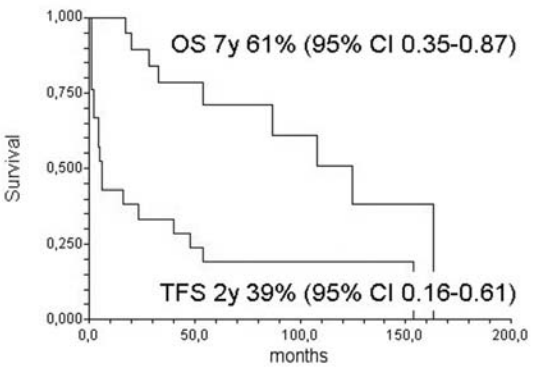

Figure.

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PERSISTENT UNEXPLAINED MILD LEUKOPENIA: HOW FAR A SEARCH SHOULD BE DONE

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Mild leucopenia is often encountered in everyday practice. In most cases it is transient, associated with smoldering viral illness or drug consumption. Persistent chronic leucopenia is usually associated with specific clinical situations but in a few cases there is no obvious explanation and there arises a question of how deep and vigorous a search should be done. In this paper we report the outcome of long follow-up of 32 patients with persistent and apparently unexplained mild leucopenia. 32 patients, 13 men, 19 women, aged 24-80 years, median 58 years presented with low (2.2-3.8x10^9/μL, mean 3.4x10^9/μL) white blood cell count (WBC) lasting more than one year. History, physical examination and the basic laboratory work-up failed to reveal the cause of the abnormality. Two to six months after the first visit all patients were re-examined with physical examination and laboratory testing including complete blood counts, biochemical and serological tests, ultrasound of the abdomen, immunophenotyping, bone marrow aspiration and biopsy and karyoryping. In most cases the findings were inconclusive and the diagnostic search was repeated every six months, until the resolution of the leucopenia or a diagnosis was reached. After follow-up of 2-6 years, median 2.4 years, in 5/32 pts the WBC was gradually restored. 4/32 patients developed autoimmune disease, 2 patients myelodysplastic syndrome, 1 chronic myelomonocytic leukemia and 10 lymphoproliferative disorders. In 11/32 pts no definite diagnosis is reached. The pts with lymphoproliferative disorders, 5 men, 5 women, aged 44-80 years, median 58 years presented the following: 3/10 hairy cell leukemia, 2/10 Waldestrom's macroglobinemia, 1 follicular lymphoma, 1 MGUS, 1 large granular lymphocytosis, 1 T- lymphocytosis with TCR rearrangement and in one pt a monoclonal lymphocyte infiltration of the bone marrow was found without further histological typing. The patient with the follicular lymphoma rapidly progressed to grade 3, stage IV and was treated appropriately. No other patient was treated. *Conclusions*. 30% of patients with persistent leucopenia eventually developed monoclonal lymphoid disorders The persistence of unexplained leucopenia may be the early sign of an emergent disease, particularly a low-grade lymphoproliferative disorder. An extended laboratory diagnostic work-up is absolutely warranted.

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THE EVALUATION OF EFFICACY AND TOXITY OF THE FIRST LINE THERAPY WITH FLUDARABINE COMBINED WITH CYCLOPHOSPHAMIDE (FC) VERSUS. 2-CHLORODEOXYADENOSINE WITH CYCLOPHOSPHAMIDE (CC) IN CHRONIC LYMPHOCYTIC B-CELL LEUKEMIA (CLL) SUBJECTS - THE OWN STUDY REPORT

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Background. Purine analogs are characterized with high effectiveness in CLL patients. The combined therapy FC/CC as the first line therapy can increase percentage of hematological response and improve quality of life and prolong survival. *Aims*. To evaluate the efficacy and safety of FC and CC therapy as first line therapy for CLL-B-1-4 grade Rai. *Design and Methods*. During 5 yr study 38 CLL-B patients (22 Male, 16 Female ; range 50 - 77 years) were randomized in the Department Of Hematology and Internal Diseases of the Institute of Hematology and Blood Transfusion to receive FC (Fludarabine-25 mg/m^2 i.v. 1-3 days and Cyclophosphamide 250 mg/m^2 i.v. 1-3 days) or CC (2-CdA-0,12 mg/kg/24h i.v. 1-3 days i.v. and Cyclophosphamide 250 mg/m^2 i.v. 1-3 days).The efficacy of therapy was performed after 3 and 6 cycles of therapy with FC / CC. Characteristics of CLL-B patients is in the Table 1.

Table 1. Number of evaluated patients 38(16F; 22M).

Age 50 - 77 years
FC 18 pts. CC
20 pts.
Number of received cycles 3-6
Time between 2 cycles 1-7 months (range 1.02 months)
Side Effects :8/38 (21%)
Leucopenia:
1oWHO after 2 cycles 2 pts.
1o WHO after 5 cycles 1pt.
2oWHO after 6 cycles 1pt.
2oWHO after 2 cycles 1pt.
Anemia after po 2 cycles 1pt.
Thorombocytopenia 4oWHO 1pt.
Electrolytes disorders 1pt.
Results are shown in the Table 2.

Table 2. Results FC (n=17/18) CC (n=18/20).

After 3 cycles :			
CR	04	02	
PR	10	14	
NR	03	02	
CR+PR	14 (77.8%)	16 (80.0%)	
Results	FC (n=13/18)	CC (n=16/20)	
After 6 cycles :			
CR	05	07	
PR	08	07	
NR	0	02	
CR+PR	13 (72.2%)	14 (70.0%)	
Deaths :	02	03	
PD	01	02	
Others	01	01	
Survival time since diagnosis (months)			8-84 months
(median 39.5 mo)	30-60 months		
(median 45.3 mo)			
Survival time since FC/CC therapy (months)			7-62 months
(median 31.8 mo)	11-59 months		
(median 40.1 mo)			

Conclusions. FC/CC combined chemotherapy induces the similar percentage hematological response (CR+PR) after 3, 6 cycles treatment. Side effects were performed in 21% of treated FC/CC CLL-B patients Overall survival time and survival time since diagnosis are longer in CC arm CLL-B patients.

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ALEMTUZUMAB IN FLUDARABINE-REFRACTORY CLL

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Background. Chronic lymphocytic leukemia (CLL) patients treated with Fludarabine have good responses in 60-65% of the cases. Alemtuzumab was approved in 2001 for the treatment of patients with CLL previously treated with alkylating agents and fludarabine. *Aims*. The trial evaluated intravenous (i.v.) alemtuzumab (Campath) 3 x 30 mg weekly in fludarabine refractory CLL. *Design and Methods*. From October 2005 to October 2008, 27 patients were enrolled and received alemtuzumab. Median age was 58 (38-80) years, 70.37% were male, 74,07% were Binet C, and a median of 3 (1-6) prior lines had been given. Unfavorable genetics were frequent (51.85%). I.V. treatment was performed on an hospitalized patients basis in 81,48% and had to be temporarily interrupted in 7 patients due to neutropenia (2 pts.), anemia (1 patient), thrombocytopenia (1 patient), infections (3pts.), and was stopped early in 3 cases due to insufficient response (1 case) and infections (2 cases). Clinical and biologic parameters (age, sex, B-symptoms, stage, ECOG, number of prior lines, node size, hepato-spenomegaly, WBC, LDH, β2-MG) were evaluated for their prognostic role. Results: Toxicity during treatment period was mostly grade I/II apart from hematotoxicity. Grade neutropenia, thrombocytopenia, anemia occurred in 8 (29.63%) patients. Grade $^{3}/_{4}$ non-cytomegalovirus infection occurred in 5 patients (18,52%). CMV reactivation was observed in 18,52% (5 cases) total, all grade. The CMV episodes were successfully treated with anti-CMV therapy and there was one CMV-related death. After a median follow-up of 30 months, there were 19 (70,37%) deaths, 56% due to disease progression, 31% due to infection, and 13% not related to CLL. Overall response rate was 33.33% (CR 7,4%, PR 25,93%), median progression free survival time was 7.7 months, and median overall survival time was 19.1 months. OS was significantly inferior for age > 65 y (10.2 vs 26.0 mo, $p<0.001$), ECOG > 1(9.8 s 19.5 mo, $p=0.001$), and β2-MG >5 (11.5 vs 25.2 mo, $p=0.004$. The median OS since next therapy in these patients was 10.3 months. *Conclusions*. Alemtuzumab was well tolerated and determined good survival in 29,63% of the fludarabine refractory advanced phase CLL patients.

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HYPOADIPONECTINEMIA IS ASSOCIATED WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA RISK

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Background/Aim. Adiponectin, an adipocyte secreted hormone, is inversely related to risk for many malignancies associated with obesity and the metabolic syndrome, including leukemia. In this case-control study, we explored the role of total and high molecular weight (HMW) adiponectin, the presumed active form of adiponectin in the etiopathogenesis of B-cell chronic lymphocytic leukemia (B-CLL). We also explored their association with several established prognostic factors for B-CLL. *Design and Methods*. Blood samples were collected from 95 cases with incident B-CLL, and 95 hospital controls, admitted for non-neoplastic and non-infectious conditions, matched on gender, age and year/month of diagnosis (±1 month) between 2001 and 2007. Serum total adiponectin concentrations were determined by radioimmunoassay (LINCO Research Institute, St Charles, MO). Serum HMW adiponectin was measured using ELISA (ALPCO Diagnostics, Salem, NH). Moreover, serum lactate dehydrogenase (LDH), β2-microglobulin (BMG), lymphocyte morphology and the surface expression of CD38 in >30% of B-CLL lymphocytes were determined. Statistical analysis of the data was performed with SAS 9.1 for Windows XP. Results: Cases presented a higher body mass index (BMI) than controls ($p=0.008$). Significantly more cases than controls presented a family history of lymphohematopoietic cancer (LHC) ($p<0.01$). Total adiponectin was positively associated with HMW adiponectin ($p<0.01$) among all study subjects. Lower serum adiponectin levels were associated with higher risk of B-CLL by bivariate analysis and after adjusting for age, gender, weight, BMI and family history of LHC (OR: 0.96; 95% CI 0.92-1.00). No significantly different adiponectin and HMW adiponectin levels were found amid different stages of B-CLL according to the Binet classification. Only LDH was weakly but significantly positively correlated with total adiponectin and HMW adiponectin ($p<0.05$) among B-CLL cases. *Conclusions*. This study suggests that adiponectin may be weakly associated with B-CLL etiopathogenesis and could represent a potential marker of disease severity. Further studies are needed to confirm these associations and to explore the mechanisms underlying adiponectin's role in lymphopoiesis in general and B-CLL specifically.

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THE RESULTS OF IFN-A AS FIRST-LINE THERAPY IN PATIENTS WITH HAIRY CELL LEUKEMIA

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Hairy cell leukemia (HCL) is a chronic B-cell lymphoid leukemia characterized by pancytopenia, splenomegaly, myelofibrosis and the presence in peripheral blood, bone marrow and spleen of atypical lymphoid cell with hairy aspect. The purine nucleoside analogs represent the first-line therapy, often associated with immunosuppression and secondary malignancies. IFN-α is an alternative therapy with significant eficacy. *Aim of study*. to evaluate the eficacy of IFN-α as first-line treatment and if the maintenance therapy with IFN-a increased progression free survival in this disease. *Design and Methods*. We studied 29 patients with HCL hospitalized in Clinic of Hematology from Craiova (Romania) between 1997-2007 treated with IFN-α as first-line therapy 3MU/m^2 three times weekly for 12-24 months, devised by age, sex. We followed the presence of splenomegaly, hepatomegaly, complete blood counts, bone marrow infiltration with atypical lymphoid cell with cytoplasmic elongations at diagnosis. Results: the median age of HCL patients was 52 years with a high frequency at men (M/F = 3/1). A half of them had splenomegaly and 14% hepatomegaly at diagnosis. Median hemoglobin value was 10.9 g/dl , leukocytes 2.7x10°/L, absolute neutrophil counts 0.86 x10°/L, platelets 83 x10°/L; the median percentage of bone marrow infiltration with hairy cell was 72% at diagnosis. Three patients were splenectomized and 26 received IFN-a treatment at a dose of 3 MU three times weekly for 12-24 months. 14% of patients achieved complete response with negative bone marrow and 63% had a partial response. 49% of patients received maintenance therapy with IFN-α. 28% of patients never needed a second therapy and 35% were retreated with IFN-α. One patient developed a secondary neoplasia. The median follow-up was 52 months and the disease free survival in patients with maintenance therapy was 28 months. *Conclusions*. IFN-α has a significant eficacy in patients with HCL and the maintenance therapy seems to increase disease free survival.

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HAIRY CELL LEUKEMIA IN YOUNG PATIENTS: RUSSIAN EXPERIENCE

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From 1995 to 2007 we followed 160 pts (m/f 1.8/1) with verified hairy cell leukemia (HCL) aged 27 - 81 (median 49) yrs. 41 (26%) pts (m/f 2. 2/1) were from 27 to 40 (median 35) yrs of age. 27 (66%) pts (m/f 1. 7/1) had a typical form of HCL, 14 (34%) (m/f 3.7/1) - a variant form of HCL. Duration of the disease covered from 1 to 30 (median 5. 8) yrs. There were no substantial distinctions in frequency and degree of cytopenia, lymphocytosis, monocytopenia and splenomegaly between old and young groups. For certain there was more frequent for the young pts enlargement of abdominal lymph nodes (32% against 9% in old group). Marrow cytology, trephine biopsy histology, TRAP test were typical for all patients. Immunophenotype was performed in 30 (73%) pts. In 60% cases clone and in 40% cases clon k were found. Differences of immunophenotyping in young and old pts showed 25% frequency of variable phenotype (CD25-, CD23+, CD10+) in young pts. Splenectomy was performed in 15 (37%) young pts. Interferon -alfa in standart doses as initial therapy for 8-12 wks received 40 (98%) pts as treatment-prophylactics of agranulocytosis after subsequent Cladribine. Cladrib-

ine received 36 pts (88%). Complete remission from 1 to 9+ (median 5) yrs was achieved in 31 (86%) pts, partial remission- in 4 (11%). Relapses after one course of cladribine were observed at 1, 3 to 6 (median 3. 5) yrs in 11 (34%) pts aged to 40. In old pts it consisted of 12%. We consider HCL patients younger than 40 yrs at risk of early relapse after Cladribine and propose the supporting therapy with monoclonal antibody anti-CD20 or 22 once in 2-3 mos during the time to be established in near future.

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DOUBLE PRIMARY HEMATOLOGICAL MALIGNANCY: REPORT OF 21 CASES

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Nowadays there are many reports of patients with two primary malignant diseases, usually two solid tumors. In 2003 we reported 57 patients with one hematological malignancy and at least one second primary malignant neoplasm, including 9 cases of two primary hematological cancers, who were admitted in our clinic from 1995-2003. In the present paper we report those patients along with 12 new cases admitted from 2004-2008, that is a total of 21 patients with two apparently unrelated malignant hematological diseases. Patients with treatment-related malignancies or disease in transformation in a more aggressive subtype were excluded. 21 patients, 13 men, 8 women, aged 8-77, median age 61, presented the following malignancies: 12 chronic lymphocytic leukemia (CLL), 10 non Hodgkin lymphoma (NHL), 7 Hodgkin's lymphoma (HL), 5 myeloproliferative disease (MPD), 5 multiple myeloma (MM), 2 myelodysplastic syndrome (MDS), 1 acute leukemia. No patient had congenital immunodeficiency, exposure to toxic agents and/or irradiation or strong family history associated with malignancy. In three patients the diagnosis of two different diseases was done simultaneously (CLL and MM, CLL and CML, NHL and AML) while in 14 patients the second disease presented 1- 7 years following the first one and in 4 patients (all with cured HD) after 13-28 years. 9/21 had received no treatment for the first disease (CLL) at the time of the second diagnosis while 12 had received the appropriate treatment for the initial disease (NHL, HL, MPD, MDS). Of these, 5 were in long term complete remission, 2 with recently achieved remission, 3 were on hydroxyurea and 2 were transfusion-dependent. The clinical and laboratory features of the second disease did not present any particular difference from the usual pattern. Treatment was administered in 16/21. 3 patients were not able to receive full-dose chemotherapy, because of the previous treatments. 12 patients responded to therapy according to their prognostic risk factors while in 4 patients the second disease was unexpectedly aggressive. These patients had rather recently -less than two years- undergone treatment for the first disease. 13/21 are alive 0.5 - 11 years after the second malignancy. 8 patients died, 6 with progressive second disease in less than 2 years, one with acute leukemia while in complete remission for 3 years and one from breast cancer. Conclusions. The prevalence of two primary and apparently unrelated hematological malignancies seems to be rising. The epidemiology and clinical and laboratory features of the second neoplasms do not differ from those of the general population, CLL and NHL being the most frequently encountered hematological malignancies. Patients with successfully treated Hodgkin's lymphoma may present after a decade a second hematological neoplasm not necessarily treatment-related. Second neoplasms in long-term survivors or patients with low-grade malignancies should be treated appropriately as the prognosis seems independent of the first disease. However, when the second neoplasm arises less than two years from the treatment of the first one, the prognosis is much worse.

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LOWER SERUM LEPTIN LEVELS ARE RELATED TO B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA OCCURRENCE

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Background and Aims. Obesity is now considered to be a risk factor for many types of cancer, including leukemia and lymphoma. Leptin, an adipocyte secreted hormone, has also been proposed to have a role in hematopoiesis and was not studied in depth in B-cell chronic lymphocytic leukemia (B-CLL). In this case-control study, we attempted to investigate the contribution of serum leptin levels to the etiopathogenesis of B-CLL, taking also into account potential confounders including the family history of lymphohematopoietic cancer (LHC) and the body mass index (BMI). We also attempted to ascertain whether a relationship between prognostic markers and levels of leptin exists among patients with diagnosed B-CLL. *Design and Methods*. Blood samples were collected from 95 cases with incident B-CLL, and 95 hospital controls, admitted for non-neoplastic and non-infectious conditions, matched on gender, age and year/month of diagnosis (±1 month) between 2001 and 2007. Serum leptin concentrations were determined using ELISA (Diagnostic System Laboratories Inc., Webster, Texas). Furthermore, serum lactate dehydrogenase (LDH), β2-microglobulin (BMG), lymphocyte morphology and the surface expression of CD38 in >30% of B-CLL lymphocytes were assessed. Statistical analysis of the data was performed with SAS 9.1 for Windows XP. *Results*. Patients in the case group on average had a higher body mass index as compared with controls (27.8 vs. 26.7 kg/m2; $p<0.01$). Significantly more cases than controls presented with a family history of LHC (13 vs. 3 controls; $p<0.01$). Serum leptin was significantly lower in cases as compared with controls (10.03 vs. 13.89 ng/ml; $p=0.004$). Among controls, BMI was positively correlated with leptin levels ($p=0.04$). Lower leptin levels were associated with B-CLL risk in unadjusted analyses as well as after controlling for age, gender, date of diagnosis, family history of LHC and BMI (OR: 0.94, 95% CI 0.90-0.97; $p=0.01$). No significantly different serum leptin levels were found amid different stages of B-CLL according to the Binet classification. However, a weak but significant positive correlation between LDH and leptin was found (r=0.22, $p<0.05$), and between leptin and β-2-microglobulin (r=0.27, $p<0.01$). *Conclusions*. Leptin was found to be lower among cases as compared to controls, despite cases having a higher BMI. These results will need to be confirmed in an even larger study population, but if reproduced may suggest that leptin becomes dysregulated, or that other factors such as inflammatory cytokines secreted by lymphocytes suppress leptin expression at later stages of the disease process.

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HEPATITIS C VIRUS-POSITIVE CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The association of hepatitis C virus (HCV) and B-cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) is not well established. Some epidemiological studies tend to rule out such a relationship. Again, little is known about the clinic-biological features and outcome of HCV-positive patients with CLL. Aims. To evaluate clinico-biological features and outcome of HCV infected patients with CLL at diagnosis compared to HCV negative CLL patients. *Design and Methods*. We retrospectively evaluated clinico-hematological characteristics of 23 HCV-positive CLL patients compared with 189 HCV-negative CLL patients seen at our Institutions. *Results*. No differences were found with respect to sex distribution and age. The HCV genotype was known only in 4 patients: in 3 cases was found 2a/2c, in one case 1b. No difference was also found in the absolute lymphocyte count, hemoglobin level, platelet count, Rai and Binet clinical stage at diagnosis, lymphocyte doubling time. Mutational status of IgVH, CD38 expression, and ZAP-70 expression did not show differences among the two groups of patients. The major cytogenetic abnormalities, detected by means of FISH in 13 HCV-positive patients, showed 7 cases of normal karyotype,3 cases of trisomy 12, 2 cases of deletion 13q14, 1 case of deletion of 11q22.3 (ATM). Finally, overall survivals of HCV infected patients and HCV-negative patients did not show any significant difference ($p=0.1$). *Conclusions*. In conclusion, HCV-positive patients with B-cell CLL seem to not differ from other patients for presentation and clinical outcome as well. However, preliminary results need to be confirmed on a large cohort of patients.

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EFFICACY OF ALEMTUZUMAB TREATMENT ON REFRACTORY T-CELL LARGE GRANULAR LYMPHOCYTIC LEUKEMIA

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Background. Lymphoproliferative disorders of large granular lymphocytes are rare (<3% of all cases of small lymphocytic leukemias) and generally indolent, so that approximately half of patients may not need therapy. When needed, standard treatment includes immonusuppresive and/or chemotherapeutic agents. However, none of these have proven universally effective in achieving durable disease control, although data are limited by patient numbers. *Aims/Design and Methods*. We describe the disease course of a 76 year-old male patient with refractory T-cell large granular CD52 + lymphocytic leukaemia, who was totally dependent on blood transfusions after failing standard treatment (continuous cyclosporin A [CSA] and low-dose oral methotrexate [MTX]), and achieved long- term (30 months) disease control with alemtuzumab. Alemtuzumab was administered first intravenously (day 1, 3 mg; day 2, 10 mg, day 3: 30 mg, then 30 mg IV thrice weekly for 6 weeks, once weekly for 3 weeks), and then subcutaneously (30 mg every third week for 16 months, and 30 mg monthly thereafter, for 21 months). *Results*. The patient became independent on blood transfusions and is still in remission, 30 months after the initial dose, of which 9 months after treatment withdrawal. No adverse effects were observed. *Conclusions*. Although MTX and CSA are effective as first-line therapy of T-cell large granular lymphocytic leukemia, alemtuzumab probably deserves further investigation, particularly for refractory disease.

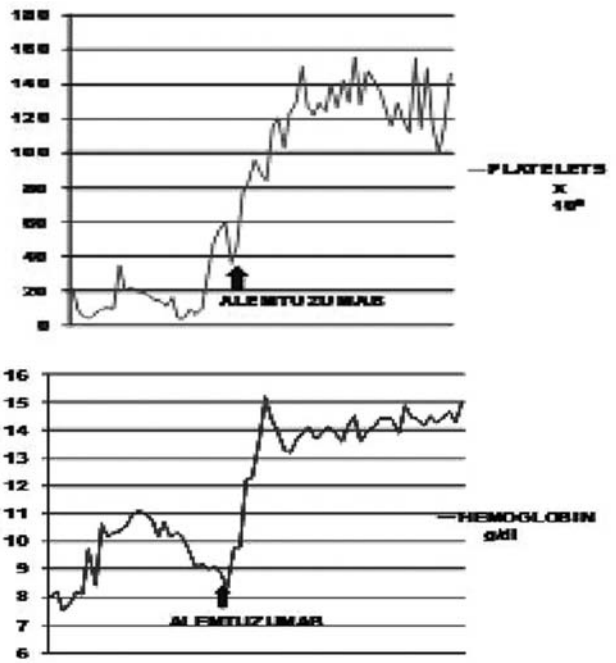

Figure.

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CHANGE IN EXPRESSION PATTERN OF TCR-CD3 COMPLEX IN PATIENTS WITH MULTIPLE MYELOMA

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Background. In hematological malignancy, cell-mediated immunity has been shown to be suppressed with advanced disease. This immune dysfunction may be due to decreased output of recent thymic emigrants, the abnormal expression of T cell receptor repertoire and, may in part, due to altered expression of TCR-CD3 complex. *Aims*. The distribution and clonality of TCR Vβ repertoire, the expression level of signaling transduction factor CD3 ζ, γ and ε genes in T cells from patients with multiple myeloma (MM) were investigated. *Design and Methods*. Specific family primers (24 VΔ subfamilies), RT-PCR and genescan technique were use to analyze the expression of TCR Vβ subfamily and the clonality of TCR VΔ T cells in 7 patients. Real-time RT-PCR with SYBR Green technique was used for detecting the expression level of CD3 ζ, γ and ε genes in peripheral blood mononuclear cells (PBMCs) from 22 patients with MM, and 17 cases healthy individuals served as control. The β2-microglobulin gene (β2M) was used as an endogenous reference. Relative mRNA expression level of genes was analyzed by using the 2Δ-Ct-100% method. *Results.* The pattern of TCR Vβ repertoire which is full expressed in healthy controls, was changed in patients. Only 5/24 Vβ subfamilies were detected in 2 MM cases, 6, 16,17, 20 and 22 Vβ subfamilies were found in 1 case respectively. The most frequent expressed Vβ subfamilies were Vβ1, Vβ9 and Vβ13 (7/7, 100%), and Vβ16 (6/7, 85.7%). Clonal expanded T cells could be identified in some subfamilies in all patient samples, while all of 24 Vβ subfamilies displayed polyclonality in healthy controls. The Vβ21 subfamily was expanded most frequently in patient samples. It could be identified in all five cases with Vγ21 expression. Vγ13 oligoclonal expansion was identified in 5/7 cases. The CD3 ζ and ε gene was expressed in all patients with MM, while CD3 γ was not detected in two cases. Compared with healthy controls, the relative mRNA expression level of CD3 γ in MM samples was significant decreased (p=0.001) and the expression of CD3 ε gene was significant increased (p<0.0001). Although the expression level of CD3 ζ is 2.23 times higher than that from healthy individuals, no significance was found compared to controls (p=0.059). We further compared the expression pattern of the three CD3 genes. There are no significant differences in the expression levels in healthy individuals. However, significant changes of ε>ζ>γ gene expression were observed in MM patient samples (p<0.0001). *Conclusions*. Skewing expression and dominant utilization of TCR Vβ repertoire may indicate T cell immunodeficiency, however frequent oligoclonal expanded TCR Vβ subfamily T cells may be also consequence of a host specific immune response to MM associated antigens. On the other hand, the altered expression of CD3 may be associated with the immune dysfunction. We here present precise data concerning changes in the variability of Vβ patterns and expression of TCR signal transduction molecules in samples from multiple myeloma patients compared to controls. This study will contribute to better understand the cellular immune features in MM patients.

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EPIGALLOCATECHIN-3-GALLATE FROM GREEN TEA INHIBITS MYELOMA CELL GROWTH, BUT ANTAGONIZES THE EFFECTS OF BORTEZOMIB

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Background. The tumorsuppressive, antiinflammatory, and antioxidant activity of the green tea polyphenol epigallocatechin-3-gallate (EGCG) is currently being investigated in a number of clinical studies including solid tumors and chronic lymphocytic leukemia. In multiple myeloma (MM), EGCG induces cell growth arrest and apoptotic cell death via binding to the 67-kDa laminin receptor-1 (Shammas et al., Blood 108, 2006). The molecular mechanisms on how EGCG exerts its effects, however, are manifold, and not fully understood. Aims: The aim of the study was to investigate the effects of EGCG on malignant plasma cells, alone and in combination with other drugs, and to delineate molecular mechanisms of action. *Design and Methods*. Six human myeloma cell lines including the IL-6 dependent INA-6 cell line were assessed for their sensitivity to EGCG in a colorimetric (MTS) based assay and by trypan blue exclusion. For primary patient tumor cells, DNA synthesis was measured by [^{3}H]-thymidine uptake. Induction of apoptosis was determined by flow cytometry upon annexin-V/7-AAD staining. Western blot analysis was performed to detect EGCG effects on intracellular signaling pathways using phospho-specific STAT3 and ERK1/2 antibodies. *Results*. EGCG inhibited the *in vitro* growth of myeloma cell lines as well as patient tumor cells in a dose-dependent manner at IC50 concentrations between 12.5 μM and 50 μM. Remarkably, concentrations as low as 1 μM were sufficient to significantly reduce INA-6 cell numbers in long-term cultures. Bone marrow stromal cells, IL-6, or overexpression of Mcl-1 and Bcl-xL did not protect from EGCG induced cytotoxicity. In INA-6 cells, EGCG dose-dependently inhibited IL-6 induced STAT3 phosphorylation, concomitant with induction of apoptosis. Interestingly, catalase, which blocks the formation of reactive

oxygen species (ROS) was not active in INA-6 cells, but abrogated the growth-inhibiting effect of EGCG in other myeloma cell lines. The combination of EGCG with doxorubicin, dexamethason, or rapamycin did not yield synergistic or additive effects. Surprisingly, growth inhibition by bortezomib was antagonized by EGCG at concentrations as low as 1 μM. This was observed in myeloma cell lines as well as in patient cells. *Conclusions*. EGCG inhibits malignant plasma cell growth by formation of ROS and abrogation of intracellular signalling pathways. Importantly, serum levels of pharmacologically active concentrations of EGCG are achievable. Using EGCG as dietary supplement, the antagonizing effect on bortezomib activity has to be considered, and may lead to choosing another therapeutic strategy. This work provides the rationale for additional studies to evaluate the optimal use of EGCG in plasma cell tumors.

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EFFECT OF COMMON ABCB1 SNPS ON OVERALL SURVIVAL IN PLASMA CELL MYELOMA

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Background. Multi-drug resistance (MDR) is a significant factor in the successful management of malignancy. One major source of this resistance is the P-glycoprotein (P-gp) drug efflux pump. Three single nucleotide polymorphisms (SNPs) which form a common haplotype have been identified in ABCB1 which, alone or in combination, may alter activity of P-gp, resulting in alterations to the MDR phenotype. We have previously reported on a single SNP rs1045642, within a plasma cell myeloma (PCM) patient cohort,1 which suggested an overall survival (OS) advantage in patients heterozygous for rs1045642 (CT) when treated with P-gp substrates. However this data was not in concordance with previous findings in Italian2 and Dutch3 studies. Further work is therefore required to determine the impact of ABCB1 SNPs on the MDR phenotype. It remains unresolved whether rs1045642, or other SNPs in the region, are providing the primary effect on OS within PCM. *Aims*. This study was designed to elucidate the impact of ABCB1 SNPs, rs1128503 and rs2032582, on the OS of PCM patients, in with the context of our previous results on rs1045642. *Design and Methods*. Patients with a diagnosis of plasma cell dyscrasia (PCD) were studied [n=92]. SNP genotyping was performed using real time PCR and melting curve analysis with fluorescence resonance energy transfer (FRET) probes and primers designed specifically for each SNP. Treatment and OS data was available on the majority of PCM patients investigated. Survival analysis was performed on each SNP with Cox proportional hazards regression analysis using R. *Results*. Previously reported results from our group have demonstrated increased OS for rs1045642 heterozygotes (CT) (p=8.4x10^{-3}, HR=0.46 (0.24-0.85)).1 In the present study, rs2032582 was nominally associated with OS (p=5.3x10^{-2}), the TT group having a poorer OS. rs1128503 was not significantly associated with OS (p=0.2). *Conclusions*. Our results suggest that rs2032582 TT genotype results in a poorer outcome in PCM patients. This is in concordance with our earlier work which also suggests that rs1045642 TT genotype was associated with a poorer response in PCM. We hypothesise that beneficial therapeutic effect of increased drug retention within tumour cells in the presence of the TT genotype is negated by increased toxicity within normal tissues. In our study rs1128503 was not associated with OS. This may be a consequence of low linkage disequilibrium (LD) between this SNP and the others in our study population or it may suggest that it is rs2032582 and rs1045642 within the common haplotype that generate the most significant effect. Further work will examine the impact of haplotypes that include these SNPs on OS, in a larger patient cohort.

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SERUM ENDOGLIN (CD105) LEVELS IN MULTIPLE MYELOMA PATIENTS IS A MARKER OF BONE MARROW ANGIOGENESIS AND DISEASE ACTIVITY

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Background. Plasma cell growth in multiple myeloma (MM) has been shown to be dependent on endothelial cell proliferation and angiogenesis in the bone marrow (BM) microenvironment. *in vitro* proliferation of endothelial cells in the presence of multiple myeloma BM mononuclear cells forms endothelial cell colonies which express endoglin (*Dominici et al. 2001*). Endoglin (CD105) is a transmembrane glycoprotein expressed on activated vascular endothelial cells. Its vascular expression is limited to proliferating endothelial cells and has been found to be overexpressed in various solid tumours, with the level of expression correlating with clinical parameters such as decreased survival and the presence of metastases. Information regarding its role in MM is limited although vessel formation is a hallmark of myeloma disease. Aim The aim of the current study was to measure the serum levels of circulating endoglin in multiple myeloma patients to determine if it could be an accurate marker of the extent of angiogenesis and the severity of MM. *Design and Methods.* 46 multiple myeloma patients and 12 healthy controls were included in the study. These patients were staged according to the Durie Salmon staging system. Serum samples were obtained at initial diagnosis from all patients and from 18 patients who had reached plateau phase after standard chemotherapy. Bone marrow biopsies were obtained from all patients before initiation of treatment and were evaluated for the expression of proliferation marker Ki67 in the plasma cell compartment and microvessel density as determined by staining with an anti-CD31 antibody. Serum levels of endoglin and of the inflammatory marker IL-6 were determined by ELISA using commercially available kits. *Results*ì. Endoglin levels were significantly higher in the myeloma patient group in comparison to the control group (p<0.05 by Mann Whitney). Furthermore, endoglin showed a higher average±SD value with increasing stage of disease (p<0.05 by ANOVA,Student Newman Keuls) (9.0±3.5 ng/mL for stage I, 9.8±2.7 ng/mL for stage II and 13.9±5.8 ng/mL for stage III). Endoglin's levels in serum decreased significantly following effective chemotherapy reaching levels comparable to controls (pre versus posttreatment values were 13.2±6.1 ng/mL versus 7.9±2.7 ng/mL respectively). Endoglin showed a good correlation with the proliferation rate Ki67 of malignant plasma cells (r:0.43, p 0.005 Spearman's rho) and bone marrow microvessel density (r:0.33, p 0.05). It also showed a significant correlation with the levels of circulating IL-6 (r:0.39, p 0.01). *Conclusions*. It was interesting to find that endoglin circulates at levels which reflect bone marrow neovascularization as expressed by marrow MVD. Serum endoglin may be the product of increased expression in the bone marrow vasculature especially in relation to an inflammatory phenotype as it couples with increased levels of IL-6. Further studies under way will determine the relative expression of endoglin in bone marrow tissues. The study also supports serum endoglin as a surrogate marker of myeloma disease activity.

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PERIPHERAL LEVELS OF OSTEOPONTIN PARALLEL DISEASE PROGRESSION IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Neoplastic plasma cell proliferation in multiple myeloma has a profound effect on bone turnover resulting in characteristic osteolysis. Many indices of bone metabolism such as pyridinoline products have been studied in MM showing increased activity of osteoclasts. Osteopontin is an osteoclast regulator produced by malignant plasma cells also involved in angiogenesis, Studies regarding the relevance of peripheral levels of osteopontin with disease activity have yielded contradicting results (*Saeki et al, 2001 and Kang et al, 2007*). *Aims*. The aim of the current study was to determine if circulating osteopontin can be used as a marker of bone disease activity in myeloma patients. *Design and Methods.* 37 multiple myeloma patients and 10 healthy controls were included in the study. These patients were staged according to the Durie Salmon staging system and bone disease was graded according to skeletal involvement on x-ray (grade O: no bone involvement, 1: one lytic lesion, 2: more than one lytic lesion, 3: bone fracture or vertebral col-

lapse). Serum samples were obtained at initial diagnosis from all patients and from 20 patients who had reached plateau phase after standard chemotherapy. Bone marrow biopsies were obtained from all patients before initiation of treatment and were evaluated for the extent of angiogenesis by staining bone marrow vessels with an anti-CD31 antibody. Serum levels of osteopontin and the angiogenic molecule VEGF were determined by ELISA using commercially available kits. Results Osteopontin levels increased significantly with increasing disease stage (median±SD 18.5±9.5 ng/mL in stage I, 37.8±11.2 ng/mL for stage II and 71.1±51.0 ng/mL for stage III). Osteopontin also increased significantly with increasing grade of bone destruction with the highest value in the subgroup of patients with grade 3 bone disease(139±67ng/ml). The levels of osteopontin in the control group were 71±65ng/ml) which was not significantly different than the myeloma group as a whole. Osteopontin levels in serum decreased significantly following effective chemotherapy in comparison to pre-treatment values in the subgroup examined. Pretreatment values of osteopontin correlated with the the angiogenesis indices bone marrow MVD (r:0.28, p:0.05) and serum VEGF (r:0.44, p:0.005). *Conclusions*, Osteopontin is a sensitive marker of bone disease activity in multiple myeloma. Its relation with angiogenic markers indicates the contribution of developed vasculature in the enhanced bone destruction seen in myeloma disease.

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OLIGO ARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION SHOWS A DIFFERENCE IN GENOMIC PROFILES OF MULTIPLE MYELOMA PATIENTS WITH AND WITHOUT CYTOGENETIC NEGATIVE PROGNOSTIC FACTORS

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Background. Multiple myeloma (MM) is an incurable disease caused by malignant transformation of the B-cell, its clone proliferation and accumulation in a bone marrow. Generally MM is characterized by high clinical and genetic heterogeneity. There is a set of confirmed prognostic factors for new diagnosed MM, but not all of them seem to have a similar impact for relapsed patients treated by new drugs (bortezomib, thalidomide, lenalidomide). *Aims*. The clinical implementation of array comparative genomic hybridization (array-CGH) analyses allow us to do a whole-genome screening and sensitive identification of new genetic markers important for diagnosis, classification and prediction of prognosis of MM. The main aim of our work was molecular diagnostic of multiple myeloma focused on detailed genomic profiling of this disease using array-CGH. Material and methods We performed high-resolution (44K Agilent) array-CGH on 19 samples from 18 MM patients with various cytogenetic findings examined previously by FISH. 8 patients had confirmed hyperdiploidy, 7 non-hyperdiploidy, 7 had gain of 1q21 and 4 of them had translocation t(4;14). 8 of the samples were DNA from bone marrow, 11 were DNA obtained from MACS separated cells. 4 samples were uninformative because of low percentage of myeloma cells in whole bone marrow samples. 11 of informative cases were relapsed MM treated by new drugs, 4 of them were newly diagnosed MM. *Results*. We have found 52 various deletions and 26 gains in our samples. According to the findings from array-CGH we confirmed previous FISH results in all informative cases. Besides that, we found another aberrations present in four or more samples, as losses of 8p, 16q, 1p13, 6q25, and gain of chromosome 19. In two patients we observed biallelic deletion in cytoband 11q22 including some potentionaly important genes. We observed a clear difference in genome profile between hyperdiploid (H, N=8) and non-hyperdiploid (NH, N=7) group of MM patients: in hyperdiploid samples, mostly whole-arm gains were present, and occurred repeatedly (19 regions, 10 in 3 or more patients in H group vs. 6 regions, one in 3 patients NH). For non-hyperdiploid samples, high numbers of smaller and unique deletions were the typical aberrations (more than 30 in NH vs. 17 in H). In all cases with FISH confirmed gain of 1q21, gain of all 1q arm was observed. Comparison of gain 1q positive and negative group showed a difference in number of sub-chromosomal deletions (42 in gain 1q cases vs. 17 in non-gains), but not in whole-arm gains (15 in gain 1q group, 16 in non-gains). *Conclusions*. The results show a difference between genomic profiles in patients with and without cytogenetic negative prognostic factors examined by FISH (gain 1q, non-hyperdiploidy). The analysis of repeating genetic changes, as well as analysis of differences in array-CGH patterns might lead to finding valuable prognostic or predictive markers especially for relapsed patients treated by new drugs, or to a better classification of stage and risk, providing the basis for improved clinical management and tailored treatment of MM.

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TETRAC, A SMALL MOLECULE INTEGRIN LIGAND, COOPERATES WITH BORTEZOMIB AND ENHANCES CELLULAR RESPONSE IN MYELOMA CELLS: A NOVEL CHEMOSENSITIZING APPROACH

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Background. Multiple myeloma (MM) is a plasma cell (PC) neoplasia accounting for more than 10% of hematological malignancies. Proteasome inhibitors represent a novel class of anticancer agents that have potent anticancer activity in both preclinical and clinical settings. Bortezomib is the first proteasome inhibitor for cancer therapy and is currently an important drug for treatment of relapsed and refractory MM. Despite all treatment approaches, this disease remains challenging with increasing chemoresistance. For more than two decades, actions of thyroid hormone in a variety of cells have been described to be relevant to proliferation and angiogenesis. In the past few years, clinical studies suggested that the pharmacological induction of mild biochemical hypothyroidism significantly improves survival time and enhances response rates to chemotherapy and radiotherapy in patients with glioblastoma multiforme (GBM), one of the deadliest refractory forms of brain tumors. Recently it was reported that thyroid hormones (T4 and T3) affect proliferation and angiogenesis through αVβ3 integrin, a common cell surface receptor. Tetraiodothyroacetic acid (tetrac), an analog of T4, was shown to block the αVβ3 integrin receptor site, mimicking hypothyroidism in the cells. Interestingly, MM cells interact with the same αVβ3 integrin for their invasion, spreading, proliferation and protease secretion activities. *Aims*. [1] To block the αVβ3 integrin utilizing tetrac and examine the effects of tetrac treatment on myeloma cell survival and proliferation. [2] To test the additive/supra additive effects of tetrac on bortezomib treatment in multiple myeloma cell lines *Design and Methods*. Proliferation (WST-1), apoptosis/necrosis (Annexin/PI), cell cycle (PI), were measured following addition of tetrac (1-100 μM) alone or in combination with bortezomib (1-100 nM) in five different MM cell lines. Results: Positive preliminary results demonstrate that tetrac treatment significantly inhibited cell proliferation/survival and increased the number of apoptotic/necrotic cells. In addition, co-treatment with tetrac and several doses of bortezomib sensitizes cells response with a significant reduction in survival and increase in apoptosis and necrosis, in a synergistic/additive manner. *Summary*. As most MM patients still relapse, new drugs combinations are needed to overcome resistance. Our novel chemosensitizing approach using tetrac, may potentially demonstrate the importance of aVb3 blocking and may be a useful unique adjunct for MM therapy

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DETERMINING THE NORMAL PLASMA CELL PROPORTIONS IN PLASMA CELL DYSCRASIAS BY FLOW CYTOMETRIC IMMUNOPHENOTYPING AND HIGHER CD27 AND LOWER CD56 AND CD28 EXPRESSION IN KOREAN PLASMA CELL DYSCRASIAS

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Background. Immunophenotyping of plasma cells (PCs) have been reported to be useful. The evaluation of immunophenotypic markers in neoplastic PCs could be easily applicable using flow cytometric methods, which have become widely available in Asian patients. Studies on plasma cell dyscrasias (PCDs) have been limited due to the relatively low incidence. However, with a slowly increasing occurrence, immunophe-

notyping of PCs becomes more valuable in diagnosing PCDs, identifying prognostic markers, and markers for minimal residual disease. AIMS : The purpose of our study was to determine the immunophenotypic characteristics of PCs to distinguish between neoplastic and reactive PCs in order to diagnose PCDs and determine the proportion of normal PCs among total PCs (nPC/tPC) in cases with PCDs. In addition, we compared the immunophenotypic expression patterns with previous studies to evaluate whether ethnic variances may exist. *Design and Methods*. A total of 52 patients that showed bone marrow plasmacytosis were included in the study. Immunophenotypic evaluation by 4-color flow cytometry was done using a combination of CD38/CD138/-/CD45, CD56/CD20/CD138/CD19, and CD27/CD28/ CD138/CD117. The CD38/CD138/-/CD45 panel was used to differentiate between reactive and neoplastic PCs. Of the 52 patients, 19 had a PCD including multiple myeloma (MM, n=16), plasma cell leukemia (n=1), smoldering MM (n=1), and monoclonal gammopathy of undetermined significance (MGUS, n=1). A reactive plasmacytosis (range, 1.5% to 31.4% of total nucleated cells) was observed in 33 patients with or without other underlying diseases such as lymphoma, leukemia, or myelodysplastic syndrome. *Results*. Neoplastic and reactive PCs were discriminated by determining the CD45 expression in CD138+ gated populations except one MM case which showed CD45+ expression. The CD138+ gated cells showed CD45+ expression in all patients with reactive plasmacytosis (n=33) whereas patients with PCDs (n=19) had a distinct CD45- population except the previously mentioned case. In PCD patients, the median percentage of normal PCs among all PCs (CD138+ cells) was 28.2% (range, 1.2 to 97.9%). Eleven patients with newly diagnosed MM had a median nPC/tPC was 19.8% (range, 1.9 to 94.0%) whereas 4 follow-up MM patients had a median nPC/tPC value of 65.2% (range, 13.6 to 91.0%). A nPC/tPC value of 97.9% was noted in the patient diagnosed with MGUS. The reactive PCs were positive for CD27 but lacked CD28 expression in all 33 patients. All but one of the patients showed CD27 expression (94.7%) whereas CD56 and CD28 expression was demonstrated in 6 patients (31.6%) and 1 patient (5.3%), respectively. *Conclusions*. We were able to differentiate neoplastic PCs with reactive PCs in most cases of PCDs. In cases of PCDs, a proportion of normal PCs were identified, which might correlate with therapeutic response and prognosis. The immunophenotyping results showed discrepancies with previous studies, such as higher CD27 expression and lower CD56 & CD28 expression. Larger studies in Asian populations is warranted to define the characteristics of neoplastic PCs that might somewhat differ from previous studies based on non-Asian populations.

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MULTIPLE MYELOMA CELLS UNDERGO DIFFERENTIATION UPON EXPOSURE TO ROSIGLITAZONE AND ALL-TRANS RETINOIC ACID

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Background. Activation of PPARg by its ligands has shown differentiating effects in solid tumors. However, few reports addressed its role in myeloma cells. Aims In this study, we investigate the effects of rosiglitazone (RGZ) as well as combined with all trans-retinoic acid (ATRA) on human myeloma cell lines and primary CD138$^+$ myeloma cells. *Design and Methods. U266*, RPMI-8226 cells and primary myeloma cells purified with CD138 immunomagenetic beads were treated with different concentration of RGZ in the presence or absence of ATRA and various biological responses were studied by the methods of [3H] thymidine incorporation, cell cycle analysis, Annexin V-PI staining, Wright-Giemsa staining and measurement of light chain protein in the culture supernatants. *Results*. Our study demonstrated that exposure to PPARg ligand (rosiglitazone, RGZ) induced proliferation inhibition and cell cycle arrest in myeloma cells. A combination of RGZ with all-trans retinoic acid (ATRA) can enhance the growth inhibition effects of RGZ. Further study shows that RGZ treated myeloma cells displayed morphological characteristics of cell differentiation, and more evident signs of differentiation were observed when RGZ was combined with ATRA. These changes were confirmed by the detection of CD49e expression and light chain protein secretion. Similar results were also observed when primary CD138$^+$ cells were treated with RGZ and ATRA.*Conclusions*. We showed that RGZ can induce growth inhibition and cell cycle arrest in multiple myeloma cells, which may be caused by induction of cell differentiation. Concomitant RXRa activation by ATRA can enhance the effects of RGZ. Therefore, RGZ may have the potential to be used as a differentiation inductor for clinical patients and ATRA may be useful as a combination therapy for myeloma. Further studies are needed to be performed to elucidate the mechanisms of the myeloma cell differentiation induced by RGZ and ATRA.

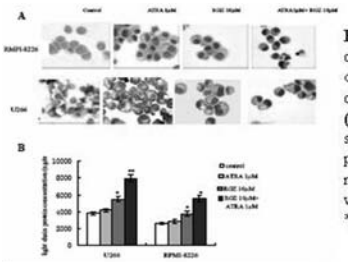

Figure 1 (A) Marked morphological changes of cell differentiation were observed in RGZ, RGZ+ATRA treated cells. Photographs are 400 magnification. **(B)** Exposure to 10 μM RGZ caused a significant increase of light chain proteins secretion in both cell lines. The most obvious change was observed when RGZ was combined with ATRA. $*p < 0.05$, $**p < 0.01$.

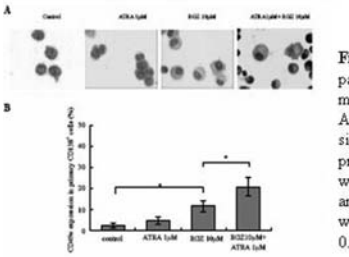

Figure 2 (A) Primary cells from myeloma patients also underwent morphological maturation after 48h exposure to RGZ and ATRA (400×). **(B)** ATRA caused no significant increase of CD49e expression in primary cells, while the CD49e expression was obviously up-regulated in RGZ group and the up-regulation was more significant when RGZ was combined with ATRA. $*p < 0.05$.

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EXPRESSION OF PRAME, WT1 AND XIAP GENES IN MULTIPLE MYELOMA PATIENTS DURING HIGH DOSE CHEMOTHERAPY AND THERAPY OF PROTEOSOME INHIBITORS

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Background. High dose chemotherapy and comprehensive drugs enable further improvement of the outcome of multiple myeloma (MM) patients. However, it is still necessary to determine prognostic factors that may influence treatment results and provide additional criteria for the precise selection of treatment approaches. A number of tumor associated genes is over-expressed in different malignancies including MM. These genes are believed to modulate cancer properties and should be taken into account during treatment. Their significance as prognostic factors require further investigation. *Aim.* To analyze the expression levels of PRAME, WT1 and XIAP genes in MM patients at diagnosis, during high dose chemotherapy and therapy of proteosome inhibitors, and to compare these data with clinical outcome. *Design and Methods.* Our study included 34 primary MM patients; all of them gave informed consent. The median age was 46 years (range 31-62). IgG MM was diagnosed in 28 cases, IgA MM in 2 and Light Chain MM in 4. 20 patients received initial treatment consisted of 3 cycles of VAD. 10 of them was given bortezomib 1.3 mg/m2 on days 1,4,8 and 11, and dexamethasone (dex) 40 mg daily on days 1-4 days (6-8 cycles) as the 2nd line therapy. In the other 14 primary MM patients VAD treatment was just started at the moment of our study and has not fulfilled yet. RQ PCR investigation of PRAME, WT1 and XIAP expression levels was performed before therapy (n=34), after VAD (n=20), after bortezomib (n=10). Results were normalized against expression of ABL gene which was used as internal control. *Results.* In primary MM patients: PRAME gene expression was found in 62% (n=21), WT1 in 20% (n=7) of patients, all of whom were exclusively PRAME-positive. In primary MM patients the XIAP gene expression was found in 100%. In the control group of healthy donors (n=8) PRAME gene expression was found in 50% (in 4 from 8), WT1 in 12% (in 1 from 8), XIAP in 62% (in 5 from 8). Median PRAME expression level in the group of MM patients was 0.3% and it dramatically exceeds not less than by 100 times the median PRAME expression in the group of healthy donors (0.003%). The level of PRAME expression in the MM group of patients was ranged from 0.001 to 132%, whereas in the donor's group it was never more than 2.5%. The PRAME hyperexpression (>2.5%+3SD) was found in 4 patients (11.7%). Median WT1 expression level was 0.01% in the case of MM patients and it was about 6 times higher than the median WT1 expression in the group of healthy donors (0.0017%). The range of WT1 expression in the group of MM patients was from 0.002 to 2.5%. Median XIAP expression level in the MM patient's group was more than 10 times as intensive if compare it with the case of healthy donors (28% vs. 2%). The minimal level of XIAP expression in MM patients was comparable with healthy donors,

but maximal level of its expression in MM patients reached 5382%, whilst in the donor's group it did not exceed 28%. The XIAP hyperexpression (> than healthy donor median 28%+3SD) was found in 27 patients (79%). It was found that PRAME, WT1 and XIAP expression did not correlate with tumor bulk and was independent of the levels of M-protein, β-2M and albumin. In the patients with high primary PRAME expression (>2.5%+3SD) the frequency of CR+PR was significantly lower than in PRAME-negative primary patients and in patients with low (<2.5%+3SD) primary PRAME expression (25% vs 75%) (p=0.06). Moreover, the higher was PRAME expression at the start of treatment, the less efficient and durable was response on the VAD treatment. The level of the XIAP gene expression significantly decreased during bortezomib treatment, in good responders nearly reaching the normal values according to healthy donor control. In the MM patients who achieved CR + PR after 4-6 courses of bortesomib + dex (n=6), expression of XIAP reduced from 11-325% (median 66%) to 1-123% (median 20%). On the contrary, in the MM patients with poor response after 4-6 courses of bortesomib + dex (n=4), we observed increased expression of XIAP from 16-127% (median 36%) to 22-528% (median 121%). The expression of PRAME significantly decreased after 3 VAD cycles to 0.001-20,7% (n=10), at the moment of auto-SCT it was 0.001 -6.1% (n=6) and after auto-SCT it was 0.004-4.9% (n=9). However, in the case of WT1, we observed that expression increased after 3 VAD cycles to 0.004-0.07% (n=5) and at the moment of auto-SCT it was 0.001-0.4% (n=6) and after auto-SCT - 0.0193-2.03% (n=7). It is worth mentioned that during the treatment in a small number of initially negative PRAME and WT1 gene patients, we demonstrated that these genes became activated. Thus, during the therapy we detected de novo activation of these genes expression up to low levels in 1 of 13 initially negative PRAME (6.1%) and in 8 of 26 initially negative WT1 (range of level 0.01-1.03%). Interestingly, the detection of de novo PRAME and WT1 gene expression did not correlate with disease status, which may be explained that period of observation was rather short. All these patients achieved still maintained CR+ VGPR. In one of the secondary positive patients (who acquired reappearance of PRAME and WT1) relapse occurred, with highly elevated expression of PRAME (104%), WT1 (0,62%) and XIAP (1264%). *Conclusions*. Expression of PRAME gene was found in 62% primary patients and the level of PRAME decreased according to tumor reduction which renders this gene to be a useful molecular marker of treatment efficiency. High expression level of PRAME turned out to be a factor of unfavorable prognosis of MM. Expression of WT1 was found in 20% of MM patients, all of whom were PRAME-positive. WT1 expression increased during treatment in a small group of pts. Some initially negative pts acquired PRAME and WT1 expression during treatment, but clinical relevance of it is not clear so far. In MM patients at diagnosis, the level of the XIAP expression is higher than in the normal control group. The decrease of the XIAP expression correlates with the chemotherapy and especially with the proteasome inhibitor treatment efficiency. XIAP expression comes to the normal values at the time of CR and PR achievement.

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CHANGES IN ACTIVATORY AND INHIBITORY NK RECEPTORS ASSOCIATED TO THE PROGRESSION OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE TO MULTIPLE MYELOMA: IMPLICATIONS FOR TUMOR EVASION OF T AND NK CELLS

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Background. Monoclonal gammopathy of undetermined significance (MGUS) is characterized by the presence of a monoclonal (M) protein in persons without evidence of plasma cell neoplasia. MGUS was considered to be benign, however a 1% of patients per year evolve to symptomatic monoclonal gammapathy constituying a premalignant state for transformation to Multiple Mieloma (MM). The molecular basis of MGUS progression to a malignant monoclonal gammopathy remains poorly understood. AIMS In this study, we examined the immunogenic differences of plasma cells from patients of non-gammopathies and plasma cells from MGUS and myeloma. We studied continuous changes in MHC class I expression, MICA and CD95 during the malignant transformation and progression of plasma cells. *Design and Methods*. We studied bone marrow (BM) of patients with diagnosis of MGUS (n=6) or MM (n=6) based on estandar criteria and no treatment were receiving at the day of the immunophenotype analysis. We also select by flow cytometry and cytology 5 BM donors. Samples were analyzed by the FACSCanto cytometer and the FACSDiva software (BD Biosciences) was used for data analysis. Were processed by direct staining using the following monoclonal antibodies (mAb): anti-CD38, anti-CD138, anti-CD95, anti-HLA-ABC and anti MICA-MICB directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC). Significance levels were determined by Student t test analysis. P values of 0.05 or less were considered significant. RESULTS We detected high levels of MHC class I expression in plasma cells from donors without monoclonal gammopathy, that are increased in MGUS and the highest levels of expression were detected in MM plasma cells. Malignant transformation in MM is associated to upregulation of MHC class I surface expression in plasmatic cells. While the activating NKG2D ligand MICA expression was the opposite: lower in MM, intermediate levels in MGUS and the highest expression in donor cells. The differences found were significant between plasma cells from donor and MGUS and between MGUS and MM cells .The expression of CD95 in MM varied considerably and appear associated with the induction of programmed cell death to anti-FAS mab. We also observed a significant reduction of expression of FAS in MM cells surface (p<0.001). Changes in these molecules expression could deeply affect the balance between activatory and inhibitory signals that regulates NK cells actitity. *Conclusions*. Our results show that the phenotype MHC class I bright, MICAdim/- , CD95 dim/- is clearly associated to progression of MGUS cells to myeloma. It is possible that, both innate and adaptative response maintains the tumor cells in an equilibrium state (MGUS) and evolve into MM only when the cells become edited promoting tumor escape from immunosurveillance. Additionally, our findings are also in agreement with previous reports that support the key role of NK cells in controlling myeloma progression.

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COMPARISON OF SERUM PROTEIN IMMUNOFIXATION ELECTROPHORESIS, SERUM FREE LIGHT CHAINS AND HEAVY/LIGHT CHAIN RATIOS FOR MONITORING IGA MULTIPLE MYELOMA PATIENTS

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Background. IgA multiple myeloma (MM) patients are difficult to monitor when the monoclonal band co-migrates with other proteins on serum protein electrophoresis (SPE) gels. While immunofixation (IFE) is more sensitive than SPE, it is not quantitative. If there are adequate concentrations of serum free light chains (FLC), they can be used to monitor responses to treatment but this leaves some pure IgA producing tumours that cannot be monitored properly. Recently, immunoassays that separately measure IgA-κ and IgA-λ molecules (targeting heavy chain/light chain conformational epitopes - HLC) have been described and IgA-κ/IgA-λ ratios can be derived in the same manner as FLCs. These assays can be used to characterise and monitor patients with IgA MM alongside conventional measurements. Aims To assess SPE, IFE, HLC and FLC ratios for analytical specificity and sensitivity when monitoring patients with IgA MM. *Design and Methods*. FLC, IgA-kappa and IgA-λ analyses were performed on a Siemens Dade-Behring BN'II nephelometer while SPE and IFE were performed on a SEBIA Hydrasys' electrophoresis system. 191 blood donor sera were used to produce normal ranges. 31 IgA MM patients (22 IgA-kappa and 9 IgA-lambda) were assessed through the course of their disease using between 3 and 45 (mean 14) time points per patient. Results By SPE and IFE, 12 patients achieved a partial response (PR), 18 patients achieved a complete response (CR) and 1 patient had no response recorded. 5 patients had no obvious paraprotein by SPE, while in a 5 further patients, the monoclonal band migrated with other proteins by SPE so accurate quantification was difficult. In all patients achieving a PR, FLC and HLC ratios remained abnormal. In 9 patients achieving a CR, FLC and/or HLC ratios were more sensitive than SPE and/or IFE. In 4 patients, changing FLC ratios or HLC ratios were discordant, indicated the presence of more than one tumour clone. In 3 patients, FLC ratios became increasingly abnormal whilst HLC ratios and SPE remained unchanged or decreased, indicating "free light chain escape." In 1 patient, the first relapse was predominately with IgA molecules whilst the terminal relapse was with both IgA and FLC molecules. *Conclusions*. Monoclonal IgA proteins can be difficult to identify and measure using electrophoretic techniques because of their co-migration with other molecules. We now report novel immunoassays that separately measure IgA-κ and IgA-λ. When applied to the study of patients with IgA MM, these assays can be more sensitive than SPE and IFE. Using these HLC assays alongside FLC tests provided greater clarity when monitoring changing monoclonal protein expression than electrophoretic techniques.

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COMPARISON OF HEAVY CHAIN/LIGHT CHAIN RATIOS WITH IMMUNOFIXATION ELECTROPHORESIS FOR THE DIAGNOSIS OF MONOCLONAL GAMMOPATHIES

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Background. In screening for monoclonal gammopathies, immunofixation electrophoresis (IFE) is a reflex test for abnormal serum protein electrophoresis (SPE). However, identification of monoclonal proteins using IFE depends upon the observer. To provide better accuracy, immunoassays have been developed that separately measure immunoglobulin-κ (Ig-κ) and immunoglobulin-λ (Ig-λ) molecules based upon recognising specific epitopes between the heavy and light chains (HLC) of immunoglobulins. Ig-κ/Ig-λ ratios can be derived that can be of use in identifying intact immunoglobulin monoclonal proteins. Aims To compare IFE and HLC ratios for identifying patients with monoclonal gammopathies. *Design and Methods*. The immunoassays for HLCs were performed on a Siemens Dade-Behring BN'II analyser. IgA-κ/IgA-λ and IgG-κ/IgG-λ ratios were measured in 150 and 142 blood donor sera respectively to generate a normal range. The 95 percentiles were used for comparison with clinical samples. 98 patient presentation samples were studied. 90 with confirmed multiple myeloma (MM) (9 IgA-κ: 8 IgA-λ: 47 IgG-κ: 23 IgG-λ: 2 free κ: 1 free λ) and 8 with monoclonal gammopathies of undetermined significance (MGUS) (1 IgA-κ: 1IgA-λ: 6 IgG-κ). Monoclonal proteins were also assessed using IFE, SPE (Sebia hydrasysTM) and individual immunoglobulin measurements (Dade Behring) Results Median and 95%ile ranges for the normal sera were: IgG-κ 7.54 g/L (3.92-12.16), IgG-lambda 3.95 g/L (2.28-6.05), IgG-κ/IgG-λ 1.86 (1.13-3.27), IgA-κ 1.41g/L (0.54-2.42), IgA-λ 1.19 (0.50-2.16), IgA-κ/IgA-λ 1.17 (0.69-1.99). Summated IgG-κ + IgG-λ and IgA-κ + IgA-λ correlated well with total immunoglobulin measurements: r2=1.01 and r2=0.97 respectively (Passing Bablock correlation). Abnormal HLC ratios were identified in 17/17 IgA sera and 2/2 IgA MGUS sera. All patient sera with IgG MM, free light chain MM and IgG MGUS had normal IgA HLC ratios. Abnormal HLC ratios were identified in 69/70 IgG MM sera which had a positive IFE. There was a discordant result between HLC and IFE in one IgG MM sample that had a reported total IgG of 18g/L. This sample needs further investigation. 3/6 IgG MGUS samples were identified with abnormal HLC ratios. High levels of polyclonal IgG seen in MGUS and smouldering MM may obscure abnormal HLC ratios and bands in SPE but the clinical relevance is unknown. 15/17 IgA and all free light chain MM had normal IgG-κ/IgG-λ ratios. The two IgA MM sera with abnormal IgG-κ/IgG-λ ratios had less than 3g/L of total IgG. One sample had a ratio of 0.99 falling only marginally outside the normal range. The remaining sample had an abnormal IgG HLC ratio of 0.39 that may indicate a monoclonal protein not detected by IFE. *Conclusions*, 86/87 intact immunoglobulin MM sera and 5/8 MGUS sera had abnormal HLC ratios. The data indicates that HLC ratios could be used as a reflex test to abnormal SPE and the uncommon remaining unclassified sample could be tested by IFE. Such a testing algorithm would remove the subjective nature of IFE.

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THE BISPHOSPHONATE IBANDRONAT REDUCES EXPRESSION OF RECEPTOR ACTIVATOR OF NUCLEAR-KB LIGAND IN BONE MARROW STROMAL CELLS DERIVED FROM MULTIPLE MYELOMA PATIENTS

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Background. Much of the morbidity and mortality associated with multiple myeloma is attributed to the severe osteolytic bone disease seen in patients with this disease. The receptor activator of nuclear factor-κB ligand (RANKL) and interleukin-1β are osteoclast activating factors which are abnormally expressed in bone marrow stromal cells and plasma cells of myeloma patients, respectively. *Aims*. In this study we assessed the effects of the biphosphonate ibandronate on RANKL mRNA steady state levels and protein production in bone marrow stromal cells obtained from multiple myeloma patients. Furthermore, we also evaluated the effect of ibandronate on the activation of MAPK signalling pathway induced by IL-1β. *Design and Methods*. Bone marrow mononucleated cells from 10 newly diagnosed multiple myeloma patients were separated by Ficoll-Hypaque density gradient centrifugation. Adherent cells were cultured and expanded in α-MEM supplemented with 10% fetal calf serum at 37°C in 5% CO2 humidified atmosphere. Cells were cultured in the presence of 5 mM ibandronate and/or 5 ng/mL IL-1β during selected times. At the end of the culture period mRNA for RANKL and protein levels were assayed by RT-PCR and western blot, respectively. Human bone marrow stromal cell line HS-5 was used to analyze the effect of IL-1β and ibandronate on the MAPK signalling pathway. All of the patients were stage III, classified according to the Durie-Salmon classification. Bone marrow samples used in this study were obtained from patients who provided a written informed consent according to Institutional Review Board Guidelines. *Results*. Ibandronate reduces RANKL mRNA and protein produced by bone marrow stromal cells. IL-1β increases the expression of RANKL mRNA and protein; nevertheless, this increase is neutralized by the presence of Ibandronate in the culture medium. Furthermore, IL-1β also significantly increases the ratio of pERK1,2/ERK1,2 on HS-5 cell line; although in the presence of ibandronate the ratio is similar to that of the control. *Summary and Conclusions*. In bone marrow stromal cells from newly diagnosed multiple myeloma patients, ibandronate may abrogate RANKL expression induced by interleukin-1β acting as an inhibitor of extracellular signal-regulated kinase.

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EFFECTS OF BONE MARROW FIBROBLASTOID STROMAL CELL ON THE PROLIFERATION AND SENSITIVITY TO CHEMOTHERAPY OF MULTIPLE MYELOMA CELL RPMI8226

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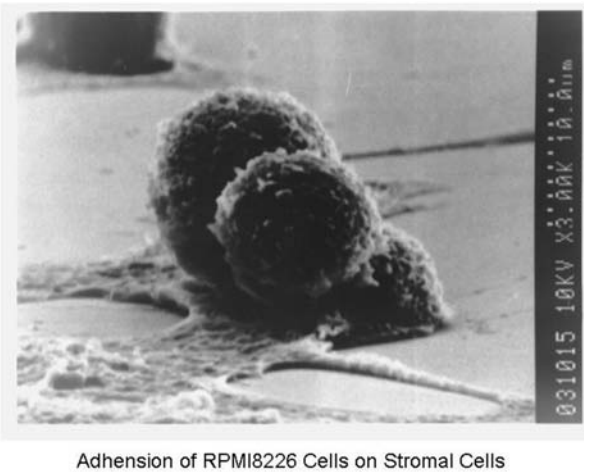
Background. Myeloma cells predominantly localize in bone marrow and rarely occur in peripheral blood and invade other organ, which is closely associated with the bone marrow microenvironment. More attention is paid to the effect of bone marrow stromal cells (BMSCs) on myeloma cells. We established co-culture system of normal human bone marrow fibroblastoid stromal cell lines HFCL cells with multiple myeloma cell lines RPMI 8226 cells (non-IL-6 dependent) and investigated the effects of HFCL cells on the proliferation and response to chemotherapeutic agents of RPMI8226 cells in the co-culture. *Aims*. To investigate the effects of HFCL cells on the proliferation and sensitivity to chemotherapy of RPMI8226 cells in the co-culture. *Design and Methods*. Setting up co-culture system of RPMI8226 cells with HFCL cells, and dividing into direct and indirect co-culture (transwell group). The adhesion ratio was determined by MTT colorimetric assay; the mitotic index (MI) of RPMI8226 cells was observed by Wright-Giemsa staining. Flow cytometer and Western Blot were used to study the changes of cell cycle and expression of proliferating cell nuclear antigen (PCNA), respectively. In cytotoxicity assays, cell viability was determined by MTT.



Adhension of RPMI8226 Cells on Stromal Cells

Figure 1. An electron microscope image.

Results. In co-culture experiment, the adhesion ratio of RPMI8226 after 1 hour was 29.4%. Growth curves show the proliferation of RPMI8226 cell in direct contact with HFCL cell was inhibited as compared with control, no obvious changes were observed in RPMI8226 cell separated by transwell inserts. After 72h co-culture, the percentage of G1, S phase of RPMI8226 cells in suspension was 33.6% , 52.1%(control group); the percentage of G1 phase of RPMI8226 cells in direct contact culture was

higher than that in transwell group (40.4% vs. 30.6%), and the percentage of S phase of RPMI8226 cells in direct contact culture was lower(40.8% vs. 49.1%) $p<0.01$. Moreover, the MI of RPMI8226 cell in transwell group was higher than that of direct contact group (1.4%±3.4%±3%±1.8%±1.6% *vs.* 1.2%±2.6%±2%±0.8%±0.4%) when cells co-cultured for 1 to 5 days. The expression of PCNA in RPMI8226 cell in transwell group was higher than direct contact group after 72h in co-culture. The dose-effect curves showed that HFCL cells diminished RPMI8226 cells death when exposure to mitoxantrone, vincristine, doxorubicin, topotecan after 72h co-culture in direct contact group. *Conclusion*. The HFCL cells could inhibit the proliferation and progression of cell cycle of RPMI8226 cells. Moreover HFCL cells could diminish the effects of chemotherapeutic agents on RPMI8226 cells in cell-cell contact co-culture.There might be cell adhesion mediated drug resistance beteen stromal cell and myeloma cell.

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FLOW CYTOMETRIC MINIMAL RESIDUAL DISEASE MONITORING IN MULTIPLE MYELOMA AFTER BONE MARROW ALLOGENIC TRANSPLANTATION

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Background: Multiple myeloma is caracterised by an increased number of clonal plasma cells in bone marrow witch produce a monoclonal immunoglobulin. Intensive treatment of patients and stem cell infusion indication results in more complete remission, even free survival and overall survival than treatment with conventional chemotherapy.*Aims*. Flow cytometry (FCM) is applicated in order to investigate whether the number of residual tumour cells in multiple myeloma after bone marrow allogenic transplantation (BMT) can predict the duration of response. *Design and Methods.* FCM investigation was used to mesure tumour load in bone marrow at six months intervals post bone marrow graft in eleven patients. 40 bone marrow samples were analysed. 4 *Results*. FCM can detect malignant plasma cells in 36 samples from 40. The number of plasma cells range from 0.01 10-2 to 7 10-2. The results of minimal residual disease(MRD) after a median follow up of five years post BMT are presented in this table Course of MRD announce clinical relapse or stability of tumour load. *Conclusions*. FCM is a reproducible and simple method of detection of MRD in myeloma, both course of MRD and quantification of malignant palsma cells are important to predict prognosis.

Table.

Statut of disease	Complete remission	Partiel remission	Relapse
Number of patients	7	1	3
MRD	$< 0.5\ 10^{-2}$	$2<MRD<5\ 10^{-2}$	$>5\ 10^{-2}$

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DENDRITIC CELL COUNTS AND THEIR SUBSETS IN MULTIPLE MYELOMA PATIENTS BEFORE AND DURING TREATMENT

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Background. Dendritic cells (DCs) are antigen-presenting cells that are pivotal for the initiation of the primary immune response by stimulating naïve resting T cells. After loading with relevant antigen, anti-tumor immunity can be induced in patients with multiple myeloma (MM). Thus, immunotherapeutic applications could be based on DCs attributes. Recent advances allow accurate quantification of peripheral blood myeloid and plasmacytoid DC populations (mDC and pDC, respectively). *Aims*. The quantitative determination of mDC and pDC subsets of DCs in peripheral blood of patients with multiple myeloma, before and after treatment using the technique of 3-colour fluorescence flow cytometry. *Design and Methods*. In this study we analysed the proportion of mDC and pDC subsets of DCs in peripheral blood of 17 patients with MM (5 newly diagnosed and 12 under treatment patients) compared to 9 healthy volunteers. Flowcytometric determination of relative and absolute cell counts in peripheral blood was based on the expression of surface antigens CD14+16 and CD85k (Beckman Coulter Monoclonal Antibodies). Depending on the expression of CD33 or CD123, we divided these cells into CD33+ dendritic cells (mDC) and CD123+ DC (pDC). The samples were analysed by BD FACS Calibur cytometer. *Results*. Flowcytometric analysis was performed by Cell Quest software and showed a sufficient reduction in both subsets compared to the healthy humans' average counts and a greater reduction in pDC subset. *Conclusions.* Although the study population was small to detect precise conclusions, we did observe a tendency of reduction in peripheral blood DCs of MM patients. Interestingly, the proportion of both DC subsets is remarkably reduced in under treatment patients compared to new diagnosed patients.

Table 1. Proportion of dendritic cells in peripheral blood.

	% mDC	CV (mDC)	% pDC	CV (pDC)
MM patients	0,254	0,130	0,160	0,124
Healthy Controls	0,481	0,199	0,548	0,224

1584

A NEW METHOD FOR DIFFERENTIATION BETWEEN AMYLOIDOGENIC AND NON-AMYLOIDOGENIC HUMAN IMMUNOGLOBULIN LIGHT CHAINS USING RIGID FIBRILLAR NANOSTRUCTURES AS MARKERS

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A number of devastating human diseases, including the amyloidoses, originate from the aggregation of structurally unstable proteins. Progressive renal dysfunction caused by the deposition of partially unfolded monoclonal proteins is, apart from infection, the commonest cause of death in patients suffering from multiple myeloma. To obtain insight into the pathogenesis of amyloid and non-amyloid renal precipitate formation we prepared a new rapid method of assessing the aggregation processes *in vitro*. The solution of human immunoglobulin light chains obtained from urine of patients with multiple myeloma was added to Congo red dye which was maintained in an electric field for the arrangement of its rod-like supramolecular nanostructures. The scaffolding efficacy was created due to higher rigidity of used dye nanoparticles which resulted from stronger stacking of molecules at low pH because of decreased charge repulsion. In this environment many of the monoclonal proteins were deposited during complex formation between the assembled molecules. This precipitation was observed by light microscopy with polarization equipment and electron microscopy. The properties of the obtained aggregates differ between light chains. Among amorphous granular material some light chains were able to form fibrillar structures with typical amyloid properties. This rapid precipitation may also suggest that the process of amyloid formation, under these conditions, may bypass the nucleation step. Our study supports this new rapid and easy diagnostic technique, which allows control and formation of immunoglobulin light chain aggregates *in vitro*. Thus the amyloidogenic tendency of Ig monoclonal light chains in urine could easily be tested.

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CYTOGENETIC ABNORMALITIES IN 40 SLOVENIAN MULTIPLE MYELOMA PATIENTSK

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Background. Multiple myeloma is a plasma cell malignancy characterised by complex karyotypes that show both numerical and structural chromosomal alterations. The non-random recurrent nature of chromosome abnormalities in myeloma suggests a role for them in disease pathogenesis. Virtually all multiple myeloma patients are characterised by the occurrence of aneuploidy, and can be divided into two major groups; hyperdiploid group, associated with recurrent gains of some, if not all, of the non-random odd chromosomes 3, 5, 7, 9, 11, 15, 19 and 21; and non-hyperdiploid group, which includes the hypodiploid, pseudodiploid and near-tetraploid karyotypes. The most recurrent chromosomal rearrangements involve deletion of chromosome 13 (del(13)), deletion of chromosome 17 (del(17)) and chromosomal translocations juxtaposing the immunoglobulin heavy chain (IGH) regulatory regions at 14q32 with a wide spectrum of partner loci (11q13, 6p21, 4p16, 16q23 and 20q11) leading to dysregulation of CCND1, CCND2, FGFR3/MMSET, c-MAF and MAFB respectively. *Aims*. Recent publications suggest that multiple myeloma is also associated with amplifications of 1q, deletions of 1p and deletions of 6q. It was also suggested that amplifications of 1q are associated with progression and drug resistance. Moreover, it was suggested that deletions of 1p impart poor prognosis. Deletions of 6q are more frequent in hypodiploid multiple myeloma patients that is associated with poor prognosis. Amplifications of 15q represent chromosomal abnormality belonging to hyperdiploid multiple myeloma that is associated with more favourable prognosis. Therefore, we investigated following chromosomal abnormalities: amplifications of 1q, deletions of 1p, deletions of 6q and amplifications of 15q in patients, where occurrence of del(13)(q14.3), translocation t(4;14), translocation t(14;16) and del(17)(p13.1) has been already tested by fluorescence *in situ* hybridization (FISH). Additionally, conventional cytogenetics with G-banding method was previously performed for majority of patients. *Design and Methods*. Chromosomal abnormalities were tested by commercially available FISH DNA probes in 40 multiple myeloma patients. Within this group of patients, subpopulation of patients had an isolated del(13) and 10 patients had no chromosome abnormalities. Remaining patients were subdivided into groups with t(4;14), t(14;16), del(17)(p13.1) and complex chromosomal rearrangements. *Results*. We were not able to confirm the deletion of 1p in multiple myeloma patients. Amplification of 1q was detected in patients that had concomitant deletion of chromosome 13. Patients with detected deletion of 6q had no other chromosomal abnormality. Patients with detected amplification of 15q were equally distributed among the two groups. *Conclusions*. The results obtained in this study indicate that amplifications of 1q and deletion of chromosome 13 are highly associated. The prognostic significance of these abnormalities highlights the importance of cytogenetic results of multiple myeloma patients.

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EPIDEMIOLOGY OF PLASMA CELL DYSCRASIAS IN EGYPT: A SINGLE INSTITUTE EXPERIENCE

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Background. Plasma cell dyscrasias (PCD) are a group of diseases characterized by the proliferation of a plasma cell clone which produces a monoclonal protein (M- protein).The most common type is Multiple Myeloma (MM) followed by Waldenstroms macroglobulinaemia (WM) and Light chain disease (LCD). Hypocalcaemia, anemia, renal damage, increased susceptibility to bacterial & fungal infections, and impaired production of normal Immunoglobulins as well as diffuse osteoporosis are common clinical manifestations of MM (*Grethlein S, medicine_MM_2004*).Thus, plasma cell dyscrasias present in different fields of clinical practice, making it of great interest to study the epidemiology of this disease. *Aims*. In the present study, we described the epidemiological aspect of PCD presenting to our oncology unit of Cairo university hospitals during eight years period of time. *Design and Methods.* We retrospectively reviewed the medical records of patients diagnosed with MM, WM and LCD in our unit from January 2001 through February 2009. Results: The study included 203 newly diagnosed and untreated PCD patients, 125 Males / 78 Females, with a mean age 45years (range 28-70). 181 were affected by MM (89.1%), 13 by WM (6.5%) and 9 by LCD (4.4%). IgG myeloma was diagnosed in 132 (72.9%) patients, IgA myeloma in 49 (27.1%).K light chain in urine was found in 139 (68.4%) patients and λ in 64 (31.6%) .The International Staging System (ISS) using β2 micro globulin and albumin as staging parameters was applied to classify 115 patients out of 203; accordingly 41 patients were found to be in stage I, 71 in stage II and 3 in stage III. *Conclusions*. This descriptive study enabled us to identify the pattern of distribution of different PCD referred to our unit, drawing our attention to the increased incidence in females & to the younger age at presentation (down to28 and 32 years old).Further collaborative studies in association with other institutions will help establish the Egyptian configuration of PCD needed for prospective individualization of therapeutic guidelines.

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TARGETING ANGIOGENESIS VIA A C-MYC/HIF-1α-DEPENDENT PATHWAY IN MM

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Background. Bone marrow (BM) angiogenesis is associated with multiple myeloma (MM) progression. *Aims*. to identify potential pathophysiologic role of Hif-1α in MM cells under normoxic conditions. *Results and Methods*. Here we report high constitutive hypoxia-inducible factor-1*Design and Methods* (Hif-1α) expression in MM cells, which is associated with oncogenic c-Myc. A drug screen for anti-MM agents which decrease Hif-1 and c-Myc levels identified a variety of compounds including bortezomib, lenalidomide, enzastaurin, and adaphostin. Functionally, based on transient knockdowns and overexpression, our data delineate a c-Myc/Hif-1α-dependent pathway mediating VEGF production and secretion. The anti-angiogenic activity of our tool compound adaphostin was subsequently demonstrated in the zebrafish model and translated into a preclinical *in vitro* and *in vivo* model of MM in the BM milieu. *Conclusions*. Our data therefore identify Hif-1α as a novel molecular target in MM and add another facet to anti-MM drug activity.

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CYTOKINES BEHAVIOR IN MULTIPLE MYELOMA PATIENTS DURING ZOLEDRONIC ACID TREATMENT

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Background. Multiple myeloma (MM) is characterized by uncoupling of bone resorption from bone formation which leads to the predominance of resorption. Bisphosphonates are chemical compounds which selectively concentrate at the interface of the active osteoclasts and the bone resorption surface where they inhibit osteoclast activity. Aim of this study was to demonstrate whether or not zoledronic acid could have an *in vivo* anti-angiogenetic property in MM as observed in solid tumors. *Design and Methods*. Serum samples from 29 (16 males and 13 females) consecutive MM patients with lytic bone lesions treated with 4 mg of zoledronic acid were tested for PDGF, VEGF, IL6, TNF α, and IGF-I any form of treatment. Basal cytokine levels were compared with the values observed after 1, 2, 7 and 21 days of treatment, using the Wilcoxon's test for nonparametric-dependent continuous variables. *Results*. A significant increase in IL-6 and TNF α was observed on days 1 and 2. As for VEGF, the levels of this cytokine did not change significantly from basal values during the entire period of observation except for day 7 (p=0.0005). Moreover, PDGF significantly decreased (p=0.005) after 2 days following zoledronic acid infusion, unexpectedly followed by a statistically significant increase of VEGF on day 7. Furthermore, these two cytokines remained mainly within the normal range throughout the entire period of study. *Conclusions*. in MM, treatment with Zoledronic acid has little activity on the angiogenetic cytokines VEGF and PDGF , therefore, the anti-myeloma effect of this drug may be operational through other mechanisms.

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INFLUENCE OF C-REACTIVE PROTEIN ON PROLIFERATION OF U266 CELLS

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Background. C-reactive protein (CRP) is a nonspecific but sensitive marker of inflammation. Interleukin-6 (IL-6), IL-1, and tumor necrosis factor α induce the synthesis of CRP in hepatocytes.It may contribute to the pathogenesis of cardiovascular disorders,and can be upregulated in many malignancies,such as multiple myeloma. As report,when CRP accessing 80 mg/L, patient's condition make advancement quickly.But whether CRP could improve the MM cells proliferation directly is unknow.Aims: The influence of C-reactive protein on proliferation of U266 cells was investigated in this experiment.*Design and Methods*. The human multiple meyloma cell lines U266 were incubated with human CRP(0, 5, 10, 20 mg/L) for 24 hours,then analyzed by blood analyser for proliferation ratio.The effects of CRP in U266 cells in various concentrations on Survivin, Hsp90α mRNA expression were examined by RT-PCR. *Results*. The proliferation ratio increase compared to the control group ($p<0.05$), further more up regulated Survivin/Hsp90α at mRNA levels in proportion for increased CRP concentrations. There were significant correlations between Survivin and Hsp90α (r=0.737, $p<0.0001$) in incubated cells. *Conclusions*. These results demonstrate that CRP could encourage the proliferation of MM cells directly by upregulating the expression of Survivin and Hsp90 in MM cells. CRP could be regarded as a potential target for MM treatment.

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EVALUATION OF PROGNOSTIC STAGING SYSTEMS FOR MULTIPLE MYELOMA IN THE ERA OF BORTEZOMIB

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Background. Multiple myeloma (MM) has an indolent clinical course with marked variability in survival outcomes. Hence, a staging system with prognostic reliability is crucial in clinical practice. *Aims*. We sought to compare the overall survival (OS) of patients with MM managed in our institution according to the Durie-Salmon Staging System (DSSS) and International Staging System (ISS), overall, and with respect to the treatment eras before and after the introduction of bortezomib. *Design and Methods*. Retrospective analysis of demographic data, clinical features and OS of all MM patients diagnosed between 1997 and 2006 was conducted. The patients were staged according to the DSSS and ISS, and OS curves were estimated by the Kaplan-Meier method, and differences compared using the log-rank test. *Results*. The OS for all patients (N=222) was 5.2 years with a median age of 62 years. 63 of the patients (28.4%) received bortezomib as treatment for relapsed disease. According to DSSS, 23 (10%) were classified in stage I, 66 (30%) in stage II and 133 (60%) in stage III, with median OS not reached in stage I, 5 years in stage II and 4.1 years in stage III (p-trend=0.01). According to ISS, 45 (20%) were classified in stage I, 101 (46%) in stage II and 76 (34%) in stage III, with median OS of 8.7, 5 and 3.3 years respectively (p-trend<0.001). Compared to DSSS, the ISS was simpler, with a higher prognostic power and more statistically significant differences in survival outcomes. However, when the survival outcome was stratified by exposure to bortezomib, both DSSS and ISS were not able to prognosticate clinical outcome. *Conclusions*. We have validated the usefulness of the ISS in clinical practice. However with the advent of bortezomib, the ISS may be insufficient for prognostication and a separate system incorporating genetic biomarkers will be essential.

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PATIENT RESPONSE TO BORTEZOMIB IN RELAPSED/REFRACTORY MULTIPLE MYELOMA: INTERIM RESULTS FROM AN OBSERVATIONAL STUDY

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Background. The Electronic VELCADE® Observational Study (eVOBS) is a non-interventional study of patients from Belgium, France, Greece, Russia, Spain, Sweden, and Turkey scheduled to receive bortezomib (VELCADE®) therapy for relapsed/refractory multiple myeloma. Data collection includes 1 year of retrospective data before and 3 years of prospective data after initiation of bortezomib. The study initiated in October 2006; this report includes interim response to treatment based on data collected from study start through 21 November 2008. Aims: In this study we assess the response rates of patients treated with bortezomib as well as other clinical parameters of importance for relapsed/refractory multiple myeloma. *Design and Methods*. Given the nature of the non-interventional study, no pre-specified response criteria were mandated to measure patient response; investigators applied several patient response criteria such as M-protein (38%), EBMT (35%), and SWOG (5%) and others (22%) and documented the patient response rates. In this analysis we report the response rate as reported by the study investigators. Response data are continuously being collected prospectively for 3 years. We also report time to response, time to progressive disease and time to next therapy by Kaplan-Meier survival analysis for all patients enrolled through November 2008, using an intent-to-treat approach. The current analysis excluded Spain and Russia due to time lag involved in data availability. *Results*. At baseline a total of 447 patients (58% male, median age 64.5 years) were evaluated. Bortezomib was initiated as 2nd-, 3rd-, and 4th-line therapy in 45%, 28%, and 17% of patients, respectively. 52% of patients received bortezomib in combination with other multiple myeloma agents. At 6 (12) months, 13.3% (17.6%) achieved complete response, 28.6%(32.5%) had at least near complete response, 66.1% (74.7%) with at least a partial response (median=91 days), and 77.7% (82.6%) had at least a minimal response (median=56 days). Within 6 (12) months, 24.1% (40.4%) of the patients progressed or died (median=296 days), 29.2% (83.3%) received alternative treatment (median=211 days) and 55.4% (88.3%) progressed or started subsequent therapy (median=192 days). *Conclusions*. The interim analysis of this current international, prospective, observational study provides preliminary evidence that bortezomib alone or in combination with other multiple myeloma agents used outside the context of a clinical trial improves overall response rates and thus is a reliable and effective option for relapsed/refractory multiple myeloma.

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BORTEZOMIB FOR THE TREATMENT OF MYELOMA - THE SCOTTISH EXPERIENCE

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Background. Bortezomib is a selective proteosome inhibitor used for the treatment of myeloma. Phase II and III trials demonstrated useful therapeutic effect; however its use was associated with a significant early discontinuation rate due to side effects. The Scottish Medicines Consortium (SMC) accepted its use within the Scottish National Health Service from October 2004 for patients who had already received two lines of therapy. Concerns about toxicity led the Scottish Haematology Society to institute a national audit of Bortezomib use between May and August 2008. *Aims*. The aim was to determine how Bortezomib was being used in Scotland: specifically, to determine the number of treatment courses patients are able to complete; to establish clinical effectiveness in the Scottish population; and to compare practice with treatment advice stipulated by the SMC. *Design and Methods.* A questionnaire was sent to all treating centres in Scotland to be completed retrospectively for up to ten patients who had completed Bortezomib treatment. *Results.* The audit was completed within a three month period with a 100% response rate. 136 completed forms from 18 centres were returned. 79% received Bortezomib in combination with Dexamethasone and 2% received Bortezomib in combination with other chemotherapy. 85% of patients received Bortezomib as third line treatment or greater. The median number of treatment cycles completed was 3 (range 0 to 8). The complete response rate was 12% and the partial response rate 44%. 44% 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 18 months. The tolerability of Bortezomib in Scotland

was less than expected from the phase III APEX trial data. In this trial, 56% of patients completed at least five cycles of Bortezomib (27% in our audit). Similarly, in APEX 29% completed eight cycles (39% in the earlier phase II SUMMIT trial), in our population only 5%. Despite this, the Scottish overall response rate was superior (56% vs 38%). The differences in response could in part be due to higher concomitant corticosteroid use in Scotland. Our higher overall response rate did not translate to an improved median overall survival. 44% of Scottish patients discontinued Bortezomib early due to side effects compared to 37% in the APEX trial. Peripheral neuropathy was the single most frequent side-effect necessitating early discontinuation in both APEX and Scottish patients. However, the prevalence was much greater in Scotland - 29% vs 8%. This may be due to greater exposure to prior Thalidomide in Scottish patients. Additionally, as Scottish patients generally received more prior therapies than APEX patients, they may have been exposed to other neurotoxins. The combination of Bortezomib with corticosteroids may be associated with higher rates of autonomic neuropathy than single agent Bortezomib. *Summary and conclusions*. In conclusion, the Scottish "real life" experience is that Bortezomib is an effective treatment. It is associated with greater toxicities than seen in clinical trials - this may be a reflection that trial patients are not always representative of "all comers".

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PROINFLAMMATORY CYTOKINES PROFILE IN THE PATHOGENESIS OF BONE DISEASE IN MULTIPLE MYELOMA

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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 some publications about the influence of different cytokines in the bone remodelling, by break the balance between osteoclastic/osteoblastic activities. *Aims*. to study a cytokine panel in a series of MM patients and to compare the results between patients with overt bone disease and without. *Design and Methods*. Between June-December 2008, we have studied a total of 39 MM patients. (IgG 23(60%); IgA 10(25%), LChain: 4; IgD 1, IgG+IgA 1) Females: 23(60%). ISS(1: 60%, 2: 32%, 3:8%) and 25 healthy controls matched by gender and age. The cytokine assay was performed in plasma according Luminex®100 technology with the Millipore human cytokines Kit, the designed panel were: IL-4, IL6, IL-7, IL-10, IL-13, MIP-1a, MIP-1b, 'NF- α. Four different degrees of bone disease was established: Osteolisis 55%, vertebral collapse 35%, osteopenia 10%. The results of the cytokine panel were compared with the variables: immunochemical subtype, ISS stage, haemoglobine concentration and free light chain ratio and with response to therapy. Descriptive analysis and mean comparative chi2 test was performed. *Results*. In this series the cytokine profile more frequent had been over expression of IL-6, IL-10, MIP-1b. The comparative analysis of profiles and variables show that there are not significant differences according immunochemical subtypes. There are significant differences in IL-10(p=0.05), IL-13 (p=0.01) in patients with low ISS vs high ISS. There are differences according the bone disease degree IL-6 (p=0.04), IL-13 (p=0.02), TNF(p=0.03). Related to haemoglobine <11 g/dL vs >11 g/dL, abnormal vs normal free light chains ratio we not observe significant differences. When we compared complete response to therapy vs failure we observed significant differences in IL-7 p=0.03),IL-10 (p=0.04). *Comments*. In our experience Luminex®100 technology is a sensible and accurate technique useful to determine cytokine profile in plasma. A different significant cytokine profile was observed in patients with different degrees of bone affectation, ISS and therapy response. It is necessary to perform more studies with more patients in order to confirm these results.

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AN OBSERVATIONAL POST AUTHORIZATION STUDY ON THE USE OF BORTEZOMIB IN MULTIPLE MYELOMA PATIENTS IN THE NETHERLANDS: RESULTS OF AN INTERIM ANALYSIS

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Background. Bortezomib (Velcade) is a novel agent in the treatment of patients with Multiple Myeloma (MM). Most data on efficacy and safety are from randomised controlled studies in selected groups of patients with MM. Aim of the study: To evaluate safety and efficacy of bortezomib in real-life daily practice as second and later line therapy in patients with MM. *Design and Methods*. In an observational, multicentre study, the efficacy and safety of treatment with bortezomib, (1.3 mg/m2 on day 1, 4, 8 and 11 with or without dexamethasone) was assessed in MM patients. All enrolled patients consented in the collection of their relevant medical data. An interim analysis was performed on data of patients enrolled from November 04 until November 06 (10^9 patients). *Results*. All results are given as median. The patients age was 67 yrs, Karnofsky Performance Score was 80%, time from initial diagnosis was 3.3 years, 62% of the patients had more than 2 previous treatment lines, 85% of the patients had disease stage III. Patients were treated with median 4 cycles (95% CI: 4.1 - 5.0) of bortezomib. Median time to initial response was 50 days and median time to best response category was 84 days. Of all patients 43.2% achieved a partial response or better (12.9% VGPR or better, 30.3% PR); 16 patients were not evaluable for response. The duration of response (DOR) was 6.3 months. Overall survival was 1.2 yrs and PFS 5.8 months. The treatment was generally well tolerated. Most common adverse events (all grades) in >15% of the patients were in order of decreasing frequency thrombocytopenia, nausea, diarrhoea, fatigue, (poly)neuropathy, constipation, malaise, anaemia, pyrexia, vomiting and anorexia. For responding patients treatment discontinuation due to an AE occurred in 46.8% of the patients. For the three most common AE's (polyneuropathy, fatigue/malaise, thrombocytopenia) resulting in discontinuation of the bortezomib treatment, the discontinuation was not preceded by a dose adjustment in almost half of the cases. In 9 patients (8.3%), Herpes zoster was reported. Twenty-two out of 10^9 patients were thalidomide naïve when starting bortezomib. 59.4% of these patients achieved a PR or better vs. 39% in thalidomide pre-treated patients, but thalidomide pre-treated patients had a longer time from initial diagnosis (3.5 vs 2.6 years) and had more previous treatment lines compared to thalidomide naïve patients (≥2 lines of previous therapy 97.7% vs 59.1% resp). *Conclusions*. Response Rates in this study were comparable to those in other community studies and higher than reported in previous clinical studies. Overall the treatment with bortezomib was well tolerated. However, it seems dose adjustment as a strategy to continue treatment and to increase the quality of response is still used insufficiently. Overall survival was as expected in this patient population. The thalidomide naïve group was yet too small to draw any conclusions on safety and efficacy as compared to the thalidomide pre-treated patients.

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FLUDARABINE BASED COMBINATION THERAPY IS HIGHLY ACTIVE AS PRIMARY OR SALVAGE TREATMENT IN PATIENTS WITH WALDENSTRÖM MACROGLOBULINEMIA

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Background. Treatment with single agent rituximab (R), alkylating agents or purine analogues is safe and moderately effective as primary treatment for patients (pts) with Waldenström Macroglobulinemia (WM). However, efficacy in relapsed/refractory WM is unsatisfactory. *Design and Methods*. We retrospectively analysed if combinations of fludarabine (F) with alkylators and/or R were safe and more effective. Results From 12/94 - 08/08, 25 courses of at least 2 cycles of intravenous F-combination therapy were administered to 22 pts with WM: FC (F 25 mg/m^2 d1-3, cyclophosphamide (C) 250 mg/m^2 d1-3; n=7); FCR (FC+R 375 mg/m^2 d1; n=14); FM (F+mitoxantrone (M) 10mg/m2 d1; n=3); FR, n=1). The median age of pts was 57yrs [range; 36-89], 18/22 (84%) male, 6 pts received F-combination as their primary treatment (24% of courses). The

19 pre-treated pts had a median number of 2 [1-7] prior treatments, prior F-exposure in 5 cases (20%), 9 pts (36%) were alkylator-refractory. The median time from diagnosis to F-based treatment was 23 Mo [0-153], baseline paraprotein (PP) 30g/L [7-64]. A total of 99 cycles were administered, median 4 [2-6] per pt. Treatment was generally well tolerated with 5% infusion reactions grade (G) 2, gastro-intestinal toxicity was mostly limited to G 1 or 2 (35% of cycles) with only one G3 episode, no renal toxicity or hepatic side effects > G 1. G 3 thrombocytopenia occurred in 5%, G ≥3 neutropenia and infections complicated 20% and 3% of cycles, respectively, none of which were life-threatening. However, 3 heavily pre-treated pts subsequently developed secondary AML/MDS (1 fatal) at 56, 61, and 91 Mo post-treatment. The overall response rate (RR) was 21/25 (84%) with a median PP reduction of 90% [30-100%]. One pt achieved a complete remission, 17/25 (68%) had a partial response, resulting in an objective RR of 72%, 3 responses (12%) were minor (PP-reduction of 25-<50%). The median times to response and maximum response (PP nadir) were 2.6 [0.7-7.3] Mo and 9.5 [0.9-47.5] Mo, respectively. The median event free survival (EFS) for all pts was 43.4 [0.9-113.6] Mo, the median overall survival (OS) was not reached. With a median follow-up of surviving pts of 59 Mo, the 5- and 10-year-actuarial survival rates were 85±8%, and 68±13%, respectively. With a median follow-up of 51 Mo [13-97], all 6 previously untreated pts remain alive and progression-free. The RR was independent of pre-treatment status whereas untreated pts had superior EFS but not OS compared to alkylator-refractory pts (p=0.01 and 0.29, respectively). Neither R administration nor the known adverse prognostic factors age >60 years or increased baseline β^2 microglobulin impacted on EFS or OS. Anemia (Hb <100 g/L) resulted in a trend for reduced EFS (43 Mo vs. not reached, p=0.05), but not OS (p=0.24). *Conclusions*. We conclude that F-combination therapy is highly active in patients with untreated as well as in those with alkylator-refractory WM, leading to high response rates and prolonged remissions. However, possible contribution to the cumulative risk of treatment-related MDS/AML in heavily pre-treated pts is a concern.

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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 cell lines and primary myeloma cells. Bortezomib therapy has become an important part of the standard of care for patients with relapsed multiple myeloma, and preliminary clinical evidence suggests that bortezomib retreatment in patients previously treated with the drug may prolong disease control. *Aims*.The aim of this retrospective review of patients with multiple myeloma (MM) was to describe patterns of retreatment with bortezomib-based therapy and responses to retreatment. *Design and Methods*. Data were retrospectively extracted from the medical records of patients treated in Russia Novosibirsk hematologycal center, who were subsequently retreated off protocol with bortezomib-based therapy. Eligible patients had 60 or more days between treatments and > or = 4 bortezomib doses during initial treatment. Response categories included very good partial response (VGPR), > or = 90% M-protein decrease; partial response (PR), 50%-89% decrease; and less than PR (< PR), < 50% decrease, excluding progressive disease (PD). Results. Retreatment response data were available for 26 patients: 3 (12%) had VGPR, 6 (23%) had PR, 8 (31%) had < PR, 7 (27%) had PD, and 2 (7%) died. Thus, the overall response rate for bortezomib retreatment was 64%. During retreatment, 15 (58%) of 26 patients received bortezomib in combination with another antineoplastic agent, 11 (42%) bortezomib alone. Median time between bortezomib treatments was 7.9 months; 31% of patients received non-bortezomib therapy between treatments. Toxicity contributed to discontinuation in 27% of patients during retreatment; rates of neuropathy contributing to discontinuation were 15%, respectively. *Conclusions*. 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 or refractory multiple myeloma, even in patients previously exposed to bortezomib.

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SERUM CONCENTRACION OF C-TERMINAL COLLAGEN TELOPEPTIDE AS AN OSTEOLYTIC LESIONS MARKER IN PATIENTS WITH MULTIPLE MYELOMA

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Background.The degradation product of collagen type I, ICTP, represents a new biochemical parameter which reflects the changes in resorption properties of skeletal system. Affection of the skeleton is one of the most important characteristics of multiple myeloma (MM). Increased resorption of bone mass can exist even in patients where osteolytic changes cannot be found by means of conventional radiography. Aims: The aim of this study was to estimate the correlation between serum levels of C-terminal collagen telopeptide type I (ICTP) and osteolytic changes seen on the conventional skeleton radiography, that is, to estimate the relevance of ICTP as a marker of osteolytic activity and survival prognostic factor, as well as comparison with known clinical parameters of prognostic significance: β2-microglobuline (β2-MG) and C reactive protein (CRP), in patients with MM. *Design and Methods*. In this study were included 25 untreated patients with MM. Following findings were determined initially in all patients: blood count, total proteins, electrophoresis with immune electrophoresis of serum and urine proteins, urea, creatinine, calcium ions (Ca++), β2-MG, CRP, percentage of plasma cells in the bone marrow, and skeleton radiography. Serum level of ICTP was determined with competitive radioimmunoassay (RIA), through ICTP marked with radioactive iodine- I125, and rabbit's polyclonal antiserum as detection reagent. *Results*. A significant difference between patients with and without osteolysis was proved in ICTP (p=0.009), β2-MG (0.011), and CRP (p=0.044) values. In univariate analysis, significante osteolysis predictor was ICTP (p=0.021), β2-MG have been on border of significance (p=0.052), and CRP have not statistical significance (p=0.069), while significant survival predictor were: ICTP (p=0.001), β2-MG (p=0.009), and CRP (p<0.001). In multivariate analysis, ICTP was, compared with β2-MG i CRP, the most powerful osteolysis predictor (p=0.021), whereas CRP was the most powerful survival predictor (p<0.001). There were statistically significant positive correlation between osteolytic changes and level of anaemia (p=0.010), uraemia (p=0.033), increased values of creatinine (p=0.034) and increased values of Ca^{++} (p=0.045). There were statistically significant positive correlations between ICTP and urea values (p=0.038), and creatinine (p=0.015). *Conclusions*. Being highly significant predictor for osteolysis and survival, ICTP can be used for identification of patients with MM who had increased risk for developing osteolytic lesions, as well for identifying patients with poor prognosis in sense of survival. Patients with increased values of ICTP, and without verified osteolytic changes by means of conventional skeleton radiography, are indicated for more thorough diagnostic procedures, e. g. skeleton IMR (magnetic resonance imaging), for more precise detection of osteolysis.

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PATHOGENETIC RELATIONSHIP BETWEEN BONE LESIONS AND RENAL FAILURE IN MULTIPLE MYELOMA IS OF 'VITIOUS CIRCLE' TYPE

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Background. Clinical manifestation and pathogenetic relationship between myeloma bone disease (MBD) and renal failure (RF) are not well- defined. *Aims*. To analyze the interactive influence of bone lesions and renal failure in multiple myeloma (MM) in clinical, pathogenetic and prognostic aspect. *Design and Methods*. 425 previously untreated patients with (223 male and 203 female) were studied for a period of 28 years (1980-2007). They were diagnosed at the University clinic of hematology, stage was defind according to Durie and Salmon system and were treated in a similar way. RF (creatinine clearance < 1,3 ml/sec) was assessed in incidence, grade and outcome after induction treatment. MBD (X-ray, CT scans) was graded according to the Merlini scale. Bone mineral density of L-spine was assessed by dual energy X-ray absorbtiometry (DXA) in anterior - posterior plan. Calciura, phosphaturia, creatininuria and the clearances of Ca, P and creatinine were determined simultaneneously. Median of survival (MS) was calculated by the method of Kaplan-Meier. Statistics were done with SPSSv13 (for Windows). *Results*. RF was found in 196 (46,1%) of the patients, most frequently in grade I (cr ≤353,0 μmol/l) - 62,7% and was completely

reversible after treatment in 91(46,4%). MBD was found in 353 (83,1%), most frequently in grade « 2» in 210 (49,1%) patients. Bone lesions had 94,4% of the patients with RF and 52,4% of the patients with MBD had RF. As MBD progressed, the incidence, grade and resistance of RF to treatment significantly increased. In patients with RF the mean Z-score showed osteoporosis (-2,32±0,40), and without RF - osteopenia (1,89±0,41). Calculating odds ratios patients with RF increase 5 times their risk to develop MBD. In 26,1% of the patients, hypercalciemia was found, in 41,1% - hypercalciuria>4.5mmol/24h, in 3,1% - hyperphosphatemia and in 26,8% - hyperphosphaturia>26.0 mmol/24h. In patients with BJ proteinuria and RF, although creatinine clearance is limited, Ca clearance (0,061±0,003 ml/sec) and Cca/Ccr ratio(0,108±0,013) are higher while tubule reabsorbtion of TRCa (89,23±1,59%) and TRP (60,16±5,34%) are reduced (p<0,05) because of proximal tubular impairment. MS of the whole group is 35 month; patients without RF and without MBD live 46 months, while patients with RF and MBD-21months *Conclusions*. The relationship between MBD and RF is based on the principle of the "vitious circle" which modulates their clinical manifestation, therapeutic outcome and prognostic significance. Influence of MBD on renal function. Hypercalciemia a) induces contraction, limits the glomerular filtration and results in RF. b) intensifies the co-precipitation of light chains and development of cast nephropathy, also resulting in RF, inhibits the adenylatcyclase activity in the tubule epithelium, induces resistance to antidiuretic hormone, resulting in polyuria + hypostenuria. 2.Influence of RF on MBD. The tandem of hypercalciuria + BJ proteinuria causes different tubular dysfunctions. When proximal tubular injury occurs, hypercalciuria and hyperphosphaturia are observed: Fanconi syndrome, light chain osteopenia /osteomalatia; intensive demineralization of the skeleton is induced and b) most probably renal osteodystrophy also plays role in advanced RF.

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EXTRAMEDULLARY RELAPSES IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Extramedullary relapses (ER) during the course of Multiple Myeloma (MM) is a truly rare finding , and is considered to carry a rather poor prognosis.This kind of MM relapse is best described after autologous or allogeneic transplantation or immunomodullatory treatment. Due to the rarity of this particular kind of relapse in MM, it has not yet been studied thoroughly. *Aims*. Purpose of this work is the presentation of the characteristics of the MM patients which had an ER. *Design and Methods.* These patients derived from a series of 406 patients which were diagnosed and followed during the last 11 years. *Results.* 14 patients (3,5% of total),8 men and 6 women with a median age of 67 years (49-76),presented with an ER during their disease course (4 liver,3 pleural effusions,2 CNS,1 lung,1 ureter and psoas muscle,1 base of the tongue).At the time of initial diagnosis of MM 43% of them were stage III , 22% were stage II and 35% were stage I according to ISS staging system, while the type of MM was IgG in 64%,IgA in 22% and non-secretory in 14% of the patients. 93% of them presented with pathologic bone fractures (7 patients) and/or bone plasmocytomas (9 patients). Serum LDH was abnormally high in 21% while for the rest of the population the percentage was 5,6% (p<0.01). Bone marrow plasma cell infiltration was <50% in 85% of patients. The median time from initial diagnosis till ER was 33 months(5-117).The mean number of therapies till ER was 2 (1-5).21% have had an autologous transplantation,43% had previously received thalidomide, 28% bortezomib, 14% lenalidomide prior to ER. During ER 28% of the patients had "B" symptoms,93% had an abnormally high LDH, while most of the patients still had a plasmacytic infiltration below 50%. 80% of patients with "B" symptoms had an ER located in the liver. The median survival after ER was 5 months and 71% of the patients died. *Conclusions*. ER is characterized by a very aggressive and treatment resistant behavior. Given the higher rate of elevated LDH and presence of plasmacytomas and extensive bone disease, it seems that patients with an ER relapse belong to a subcategory of MM with a more aggressive kind of biological behavior since the time of initial diagnosis.

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INCIDENCE AND OUTCOME OF OSTEOLYTIC LESIONS IN THE JAW IN PATIENTS WITH MULTIPLE MYELOMA (MM)

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The increase incidence of Osteonecrosis of the jaw (ONJ) in patients receiving intravenous bisphosphonates, and the higher prevalence in patients with MM, suggest a predisposition in these patients to suffer this complication. This fact justifies the interest in understanding the impact and outcome of the jaw bone lesions associated with MM. Aims: to analyze the incidence and location of lytic lesions in the jaw in patients with MM at diagnosis, and to analyze their progress after the induction treatment. *Design and Methods.* A retrospective study of patients diagnosed of MM was performed at diagnosis or during the 1st line treatment of MM without clinical o analytical data of progression. Additionally, 10 patients were reevaluated after completing the induction therapy. A single examiner made the visual inspection of oral cavity and the orthopantomography in all the patients. To set the location of the lesions, the jaw was divided into two halves (right and left) and, in turn, each half was divided into 10 anatomical regions (Rouvière). Statistical analysis was performed using SPSS program version 15.0. Results: 48 patients were included. The median age was 61 (range 30-73 years). 22 were male, 26 female The clinical characteristics of the group were: 1) Type of M protein: IgG: 48% (23), IgA: 25% (12), BJ 27% (13). 2) Skeletal survey with plain radiography: without osteolytic lesions 15% (7), < 4 affected regions 46% (22), > 4 affected regions 29% (14), and not available in 5 cases. 3) Durie-Salmon staging (DS): I 13% (6), II 31% (15) and III 56% (27), 4) ISS: I 27% (13), II 17% (8), III 39% (19), and not available in 8 cases. The jaw was affected at the diagnosis in 28 patients (58%), with no differences between right and left jaw. We identified a higher frequency of osteolytic lesions in 5 anatomic areas. We found no statistically significant differences when comparing the presence or absence of osteolytic lesions in the jaw with the type of M protein, skeletal survey with plain radiography, DS staging or ISS. The comparison of the initial study with the survey after completing the induction treatment was performed in 10 patients. The 1st study show that 90% of patients (9 / 10) had at least one osteolytic lesion of the jaw, with a total of 66 affected areas; At the 2nd revision jaw lesions were detected in at least one region in 7 patients (70%), with a total of 54 affected areas. Affected regions decreased in 5 cases (50%), remained stable in 3 cases (30%), while increased in 2 (20%). The inferential analysis (t-student for paired data and chi-square), showed no statistically significant differences between both evaluations. *Conclusions*. Osteolytic lesions in jaw are common in patients with MM, affecting more than half of patients at diagnosis. Our study suggests an improvement in osteolytic lesions of the jaw after induction treatment.

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BORTEZOMIB RETREATMENT IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA: RESULTS IN A COHORT OF 52 PATIENTS

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Background. Bortezomib is a drug with significant activity in relapsed or refractory multiple myeloma (MM) although there is limited experience in retreatment in the daily clinical practice. *Aims*. To evaluate bortezomib efficacy and safety as retreatment in non selected relapsed or refractory MM patients. *Design and Methods.* All the patients with MM followed in our department from June 2004 to January 2009 were retrospectively reviewed. The patients with relapsed or refractory disease after one or more therapeutic regimens that were treated with bortezomib (both as first and subsequent rescue therapy) were selected. Bortezomib was combined with other drugs (accordingly to the clinical features of each patient) when previous response was shorter than six months, minor or nonexistent. Efficacy was evaluated as response and time to symptomatic progression (TTP) and clinical features were assessed as prognostic variables. Complete response (CR) was defined as the disappearance of both monoclonal serum protein (tested by IF) and urinary component. Partial response (PR) was defined as a 50-99% monoclonal serum protein reduction and 90% reduction in urinary component. Minor response (MR) was defined as a reduction of 25-50% and non response (NR) as an inferior reduction or progression. Response was

assessed after at least two cycles of bortezomib unless clinical progression during treatment and retreatment occurred. Safety evaluations were coded using the WHO scale. Efficacy and safety of first rescue treatment with bortezomib was compared with subsequent retreatment. Statistical methods: descriptive, χ^2, Fisher exact test, Kaplan-Meier tables, log-rank test and Cox binary logistic regression. *Results*. Fifty two patients received first rescue treatment with bortezomib or bortezomib/dexametasone (B/D): mean age was 69.8 years (49-85), 44% had refractory MM and 56% relapsed MM. Median time from diagnosis was 2,6 years (0-15) and the median number of previous therapies was 2 (1-9), eighteen patients (35%)had undergone an ASCT. After a median time of 4.5 months (1.5-6.5), 46 patients were assessable for response. 19 had CR (41%), 13 PR (28%), 4 MR (9%) and 10 NR (22%). There was progression in 36 cases (78%) in a median follow up of 2.1 years in living patients, with median TTP of 9.5 months and median survival of 2.5 years. ISS was the only variable associated with response (1 versus 2-3) (p:0,02;OR=0,1; 95% C:I: 0,01-0,94). A longer TTP was significantly associated with relapsed versus refractory disease (13 months versus 7 months. p=0.03). As for subsequent rescue treatment, 16 patients received a second bortezomib therapy for relapsed or refractory MM (7 B/D, 2 B/D with infusional doxorubicin and 5 B/D with endovenous melfalan). Their median age was 73 years, the median number of previous therapies was 4 (2-10) and time from the first bortezomib course was 8 months (2-20). 13 patients were evaluable for response: 3 achieved CR and 4 PR and the median TTP was 7 months. In these patients no statistical difference was found between response and TTP in the first and second treatment with bortezomib, although the probability of response was greater in those who responded to the first treatment (p=0,07). Safety: We detected adverse events in 71% of patients in the first treatment and 69% in the second, without significant differences (thrombocytopenia: 17% versus 19%, peripheral neuropathy: 33% versus 19%, others: 40% versus 33%). Two patients in each group required discontinuation of treatment for toxicity. *Conclusions*. In our experience bortezomib is an active drug in relapsed or refractory MM both as first rescue treatment and as subsequent therapy without changes on its safety profile.

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THROMBOPOIETIC CYTOKINES AND PLATELET COUNT IN MULTIPLE MYELOMA

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Background. Platelet counts are frequently in normal range among patients with multiple myeloma (MM) despite the disease activity. Cytokines like interleukin-6 (IL-6) and IL-1a are both implicated in myeloma pathogenesis and megakaryopoiesis. *Aims*. We analyzed plasma levels of TPO, IL-1α, IL-11 and IL-6 levels in myeloma patients with various stage and activity. *Design and Methods*. Sixty eight patients with multiple myeloma [30 female and 38 male, median age 58 (40-79), 38 newly diagnosed, 15 in plateau, and 15 relaps and/or refractory patients] were incluted in the study. Twenty one healthy adult subjects [10 female and 11 male, median age 58 (41-71)] were studied as control group. Plasma TPO, IL-6, IL-1α, IL-11 levels were measured by ELISA. *Results*. Platelet counts were not different between control group and myeloma patients with various disease stage and activity. Platelet counts inversly correlated with TPO levels (r=-0.577; p<0.0001), and percent of bone marrow plasma cells in bone marrow aspiration (r=-0,268; p=0.06) and a positive corelation was detected with IL-6 (r=0.263; p=0.04) levels in MM patients. TPO and IL-6 levels were significantly correlated (r=0,305; p<0.001). TPO (p<0.001) and IL-6 (p<0.001) levels were higher in MM patients than healthy subjects, but IL-1β and IL-11 levels were not different. Disease activity has no effect on plasma cytokine levels. TPO levels were higher in stage III than stage I (p=0.05) and stage II (p=0.03) patients in newly diagnosed MM. Cytokine levels and platelet count were not different between stage A and B disease. *Conclusions*. In this study, IL-6, and TPO levels were higher in myeloma patients than healthy subjects. High TPO levels induced by IL-6, stimulate platelet production and may sustain normal platelet counts despite bone marrow infiltration by plasma cells.

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FERRITIN AS A PROGNOSTIC FACTOR OF MULTIPLE MYELOMA

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Background. Serum ferrtin is a known marker of acute phase reactions and iron storage. A previous study showed that hematologic malignancies had significantly elevated serum ferritin levels. Another study suggested that ferritin may be a surrogate for more advanced disease and thus have an impact on relapse because elevated serum ferritin levels are associated with overall survival (OS) and relapse-free survival following autologous stem cell transplantation for lymphoma. *Aims*. This study was planned to examine serum ferritin levels in newly diagnosed MM patients to determine whether the level would be correlated with outcome and be an independent factor of survival in MM.

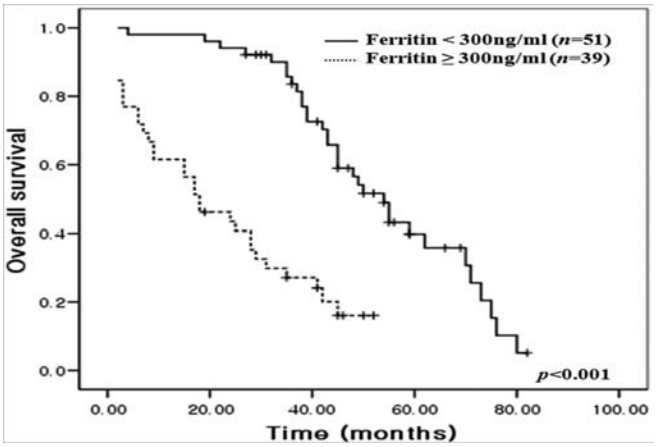

Figure 1. Overall survival according to serum ferritin level.

Design and Methods. Between 2001 and 2006, 121 consecutive patients with MM were evaluated at the Pusan National University Hospital. Ninty patients were eligible to partcipate in the study. The median follow-up duration was 35 months (range, 2-72 months). The international staging system was used for initial staging, Eighteen patients were stage I, 31 were stage II, and 41 were stage III. Twenty-seven patients underwent autologous stem cell transplantation (ASCT). The primary endpoint was to assess the impact on overall survival (OS) based on the serum ferritin level at the time of diagnosis in newly diagnosed MM patients. The secondary endpoint was to determine whether the serum ferritin level at the time of diagnosis was an independent prognostic factor for OS in MM patietns. *Results*.We studied 89 consecutive patients with multple myeloma at the time of diagnosis to determine the value of serum ferrtin levels in comparison with previous known prognostic factors. During a median follow-up of 35 months, we found that the OS in the elevated serum ferritin level group (≥300 ng/mL) was longer than the normal serum ferritin level group (< 300 ng/mL; p<0.001). Multivariate analysis showed that an elevated serum ferritin level was an independent prognostic factor of mortality in patients with multiple myeloma (RR=3.610, 95% CI=1.608-8.105, p=0.002). *Summary and Conclusions*. In conclusion, the serum ferritin level is expected to be a prognostic factor related to OS of the patients with multiple myeloma. A multicenter study is needed to evaluate the usefulness of the ferritin level.

1604

LONG-TERM FOLLOW-UP OF MULTIPLE MYELOMA PATIENTS TREATED BY VELCADE

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We evaluated the immediate and long-term efficacy, tolerance, survival and time to progression of MM patients who received VELCADE

as induction and in those who relapsed or were refractory after standard therapies. 92 pts included, 62 M, 30 F. Median age = 60 years [35-79]. Patients= group1: 16(17%) pts who received Vel. as induction, group2: 58(63%) pts who received Vel. as 2nd line n=41 or 3rd line n=17, group 3: 18(12%) pts who received Vel. as ≥ 4th line. Median interval diagnosis-Velcade. initiation = 28.3 months (0.2-125). Median number of Vel. cycles in group 1, 2 and 3 were 4[2-9], 5[2-12] and 5[3-12] respectively with a standard dose of 1.3 mg/m^2. Peripheral neuropathy occurred in 61 pts(66%). The overall response rate was observed in 67(73%) pts [13 (14%)CR, 22 (24%)VGPR and 32 (35%)PR]. With a median follow-up of 23.4 months, the median overall survival (OS) for the whole population and for group 1 and 2 was not reached, and was 17 months for group 3. The probability of OS at 3 years for the whole population was 61%[50-73.5] and were: 80%[62-100], 66%[54-81] and 45%[25-82.5] for groups 1, 2 and 3 respectively. The probability of OS at 5 years for the whole population from MM diagnosis was 72.5%[63-84]. The median time to progression was 25.5 months for the whole population; it was not reached for group 1, were 25.5 and 13 months for group 2 and 3 respectively. The probability of progression-free survival (PFS) at 2 years was 51%[40-64,5] for the whole pop. and 66%[45-96], 52%[39-70] and 29%[12-71] for group 1, 2 and 3 respectively. We showed no significant difference in term of OS and PFS between the patients with del(13) [51%(31-83) and 42%(23-78)] and without del(13) [83%(68,5-99,6) and 58%(40-85,5)] (p=0.006). The multivariate analysis showed a significant negative impact of thalidomide HR=3.06 and a positive trend for short interval diag-Vel. We performed a study of MRD by free light chains analysis and the results will be presented.

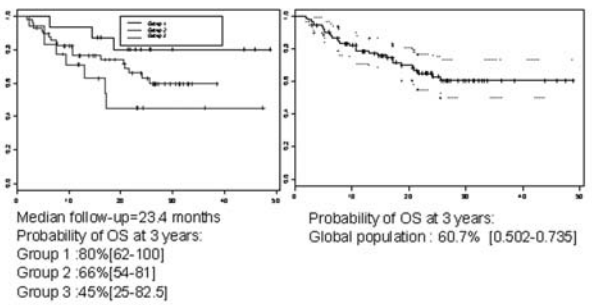

Figure.

1605

THALIDOMIDE THROMBOPROPHYLAXIS: IS ASPIRIN COVER ADEQUATE TO PREVENT VENOUS THROMBO-EMBOLISM. A 2 CENTRE RETROSPECTIVE AUDIT

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Background. Thalidomide therapy has changed the landscape of myeloma treatment. One major side effect of Thalidomide is increased venous thrombo-embolism (VTE) when used in conjunction with Dexamethasone, with one meta-analysis showing an incidence of 15.7% if not on prophylaxis. This increased VTE risk is related to decreased thrombomodulin levels and an increased resistance to activated Protein C. No clear guidelines for thromboprophylaxis in Thalidomide treated patients exist. BCSH Guidelines note that although studies exist looking at Thromboprophylaxis including Aspirin, Low Molecular Weight Heparin (LMWH) and Warfarin, no consensus opinion has been reached. We have noted heterogeneity in our own prescribing practice. Historically we opted for no thromboprophylaxis or Aspirin 75mg OD, though recently a move has been made towards LMWH. In our clinical practice we suspected the number of VTE events in our population was higher than rates quoted and therefore decided to audit our practice. Aims Collect data on Thalidomide treated patients at Worthing and Southlands Hospital (WASH) and Brighton and Sussex University Hospital (BSUH). Determine incidence of VTE and compare against mode of thromboprophylaxis used. Alter prescribing practice if a clear advantage is found. *Design and Methods*. We retrospectively audited 71 patients who have received Thalidomide at WASH, a further 11 patients from BSUH were also included. Data was collected on patient demographics, diagnosis, past history of thrombosis, dosing and duration of thalidomide, thromboprophylaxis and subsequent VTE. *Results*. Age range: 54-89 Age mean: 73.6 Sex: 45♂, 37♀. *Diagnosis.* Myeloma (74), Mantle Cell (3), LPL with Amyloid (1), Primary Amyloid (1), Myelofibrosis (1), SLVL (1), MDS with Epistaxis (1) Prophylaxis used: LMWH (30), Aspirin (29), Nil (17), Warfarin (6) Events: P.E. (5), DVT (3), CRVO (1), CVA (1) Deaths: Unknown cause (8), Sudden death (2), Congestive cardiac failure (1) Events details: Event 1 - DVT on Aspirin Event 2 - DVT on LMWH Event 3 - CRVO on Aspirin Event 4 - DVT on Nil Event 5 - PE on Aspirin Event 6 - PE on Nil Event 7 - PE on Aspirin Event 8 - PE on Nil Event 9 - CVA on Warfarin Event 10 - PE on Aspirin. *Summary and Conclusions.* Incidence of VTE regardless of anticoagulation was 12.2%. Given the quoted VTE incidence of 15.7% with no prophylaxis, our population shows a higher than expected rate of events. We observed 10 VTE events, though this number may be higher if we consider the unexplained deaths. Of these, 2 patients have documented sudden deaths where the possibility of a fatal VTE is high. These patients however have not been included in our analysis. Of the 10 events only 1 patient was on LMWH prophylaxis and 1 on Warfarin. This suggests that no prophylaxis/Aspirin is inferior at preventing VTE compared to LMWH (or Warfarin). However no head to head studies comparing the different thromboprophylaxes have been undertaken. Given the difficulties maintaining Warfarin levels in patients receiving pulsed chemotherapy we recommend LMWH as thromboprophylaxis. We recognise for some individuals this may be unsuitable i.e. chronic renal failure, high bleeding risk or patients unable to tolerate/administer daily injections.

1606

LOW-DOSE THALIDOMIDE THERAPY IN PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA

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Background. Multiple myeloma is a clonal disorder of plasma cells, accounting for 10% of hematological malignancies. The median overall survival has been improved by high-dose chemotherapy with autologous stem cell transplantation. However, treatment of relapsed patients after conventional and/or high-dose chemotherapy remains unsatisfactory. Treatment with thalidomide for patients with relapsed/refractory myeloma has been initiated in many countries. Some studies have shown that the response rate and survival were improved by a standard dose of thalidomide. However, treatment with thalidomide at a standard dose has also caused many adverse events involving the nervous, gastrointestinal and vascular systems. We conducted a retrospective chart review of 73 patients who had multiple myeloma in 6 facilities in Hokkaido, Japan. *Aims.* The aim of this study was to determine of the efficacy and safety of low-dose thalidomide (100-200 mg). *Design and Methods*. Between July 2003 and June 2008, 73 patients with relapsed/refractory multiple myeloma were treated with thalidomide in those facilities. Sixty-one of the 73 patients were evaluable. The diagnosis of multiple myeloma was made according to the Southwest Oncology Group (SWOG) criteria. The male/female ratio was 1.03 (31/30) and the median age was 69 yr (range, 48-90 yr). Immunoglobulin subtypes were as follows: IgG (n=30), IgA (n=19), IgD (n=1) and light chain (n=9). One patient had nonsecretory type. The staging of multiple myeloma was done according to the crassification of Durie and Salmon as follows: clinical stage I (n=8), II (n=16), III (n=37). Thirteen patients received stem cell transplantation (SCT). Most patients were administered thalidomide orally at maintenance and maximum doses of 100~200 mg/day. Only three patients were administered a maximum dose of 300~400 mg/day. *Results*. The overall response (nCR+PR+MR) rate was 39%.There was significant difference between age of responder and that of non responder (65.5±9.7 yr vs 71.4±9.3 yr, p=0.02). There was no significant difference when thalidomide was used alone or in combination with steroid and/or chemotherapy (p=0.56). The actuarial overall survival rates were 50.4% at 2 years and 25.0% at 5 years. Overall survival was longer for patients who received SCT than for patients who did not receive SCT (p=0.002). Frequent non-hematological events included constipation (39%), peripheral neuropathy (31%), edema or weight gain (18%), drowsiness (18%) and skin eruption (11%). Thrombosis (deep vein thrombosis: 2 patients, pulmonary embolism: one patient) was observed in only three cases (4.9%). Hematological adverse events

included leucopenia (6.6%), anemia (3.3%) and thrombocytopenia (3.3%). Grade 3 to 4 adverse events were observed in 18 patients. Frequency of adverse events was significantly lower than those in previous series used standard-dose thalidomide. *Summary and Conclusions*. Low-dose thalidomide therapy was as effective as standard-dose thalidomide in patients with refractory/relapsed multiple myeloma, and the incidences of adverse events were lower than these in the case standard-dose thalidomide.

1607

BONE MARROW SAMPLING USING A ROTARY POWERED DEVICE YIELDS EXCELLENT BIOPSY SPECIMENS IN AN ANIMAL MODEL

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Background. The importance of bone marrow examination in the evaluation of hematopoietic and non-hematopoietic diseases is well established. The most common method of accessing the bone marrow for diagnosis and monitoring disease and treatment is the core, or trephine, biopsy of the bone within the medullary cavity. Recently a new FDA-cleared battery-powered bone marrow biopsy system was developed to allow operators to access the bone marrow space quickly and efficiently. *Aims*. Using a swine model, a study was designed to compare the quality of bone marrow biopsy core specimens using the new powered device to specimens obtained using a traditional manual device, with particular attention to the possibility of cellular damage resulting from a thermal effect caused by the rotation of the needle attached to the powered device. *Design and Methods*. The study was reviewed and approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee (IACUC) prior to implementation. Three anesthetized mature pig were used for the study. The powered device (OnControl®, Vidacare Corporation, San Antonio, TX, USA) was comprised of two basic components: the battery-powered driver and the needle set with an 11 gauge x 4 inches (2.3mm x 102 mm) outer cannula. The manual device was an 11 gauge x 4 inches (2.3 mm x 102mm) T-Handle Jamshidi® bone marrow biopsy needle (Cardinal Health, Dublin, OH, USA). Following cut-down to the iliac crest by the veterinary staff, operators performed the bone marrow biopsy procedures. Core biopsy samples were measured for length, assessed for gross sample quality, fixed in preservative, and submitted to a pathologist blinded to the biopsy device for analysis. The primary endpoint of the study was quality of bone marrow core biopsy samples, and the secondary endpoint was biopsy sample length.

Figure 1. OnControl Rotary-Powered Bone Marrow Biopsy Device.

Results. Thirty-three bone marrow samples were obtained from three swine on two different dates, including 23 samples using the powered device (Powered) and 10 samples using the manual device (Manual). To date, pathology results have been received for 13 samples obtained from the first swine. Pathology reported no cellular damage or other significant artifact for any of the samples captured from either device. For eight Powered samples, pathology reported a mean length of 22.2±10.8mm. For five Manual samples, pathology reported a mean length of 12.7±6.8mm. *Summary and Conclusions.* In this study, preliminary results showed Manual and Powered biopsy samples were equivalent in specimen quality at the microscopic level and the samples by the Powered method were greater in quantity of sample obtained. The new Powered bone marrow biopsy system appeared to be capable of quickly and accurately capturing core biopsy samples of suitable length and quality for analysis, without significant artifact. The Powered device may reduce the time and effort required to obtain adequate bone marrow specimens; and thereby lessen the procedural pain experienced by patients. This should be confirmed in randomized, controlled clinical trials.

1608

AUTOLOGOUS STEM CELL TRANSPLANTATION AND MAINTENANCE TREATMENT IN MULTIPLE MYELOMA PATIENTS. A SINGLE CENTER EXPERIENCE

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Background. During the last two decades autologous stem cell transplantation (ASCT), is the standard management for the younger patients with multiple myeloma (MM), followed by maintenance therapy (thalidomide or α-interferon). AIM: The purpose of this retrospective study was to evaluate the effect of ASCT in MM patients, in terms of event free survival (EFS) and overall survival, in correlation to thalidomide (THAL) and α-interferon (IFN) as regimens for maintenance treatment in the post transplant period. *Design and Methods*. one hundred twelve patients with MM undergone ASCT in our Department, since 1989 until 2007. Females are 53, males 59 with median age 53 years (30-65). At the diagnosis they were at stage (DS) IA=9, IIA=33, IIIA=49 and IIIB=21 patients. The subtype is IgG=69, IgA=31, IgD=3, free light chain=8 patients and one patient had non secretory myeloma. At the time before the ASCT, 37 patients are in CR, PR =51, VGPR=12, PD=5, SD=7 patients. Response was assessed by international uniform response criteria for multiple myeloma. Conditioning regimen for 101 patients was melphalan 200 mg/m^2 and for the rest Bu/Cy/VP-16. After ASCT 47 patients received IFN and 28 received THAL as maintenance treatment. The remaining 37 patients did not received any regimen as maintenance treatment. *Results*. After ASCT 63 patients (56%) are in CR, 36 in PR (32%), 8 in VGPR, 2 in SD and 3 patients developed PD. The transplantation related mortality (TRM) is 0.89%. The median value of OS is 84 months (16-192). The median value of EFS since ASCT for all the patients is 27 months (4-137). The EFS since ASCT for patients received IFN as maintenance is 38.5 months (5-111), for the patients received THAL the EFS is 23.5 (4-61) months (p<0.001) and for the patients who did not received maintenance therapy is 17 months (4-47). The ten years OS is 30.7% for all the patients. *Conclusions*. 1) ASCT is a safe procedure with very low TRM, 2) ASCT followed by maintenance treatment may offer long lasting remissions in a previously difficult responded disorder, 3) in our study group there is a slight superiority of IFN in EFS rate (this result maybe affected by differences between the groups of THAL and IFN), 4) the ten years OS in MM patients received ASCT and maintenance therapy is 30.7%.

1609

PERCUTANEOUS KYPHOPLASTY IN THE TREATMENT OF PATHOLOGICAL VERTEBRAL BODY FRACTURES IN MULTIPLE MYELOMA PATIENTS

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Background. Percutaneous kyphoplasty is a minimal invasive procedure for stabilizing pathological vertebral compression fractures caused by osteoporosis or osteolytic tumours such as multiple myeloma and metastatic diseases of the spine. This technique involves the injection of thick viscous bone cement (poly-methyl-methacrylate) into the preformed cavity through special instruments. In this way rapid pain relieve and stabilizing of vertebral fractures can be achieved. *Aims.* Retrospective evaluation of clinical and radiographic outcome of percutaneous kyphoplasty in the treatment of vertebral fractures caused by multiple myeloma. *Design and Methods*. Between May 2005 and August 2008, fourteen consecutive kyphoplasty procedures were performed in 8 patients with multiple myeloma vertebral fractures. Median of patient age was 61 years (53-67). The kyphoplasty was performed in thoracic and lumbar spine location, under general anesthesia and continuous skiascopic control. For the evaluation of the analgetic effect a 10-point subjective visual analog scale (VAS) has been done during the period of 6 months. Further, plain X-ray pictures were taken before the surgery, 1 day after the surgery and 6 months after the surgery in order to measure the vertebral body height (anterior, mid-vertebral and posterior) and assess the restoration of the sagittal alignment. *Results.* The average pain

scores (VAS) decreased significantly ($p<0.01$) from 5,6 points (before the kyphoplasty treatment) to 2,5 points (after 3 months) and 1,2 points (after 6 months). Improvement of vertebral height was achieved in 12 of 14 fractured vertebral bodies (85,7%). A significant elevation ($p<0.05$) was observed only in the mid-vertebral body height, where a mean increase of 5 mm (pre- to postoperative value) was achieved. Requirement of analgetics decreased in all patients immediately after the procedure. Clinically asymptomatic peroperative cement leakage occurred in 4 cases (28,6%). During median of the follow-up of 20,5 months, we didn´t observed any late complication or secondary fracture. *Summary*. With respect to our results we can support the percutanous kyphoplasty as an effective and safe procedure for the treatment of painful pathological fractures of multiple myeloma patients.

1610

BORTEZOMIB IN COMBINATION WITH DEXAMETHASONE FOR THE TREATMENT OF ELDERLY PATIENTS AFFECTED BY REFRACTORY MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a neoplastic disease especially affecting elder patients even if in recent years it has been also observed in younger patients. The use of the proteasome inhibitor bortezomib has been recently introduced in the treatment of relapsed and/or refractory MM. In fact, bortezomib has proven to be safe and effective in MM patients not only as monotherapy but also given in combination with cytotoxic agents. Bortezomib-based combination regimens have induced clinical benefits with manageable toxicities and may ultimately lead to improvement in the duration of response and survival of patients in the first-line setting. Aims. The objective of study was to evaluate the efficiency and safety of bortezomib in combination with dexamethasone for the treatment of elderly patients affected by relapsed/refractory MM. *Design and Methods*. In our institution we are following 33 elderly patients with stage II/III MM (18 F and 15 M, median age: 73 years, r.: 67-84 years). As first-line treatment all patients received Melphalan and Prednisone chemotherapy (10 out of 33 plus thalidomide). However, patients suspended chemotherapy after a maximum of 6 cycles of Melphalan and Prednisone (with or without thalidomide) for excessive toxicity even if they presented progression disease (PD) at the clinical re-staging performed with both serum marker evaluation and cytological examination of bone marrow blood. All the 33 patients underwent a treatment with bortezomib (1,3 mg/m^2 i.v. d. 1,4, 8, 11 every 21 days) together with dexamethasone (40 mg i.v. d. 1,4, 8, 11 every 21 days). *Results.* At a clinical re-staging performed after four courses from the beginning of bortezomib-dexamethasone combined administration a partial remission (reduction of M-component > 50-75%) was recorded in 28 out of 33 patients while the remaining was in steady disease (SD). Thereafter all patients received further four courses of therapy. At one month from the end of treatment 5 out of 33 patients achieved a complete remission (negative immunofixation) and the remaining showed a partial remission (PR). At the present, (month +24) nine patients show a progression disease, while four patients are in CR and the remaining in PR. *Conclusions*. Our results suggest that the combination of bortezomib and dexamethasone is effective and well tolerated in the treatment of refractory MM in elderly patients. Although there are several published data on the activity of the therapy based on the combination between bortezomib and dexamethasone, little is still known about the improvement in the duration of response and survival of elderly patients in the first or second line therapies.

1611

ALGORITHM FOR MONOCLONAL GAMMOPATHIES DIAGNOSIS. TO PROSPECTIVE STUDY OF INCIDENCE FROM OUR POPULATION

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Background. A protocol with a flow diagram form has been designed for the study of Monoclonal Gammopathy (MG), it follows the diagnostic criteria developed by "The International Myeloma Working Group 2003" and uses standard laboratory methods. Review of the samples sent to their Laboratory for protein electrophoresis to over to a 3-year period, between January 1, 2006 and December 31, 2008. *Design and Methods*. The study includes: 1.Protein: quantification of total proteins, albumin, globulins, ratio albumin/globulin and inmunoglobulins in serum and urine. 2. Serum protein electrophoresis (SPE), identification of the M-component (MC) by Inmunotyping (IT) to determine the isotype of heavy and slight chain. 3.Measures in serum levels of free lambda and kappa immunoglobulin light chains (FLC). 4.Estimation renal function: urea, creatinine and glomerular filtration ratio (GFR) in serum. β-2 microglobulin, cystatin and slight chains in serum and urine. 5.Anemia study: complete hemogram and erythrocyte sedimentation rate. 6.Osteolysis activity: calcium, total alkaline phosphatase and, if appropriate, determination of the alkaline phosphatase bone isoenzyme. 7.Protein electrophoresis in urine, with or without previous concentration to determine the presence of MC. Inmunotyping and measures levels FLC in urine. Modular Analytics D/P Hitachi/Roche, Capillary Electrophoresis/Sebia, Nephelometric BNII/ Dade Berhing, Advia120/Bayer Cytometer, Hydragel Iso-ALP/Sebia Results Following this protocol in order to detect MG, we extracted huge amount of dates from to single sample. These dates were than available for the clinician on the same day. No additional analytical applications or to parameter repetitions were needed. Incidence: Total SPE 10500, MC 3,7%: Monoclonal Gammopathy of Undetermined Significance (MGUS) 54%, Multiple Myeloma 28%, Lynphoma 4%, Waldeström´s Macroglobulinemia 3%, Amyloidosis 2%, Chronic Lymphoid Leukemia 2%, Light Chain Disease 1%, Others 6% *Conclusions*. The importance of the Laboratory in the monoclonal gammopathy diagnosis is: 1) To search and eventually characterize the M-Component. 2) To complete a study whose protocol includes: MC concentration, grade of anemia, estimation and monitoring of renal function and evaluation and monitoring of bone osteoclastic activity. These latter analyses are relevant to exclude myeloma and to detect a possible progression of malignant disease in the case, more frequent, of MGUS. This algorithm allows for efficient diagnosis or exclusion of multiple myeloma.

1612

ASSESSMENT OF DISPOSITIONAL OPTIMISM AND PERCEIVED STRESS RELATED TO A HEMATOLOGICAL DISEASE: MULTIPLE MYELOMA

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Background. Based on the observation of daily interactions with our patients, physicians and psychologists have noted a significant emotional distress in patients with multiple myeloma (MM), more evident than in other similar diseases. There are no data on literature assessing this important issue. With this in mind, we compared possible variables related with emotional distress in two hematological diseases, MM and chronic lymphocytic leukemia (LLC). *Aims*. To characterize the perceived experience in relation with the suffering of the disease and to identify the significance of the pain as aggravating factor of mod. *Design and Methods*. Patients where asked to answer a questionnaire on: 1) two pain scales, one related to the pain at the moment of the survey and the other one related to the pain in the last month, 2) two validated test, one about optimism (LOT-R)1 and the other one of perceived stress (PSS)2. Questionnaires where given to the patients at the outpatient clinic of MM of our center. Patients with chronic lymphocytic leukemia where used as controls. For statistical analysis SPSS 15.0 software was used. Chi-square test was used to compare qualitative variables and Mann-Whitney U-test was used to compare means between the two groups. Results Forty-one patients answered the questionnaire: 21 with MM and 20 with CLL. The mean age was 67 (MM: 65, range: 54-84; CLL: 70, range: 60-82, $p>0.05$). Fifteen percent of MM had more than 5 years of disease, 40% between 3 and 5 years, 40% between 1 and 3 years and 5% less than a year of disease. Of patients with LLC, 50% had more than 5 years of disease, 25% between 3 and 5, 20% between 1 and 3 and 5% had less than a year of disease. For the pain at the moment of the survey, MM patients had 3.75/10 points, whereas CLL had 0.5/10 ($p<0.0001$). With regard to the pain over the previous month, the mean value for MM was 4.5/10 and 0.75/10 for CLL ($p<0.0001$). The mean value of MM patients for optimism was 19.2/40 and for CLL patients was 25.9/40 ($p<0.0001$; statistic power: 96%). With regard to the stress perception, patients with MM had a mean of 19/28 points and patients with CLL, 15/28 points ($p=0.07$). *Conclusions*. Patients with MM show an important abnormality of variables that negatively influence the mod as well as the optimism and the perception of the stress (despite the fact that this variable was not significant, there was a clear tendency), probably related with pain that is difficult to control. Patients with MM show a pessimistic vision of their disease, with emphasis in the inability and frustration. We think that obtaining these of kind of objective data is of

paramount importance to the better understanding of our patients. In addition, it is necessary to emphasize the importance of the interdisciplinary management of the myeloma-related pain to improve the quality of life of these patients.

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1613

AGGRESSIVE THERAPEUTICS APPROACH INCLUDING IN HOME ASSISTANCE CHEMOTHERAPY IN MULTIPLE MYELOMA

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Background. Multiple Myeloma (MM), is a B-cell neoplastic disease characterized by bone marrow infiltration by malignant plasma cells which secrete monoclonal immunoglobulin fragments. Although several parameters such as β-2-microglobulin, serum creatinine, haemoglobin, calcium levels or cytogenetics abnormalities have been considered as predictive factors of the outcome of patients (pts) with MM, the molecular features of this disease remain still unclear. Consequently therapy for MM remains empiric. New agents have improved the possibility of obtaining disease response and better quality and duration of responses. Although response to therapy traditionally has not been considered a good predictor of long-term outcome, most recent studies suggest that response is a surrogate of improvement in survival. Aim:Here, we want to show that, despite relapse and therapy-toxicity, pts with MM must be always treated after relapse or in presence of progressive disease, and if possible also in home assistance (HA) chemotherapy, especially elderly ones, because survival increases in presence of PR, VGPR or SD rather than in presence of PD.*Design and Methods*. We report a retrospectively analysis of 181 pts (94 females/87 males, median age 58 ys) diagnosed with MM in our institution from 1980 to 2007. *Results*. Overall survival (OS) was influenced significantly by ISS (1 > 3 and 2 >3; and 1+2 >3), renal impairment (cr >1.5 mg/dl), β-2 microglobulin (>3.5 mg/dl), LCh-MM vs not LCh-MM, and haemoglobin <10 g/dl, autologous or allogeneic transplamtation ; bone lesion or fractures, isotype κ/λ, precedent MGUS and the presence of Amiloid AL didn't influence survival. Pts who achieved PR or VGPR after first line therapy (47%) had an OS longer than pts in progressive disease (PD) (31.5%) or stable disease (SD) (21.5%), as pts with PR + VGPR vs PD; also after second and third line therapy. OS of pts in HA chemotherapy was 13 months from the moment of enrollment in home assistance chemotherapy. We treated 35 pts in HA chemotherapy and the median survival (ms) was 52.2 months vs 36 months of pts not in HA chemotherapy, matched for the same characteristics. 18 pts of these are still alive (ms 65.7 months vs pts not in HA chemotherapy ms 47.5) but our data are still limited and the comparison between the two groups doesn't achieve statistical evidence(p=0.07). *Conclusions*. We can only suggest that pts with PD or relapse must be treated also in HA chemotherapy, if elderly, to achieve at least a SD.

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VERTEBROPLASTY IMPROVES QUALITY OF LIVE IN MYELOMA PATIENTS

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Background. Back pain is common in myeloma patients and the incidence was found to be 40-60% (1) and new patients may even present with vertebral collapse (2).Treatments for this include analgesia and or invasive orthopaedic surgery. A new and considerably less invasive intervention is percutaneous vertebroplasty (PV) (3). In PV acrylic bone cement is injected into the vertebral body by a percutaneous approach under X-ray guidance in order to stabilize the vertebra and ultimately to reduce pain. Whilst there are trials in osteoporosis ongoing (4) we are not aware of any studies published so far looking into quality of life (QOL) issues, the good pain control has been demonstrated in several other studies in myeloma patients (7,8,9), some with larger patient groups. *Aims*. to assess the effectiveness of PV for pain relief and QOL in myeloma patients. *Design and Methods*. We assessed QOL and effectiveness of pain control via the EQ-5D health questionaire. The EQ-5D, established in 1987, is a simple measure of health status comprising a visual analogue scale(VAS) and a descriptive system with the five dimensions mobility, self-care, usual activities, pain, anxiety/depression(5). This information was obtained by direct telephone contact or in presence of the patient at pre-assessment, 12-24 hours post procedure, 1 week post-procedure and at 1,2,6 and 12 months post-procedure. In addition we gathered the following information from the patients' clinical case notes: age at procedure, time from diagnosis to procedure, type of myeloma, number of previous chemotherapy regimens (including bone marrow transplant), time from referral to X-ray to procedure, reduction in analgesia after the procedure, complications from the procedure. Results: The number of patients who had PV is n=8, the mean age was 70.4 years (range from 60-85) and the time from diagnosis of myeloma to the procedure was 51.4 months mean (range 7-154 months). Four patients were IgA and four IgG myeloma, the mean number of previous treatment course was 2.75 with 4 patients having had a autograft and one had auto- and allograft. The mean time from referral to procedure was 3.8 months. Most important for our study: 63% of patients (n=5) subsequently were able to reduce their analgesia with 3 patients coming completely off any analgesia. There were no complications in any patient. The EQ-5D results showed an average VAS score of 59.0 prior to procedure (the average for healthy people in that age group ranges from 79.8 to 72.5 in the UK population(6)) and increased to 72.7 immediately after and to 79.0 at 1 week after the procedure. There is then a slight drop to 78.5, 77.8, 74.3 and 70.0 at 1month,2 months,6 months and 12 months (but there were only 3 patients in this group) respectively. The mobility index increased by one point in 4 of 8 and was sustained for 6 months. The self-care index did not change due to the procedure(but all our patients were outpatients anyway which may explain their relatively high degree of independence). The usual activities index showed improvement over time from an increase in 3 patients to 6 patients at 2 months but then declined again to 3 patients. The pain index improved in 5 patients and was sustained in 4 patients for up to 6 months, at 12 months still 2 of 3 patients felt an improvement in their pain score. The anxiety/depression showed improvement in 4 patients which was sustained up to 6 months and at 12 months still 3 of 3 patients showed improvement. *Conclusions*. PV showed the potential to reduce their objective analgesia requirement in more than 60% of our patients confirming other studies. Nobody however has bevore shown an improvement in Qol which we were able to demonstrate with the VAS score returning to normal for the respective age group as well as an improvement in their subjective health status regarding pain and anxiety/depression. This was sustained for at least 6 months. Our group is small with so far short follow-up: however with additional information on 8 more patients pending, these results will make PV results especially interesting from a pharmaco-economical and health-economical point.

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PREDICTIVE VALUE OF SERUM FREE LIGHT CHAINS RATIO IN RELAPSED OF MULTIPLE MYELOMA AFTER AUTOLOGOUS TRANSPLANT

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Background. The International Myeloma Working Group Uniform Response Criteria, included the normalization of serum free light chain ratio (FLC) as a criteria of stringent complete response. The quantitative assay Freelite®, is an easy and reproducible method to determine serum concentration of FLC, and could be a good biomarker to predict the early relapse in Multiple Myeloma patients in apparently complete response. *Aims*. To determine the predictive value of the abnormal ratio serum FLC in detect early relapsed in MM patients in complete response after autologous stem cell transplant. *Design and Methods*. Observational, analytical study performed in 27 MM patients included in an autologous stem cell transplant program in 2004-2008 in one center. All patients receiving induction therapy with alternant VBCMP/VBAD (x4), mobilization and collection of $CD34^+$ cells was performed after the second VBAD therapy and conditioning with high doses of Melphalan. The infussion of $CD34^+$ cells was performed with a mean of cells In all cases were recollected serum samples in days 0,+4, +7, +21, +30, +60 y +90 and stored frozen at -80ºC. We have analyzed serum FLC in a nephelometer system following the manufacturer instructions. We have compare the increase of FLC according M-component subtype, number of $CD34^+$ cells administered, time to immune reconstitution, administration of coadyuvant AM3-glycopeptical therapy, infections in early postrasplant period, maintenance therapy and time free relapsed survival. Descriptive statistical analysis, ANOVA comparative test and Kaplan-Meier survival study was performed. *Results*. 27 patients, 47.0% females, mean age 63.2 y (38-72), IgG(46.1%), IgA (15.4%), light chains (23.0%), non secretory (7.7%), mean CD34+ cells infused $5.39x10^6$/kg (2.24-18.8), time to immune reconstitution 15-17 days, ratio of κ to λ at baseline 57.0 (CI95% 0.8,20.3), after 4 days: 37.3(CI95% 0.1,3.2), +7: 1.85(CI95% 1,3), +21 2.37 (CI95% 0.4,2.0), +30 0.87 (CI95% 0.2,0.9), +60 0.4 (0.2, 2.1) and +90 1.26 (CI95% 0.3, 2.9). Mean relapsed free survival 27.6 m (CI95% 9,55), OS 59.0 m (CI95% 27,76), 7 patients have dead for progression. At day +21 correlation κ/λ ratio and RFS was observed. *Comments*. The early determination of the ratio free serum free light chains could be a good biomarker to predict RFS and OS in patients with Multiple Myeloma in complete response after autologous transplant.

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A CASE SERIES OF SIXTH NERVE PALSY ASSOCIATED WITH INTRACRANIAL PLASMACYTOMA.

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Background. Multiple myeloma (MM) are rare causes of lesions of skull base and cavernous sinus. We present a series of four patients whose initial presentation included a VI cranial nerve (CN VI) palsy. Aims: We analyzed clinical presentation and outcome of patients with MM and CN VI palsy. *Results*. Over the period of 3 years we collected data of 4 patients with MM and CN VI palsy. Case 1: A 49 year old patient presented with a right CN VI palsy. CT and MRI brain revealed an enhancing soft tissue mass in the right side of the brain stem with extensive bony destruction with invasion of the right cavernous sinus and numerous osteolytic lesions and was diagnosed with MM. Patient had radiotherapy and 4 cycles of bortezomib and dexamethasone. Her CN VI palsy completely resolved. Stem cell collection and autologous stem cell transplant followed and patient is doing very well, her disease markers are stable. Case 2: A 64 year old lady with IgA myeloma completed 8 cycles of VMP while developed signs of CN VI palsy. MRI confirmed the presence of myeloma deposit in the left cavernous sinus associated with bone destruction. At that time she had stable that rapidly progressed. Patient had radiotherapy and afterwards was started on systemic therapy. She died shortly afterwards with a progressive disease and sepsis. Case 3: A 69 year old man had been admitted to regional hospital with double vision. He had multiple co-morbidities. MRI brain showed massive tumour of base of the scull. The bone marrow aspirate and skeletal survey were consistent with MM. Initially patient was treated with radiotherapy, however developed acute renal failure complicated by multiorgan failure and died in intensive care unit. Case 4: A 70 years old female with 2 years history of IgG MM presented with diplopia and progressive right CN VI palsy. Subsequently she developed right CN III palsy. First MRI of brain was not diagnostic. A repeated MRI revealed a cavernous sinus lesion. Initial treatment consistent of radiotherapy and dexamethasone and subsequently bortezomib. Due to complications she was changed to thalidomide. Due to progression patient died shortly. Discussion: In our series, three patients had a lesion of cavernous sinus. In one patient there was a direct extension of the skull lesion. The initial MRI was not diagnostic in one case. Two patients who developed CN VI palsy in the course of disease progressed rapidly and had a poor outcome. One patient who presented with *de novo* MM and CN was treated successfully with regimen containing bortezomib and stem cell transplant. As MM is a potentially treatable disease, it should be included in the differential diagnosis of cranial nerve palsies, especially among those who demonstrate a progressive course or multiple cranial neuropathies. MRI is a diagnostic modality of choice, however a negative result does not exclude the cavernous sinus lesion and the imaging should be repeated if clinically indicated. Therapeutic approach can incorporate local radiotherapy as well as systemic chemotherapy including bortezomib.

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THERE IS NO PLACE LIKE HOME: A PILOT PROGRAM OF HOME ADMINISTRATION OF BORTEZOMIB AT GALWAY UNIVERSITY HOSPITAL

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Introduction. Evidence shows that combinations of previously standard therapies with novel agents have improved outcomes for patients with multiple myeloma. Bortezomib is one of the novel therapies that is proven to improve overall response (OR), complete response (CR) rates and progression free survival. Routinely bortezomib is administered intravenously in the day ward facility, twice a week over a two week period with a ten day rest period. In the West of Ireland many patients need to travel long distances to the regional haematology day ward and as a result experience significant inconveniences. Aims: As bortezomib is increasingly used and has significant impact on capacity of haematology day-ward we decided to look for an alternative arrangement for the administration of bortezomib. The purpose of this was to increase the accessibility for the patients, reduce inconvenience related to treatment and ultimately improve the patients' quality of life. After two years of preparation we established a pilot scheme for the administration of bortezomib at home. *Design and Methods*. Organisation of the home administration of bortezomib: Home administration of bortezomib is coordinated by a consultant haematologist. Administration is delegated to an external company, which employs qualified nurses and provides complete evaluation of home conditions, and suitability for administration of bortezomib at home. Bortezomib is supplied from the hospital pharmacy. Patients who are identified as being suitable for the home administration of bortezomib attend the Haematology Day Ward on day 0. Clinical examination and evaluation of relevant laboratory results are carried out and chemotherapy is prescribed. The prescription is sent to pharmacy and the administering company. Patient receives information regarding possible side effects and therapy related risks. On the days of administration the nurse collects bortezomib and performs systematic clinical evaluation for side effects and detection of contraindications for the administration of bortezomib. In addition, on the day 8 of each cycle full blood count and biochemistry samples are collected. Relevant documentation of each administration is provided and results of laboratory tests are sent to the treating consultant haematologist. Patient is routinely evaluated on day 21 prior to next cycle of therapy or more frequently if clinically required. Results: To date 9 patients have been enrolled to the program and there has been 60 home administrations of bortezomib. All of them were successful. Two patients were withdrawn from the program due to non-myeloma related medical issues. No significant complications of therapy were observed. The feedback from patients is very satisfactory and encouraging as they find the service is convenient and benefits their quality of life. *Conclusions*. In our experience the program of home administration of bortezomib is safe and efficient in administration of a potent chemotherapeutic agent to more patients, who otherwise could potentially experience delays in accessing the treatment.

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MULTIPLE MYELOMA: EVALUATION ON THE KNOWLEDGE OF PRIMARY HEALTH ATTENTION DOCTORS IN RESPECT TO THE DISEASE

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Background. Multiple Myeloma (MM) patients frequently show non-specific and high prevalence symptoms such as bone pain and anemia. These symptoms can be treated as distinct medical circumstances if the professional doesn't consider MM as an option for distinguishing diagnosis, leading to unnecessary interventions and delaying the correct diagnosis. Being the doctor who works in primary attention the one that generally first sees the patient, it is important that he recognizes the most common characteristics of this pathology, requests examinations for diagnostic confirmation and carries through the correct guiding. Aims:To verify the clinical knowledge on MM in general clinical doctors from the units of primary health attention in Belo-Horizonte, MG/Brazil. *Design and Methods*. Observational and transversal study of field research through the use of "best answer" multiple choice test to be answered by a doctor from each one of the 137 units of primary health attention in Belo-Horizonte from October to December, 2006. The applied instrument was composed of 5 questions that approached basic aspects in the diagnosis and handling of the patient suffering from MM. The descriptive analysis of the variables for characterization of the studied group was made through the distribution of frequency, medium and average. The knowledge on the disease was defined on the basis of the percentage of correct answers to the questionnaire, as well as the questions with higher rates of rightness and errors, delimiting the difficulties and the previous knowledge of the interviewed ones. The test of quisquare was used to compare the frequency of the categorical variables and the analysis of variance (ANOVA) with LSD correction for the continuous variables. *Results*. The time since graduation of the evaluated doctors varied from less than 1 year to 37 years (M=11,1/ D*p*=9,3). In relation to how long doctors had been working in primary attention, it varied from 1 month to 30 years (M=7,84/ D*p*=7,37). Most of them (61,5%) had some type of specialization, being The Medicine of Family and Communities (27,7%) the most frequent. In the evaluation on knowledge, most of the studied population (94,5%) correctly concluded that for an aged patient with anemia, the evaluation of chronic and neoplasics diseases is necessary. 61,4% of the evaluated doctors could not identify MM as the main diagnosis for an aged patient with characteristic osteolytic bone injuries in the x-ray.59,3% had not known how to diagnose hypercalcemia for a clinical case with classic manifestations of this metabolic clutter. 71,1% had not known the correct interpretation of eletrophoresis of serum proteins in MM. Only 37% had formulated hypothesis of MM for a typical clinical case. These results had not been influenced by the time since graduation, how long doctors had been working in primary attention or presence of specialization. *Conclusions*. The evaluated professionals had shown laboratorial and clinical unfamiliarity with MM; this unfamiliarity was homogeneous in all net of primary attention in Belo Horizonte, not being influenced by time since graduation, how long doctors had been working in primary attention or presence of specialization.

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SAFETY AND EFFICACY OF LIPOSOMAL DOXORUBICIN ADDED TO LOW DOSE BORTEZOMIB AND DEXAMETHASONE IN ADVANCED REL/REF MULTIPLE MYELOMA

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Background and Aims. Despite the significant improvement related to the introduction of new drugs, the treatment of relapsed multiple myeloma (MM) represents a challenge, especially in patients with a low performance status, due to age, concomitant diseases and toxicity due to previous chemotherapy. In order to improve outcome in this subset of patients and reduce the therapy related toxicity we planned a 3-drugs combination adding liposomal encapsulated doxorubicin to dexamethasone and low dose Bortezomib. Bortezomib and anthracyclins can reciprocally increase their efficacy, as already demonstrated *in vitro* and *in vivo*. In comparison with standard doxorubicin, liposomal and pegylated liposomal doxorubicin show comparable efficacy and lower cardiac toxicity. Furthermore liposomal doxorubicin is reported to reach higher concentrations in bone marrow and is not associated with severe palmar-plantar erythrodysesthesia (hand-foot syndrome) reported with the pegylated form. *Design and Methods*. Starting from July 2006 14 patients with poor performance status referred to our institution for relapsed MM were treated with bortezomib 1.0 mg/mq i.v. twice weekly for 2 weeks (days 1,4,8,11 of each cycle) in a 21 days cycle, oral dexamethasone 20 mg on the day and the day after bortezomib and liposomal doxorubicin (Myocet®) 50 mg/mq i.v. on day 4 (30 mg/m^2 for patients older than 75 yrs). Response was defined according to modified EBMT criteria (*Bladé et al, 1998*). Responsive patients continued treatment until a maximum of six cycles. After the completion of the schema Thalidomide 100 mg/die p.o.continously was added for patients who had not presented previous unacceptable toxicity.

Table 1. Patients characteristics.

Age (years), median		65 (55-79)
Relapsed		11 (79 %)
Refractory		3 (21%)
Durie-Salmon stage III		9 (64%)
Prior tratment		2 (1-6)
Treatment results:		2 CR (14%)
		1 nCR (7%)
		2 VGPR ((14%)
		7 PR (50%)
		2 SD (14%)
Toxicity: haematological	gr 1-2	2 (14%)
	gr 3-4	4 (29%)
Toxicity: neurological	gr 2	4 (29%)

Results. Clinical characteristics of the patients are described in Table 1. Hematological toxicity was present in 42% of the patient, whereas transient neurological toxicity developed in 4 patients (29%). No case of DVT or cardiac failure was recorded. Overall 12 out of 14 patients (86%) responded, with 2 CR, 1 nCR, 2 VGPR and 7 PR. All partial responses (PR) exhibited a >70% reduction in monoclonal component (MC) ; 2 patients showed SD. Despite the high response rate, PFS was very short for 8 out of 14 patients, who experienced disease progression in a median of ten weeks after completion of therapy. Four patients maintained response at 6+ (CR),8+ (VGPR), 8+ (VGPR) and 20 (CR) months. *Conclusions*. According to our experience the combination of Bortezomib, Dexamethasone and Myocet® is a very feasible treatment for relapsed MM in frail and heavily pretreated patients, showing a high overall response rate (86%). Hematological toxicity was manageable and extrahematological toxicity was negligible so that all patients were treated on an outpatient basis. Nevertheless a high proportion of responding patients (67%) experienced early progression of disease, despite the apparently satisfactory quality of response (6 PR with >70% MC reduction, 1 VGPR, 1 nCR). Conversely four patients (33%) experienced a durable and stable response. Whereas the early relapsing patients reached their best response after 4-6 cycles of therapy, all the long lasting responders had reached at least a VGPR (>90%) after only 2 cycles, suggesting that early acquisition of VGPR rather than quality of best response could predict progression free survival.

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DOUBLE AUTOLOGOUS STEM CELL TRANSPLANTATION IN POEMS SYNDROME

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Background. POEMS syndrome is a rare multisystemic disease related to a plasma cell dyscrasia and characterized by the association of Polyneuropathy, Organomegaly, Endocrinopathy, M protein and Skin changes. POEMS is a potentially fatal disease and patients' quality of life deteriorates because of progressive neuropathy, massive peripheral edema and multiorgan failure. We described a case of POEMS syndrome treated with high dose chemotherapy and double autologous peripher-

al stem cell transplantation (PBSCT). *Design and Methods*. We observed a 30 years-old woman with POEMS syndrome associated with Castleman disease. She presented a severe and rapidly progressive sensorymotor polyneuropathy with inability to walk, monoclonal component IgA l, splenomegaly, melanosis and thrombocytosis. Results of bone marrow biopsy showed mild plasmacytosis (10%). She was previously treated with high dose intravenous immunoglobulin in the neurologic unit without any clinical benefit. The patient received cyclophosphamide 2 gr on day 1,3, dexamethasone 40 mg on days 1-4 for 2 cycles and G-CSF 5 mcg/kg/day was added for mobilization. Time from diagnosis to first transplantation was three months. At time of first PBSCT the patient showed a severe neurologic involvement (Modified Rankin Scale 4). Melphalan 140 mg per square meter of body surface area was administered intravenously as conditioning regimen followed on day 2 by reinjection of the cells collected by leucapheresis. The number of CD34+ cells infused was $7.1x10^6$/kg. All the procedures for PBSCT were well tolerated and the post-transplantation period was uneventful, except for an episode of neutropenic fever without bacteriological documentation that resolved under broad-spectrum antibiotics. Five months later, the patient underwent a second consolidation PBSCT using the same conditioning regimen. The same number of CD34+ cells were infused. Any complication occurred in the period after the transplantation procedure. *Results.* After the first PBSCT we observed slow but progressive improvement in neurological disease, skin changes and performance status. After the second PBSCT splenomegaly was resolved and laboratory analyses showed negativization of monoclonal component, absence of plamacytosis at bone marrow biopsy and absence of trombocithosis. After one year from transplantation, the patient could perform their daily activities autonomously and had gone back to their social and working life. *Conclusions*. To our knowledge, between 1998 and 2008, 42 patients with POEMS have been successfully treated with autologous bone marrow transplantation. The goal of PBSCT in POEMS syndrome is to achieve remission of both the haematological and the systemic manifestations. Our case confirms usefulness and safety of this procedure in this disease and supports the idea that PBSCT should be considered early during the disease course to obtain the best response.

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EFFICACY AND SAFETY OF LENALIDOMIDE IN COMBINATION WITH DEXAMETASONE IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA

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Background. Lenalidomide is an active immunomodulatory agent recently approved for relapsed or refractory multiple myeloma (MM) in combination with dexametasone but there is yet little experience of its use outside of clinical trials. Aims: To evaluate the efficacy and safety of the combination lenalidomide-dexametasone (L/D) as rescue therapy in relapsed or refractory MM in unselected patients. *Design and Methods*. All the patients with MM followed in our department from the 1st January 2007 to the 31st January 2009 were retrospectively reviewed. The patients with relapsed or refractory disease after two or more therapeutic regimens that underwent treatment with L/D (25 mg of daily oral lenalidomide on days 1 to 21 of each 28-day cycle and 40 mg of weekly oral dexametasone) were selected. They all received anti-thrombotic prophylaxis with low molecular weight heparin. Treatment was continued until the occurrence of disease progression or unacceptable toxic effects. Efficacy was assessed by the response rate and time to progression (TTP). Complete response (CR) was defined as the disappearance of both monoclonal serum protein (tested by IF) and urinary component. Partial response (PR) was defined as a 50-99% monoclonal serum protein reduction and 90% reduction in urinary component. Minor response (MR) was defined as a reduction of 25-50% and non response (NR) as an inferior reduction or progression. Response was assessed after at least one L/D treatment course. Safety evaluations were coded using the WHO scale. Statistical methods: descriptive, χ^2, Fisher exact test, Kaplan-Meier tables, log-rank test and Cox binary logistic regression. Results. Seventeen patients were included, with a median age 76 years (47-80) and a median time from diagnosis of 3,1 years (0,3-14,5). The median number of prior therapies was 4 (2-13): 53% had previously undergone an ASCT, 100% had been treated with Bortezomib and 23% with thalidomide. The reason for therapy with L/D was relapse in 7 cases (41%) and refractoriness to prior therapy in 10 (59%). 16 patients were assessable for response: 2 had obtained CR, 12 PR (CR+PR: 87%) and 2 NR. Two patients with PR underwent a ASCT after 4 cycles of L/D, in one case and after mobilization with cyclophosphamide and filgrastim. The median TTP from the beginning of L/D was 10 months and median survival of 15 months; 7 patients are alive at end of study with a median observation of 8 months. Association of response, TTP and overall survival with clinical variables such as age (>76 years versus equal or minor 76), gender, reason for treatment, monoclonal component type, performance status and previous therapies was analysed. No statistically significant relationship was found in any case. Nevertheless there was a trend of a superior median TTP in patients with 4 or less prior lines of treatment (11 versus 4 months) and in the previously treated with thalidomide (11 versus 2.6 months). The most common toxicity was hematological with neutropenia or thrombocytopenia grade 3 or 4 in 6 patients (35%). It was more frequent in those with 4 or more prior lines of therapy (p=0.04) or prior ASCT (p=0,009). However age was not associated with hematological toxicity. There was a pulmonary embolism in one case, but therapy with L/D could be continued. On the other hand, therapy was discontinued due to facial angioedema in another case. No febrile neutropenia events were registered. G-CSF support was required in 5 patients. *Summary and Conclusions*. In our experience L/D has a remarkable efficacy in response rate and TTP in relapsed or refractory MM, even in elderly patients with several previous treatment lines (half of them with prior ASCT). Main adverse events were mostly hematological and controllable.

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AN IN VITRO CULTURE SYSTEM FOR HIGH-THROUGHPUT SCREENING OF ANTI-MYELOMA DRUG CANDIDATES

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Background. Long-term cultures of MM cells are hard to establish in the absence of additives like cytokines or feeder cells that could alter the outcome of drug tests. Similarly, their engraftment in animal models has not been prominent. Hence, a high number of drug candidates with anti-myeloma potential are accumulating waiting to be tested. *Aims*. Establishment of primary BM cultures from MM patients in the absence of additives to improve our capacity to evaluate drug candidates in high-throughput assays leading to a more personalized medicine. *Design and Methods*. Bone marrow aspirates samples from 10 MM patients at different disease stages were cultured in complete medium (RPMI1640, 10% FCS, 1% pen-strep, non-essential amino acids, L-glutamine, and sodium pyruvate) in 75 cm^2 flasks following red cell blood lysis. Confluent cultures were trypsinized and expanded in larger flasks. Subsequently, adherent cells were tested for CD138 expression. In parallel, a cell drop was cytospined and stained by May-Grunwald Giemsa. *Results*. The bone marrow cells expanded *in vitro* generating confluent cultures of adherent cells after 3-4 wks. Subsequent trypsinization and replating of primary BM cells generated 3 weeks later a new layer of adherent cells. BM cells derived from patients at partial remission (PR) or relapse contained a large population (24% to 42%) of CD138 positive cells, whereas BM cells from patients in remission show only background levels of the plasma cell marker. Histology revealed the presence of plasmacytoid cells with typical eccentric nuclei and golgi halo in the BM cultures derived from patients in PR and relapse, but not from patients in remission, suggesting that the previous (before culture) disease state was conserved. *Conclusions*. This *in vitro* culture system will be useful for screening novel drug candidates in high-throughput assays and also will facilitate studies on several aspects of myelomagenesis.

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A PHASE I/II CLINICAL STUDY TO INVESTIGATE EFFICACY, SAFETY AND PHARMACOKINETICS OF LENALIDOMIDE WITH/WITHOUT DEXAMETHASONE IN JAPANESE PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA

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Lenalidomide is an immunomodulatory drug which has been approved in USA, EU, and other countries for the treatment of relapsed and refractory multiple myeloma (MM), but it has not yet been introduced into clinical practice in Japan. We conducted a multicenter phase I/II clinical study to investigate the maximum tolerable dose (MTD), efficacy, safety and pharmacokinetics of lenalidomide administered alone or in combination with dexamethasone (Dex) in Japanese patients

with MM. Fifteen patients with relapsed and/or refractory MM were enrolled. This study was composed of two stages, i.e., the MTD-determining stage and the subsequent treatment stage. In the MTD-determining stage, lenalidomide (10 mg or 25 mg) alone was administered once a day for 21 days to confirm the safety. In the treatment stage, lenalidomide was administered alone or in combination with Dex for 21 days in each 28-day cycle to investigate the efficacy, safety and pharmacokinetics. Coadministration of Dex was started after determining the MTD. In the MTD-determining stage, 3 patients were enrolled to the 10-mg single cohort and then 6 patients to the 25-mg single cohort. In the treatment stage, 6 patients were newly registered to the combination cohort. In the 10-mg cohort, no dose-limiting toxicity (DLT) was observed, and in the next 25-mg single cohort, grade 3 toxicity (hypoxia) was observed in one out of 6 patients. Based upon the observation, 25mg was chosen as the dose for next stage. Regarding the efficacy evaluation throughout the MTD stage and the treatment stage, all of the 6 patients who received the combination treatment showed good response; two of the 6 patients achieved complete response (CR), 4 patients achieved partial response (PR). One patient of the 10-mg single cohort achieved minimum response (MR). Three patients of the 25-mg single cohort achieved PR, and one patient did MR. In safety evaluation, hyperglycemia of grade 3 was noted in one patient of the combination cohort, but neither grade 4 adverse event nor thromboembolic event was observed. In pharmacokinetic evaluation, it was found that the pharmacokinetic profile of lenalidomide was similar between Japanese and Caucasian MM patients and it was also confirmed that coadministration of Dex did not have an significant impact on the pharmacokinetic profile of lenalidomide. In conclusion, this study demonstrated the MTD of lenalidomide to be 25 mg daily in Japanese as well as Caucasian MM patients and the combination therapy of lenalidomide with Dex could be sufficiently effective in the clinical practice. In addition, the adverse events associated with this treatment were well-known and manageable

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THE USEFULNESS OF INTERPHASE FISH AT DIAGNOSIS OF MYELOMA IN ADDITION TO METAPHASE CYTOGENETICS

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Background. Multiple myeloma is characterized by the accumulation of malignant plasma cells within the bone marrow and regarded as incurable, but remissions may be induced with steroids, chemotherapy, thalidomide and stem cell transplants. The clinical heterogeneity of myeloma is dictated by the cytogenetic aberrations present in the clonal plasma cells. Fluorescence *in situ* hybridization (FISH) overcomes the limitations of standard cytogenetics and allows for the detection of numerical and structural chromosomal abnormalities in both metaphase spreads and interphase nuclei. *Design and Methods*. We evaluated the chromosome abnormalities in 34 MM patients using conventional cytogenetics and interphase FISH with 6 probes such as IGH/CCND1, IGH/FGFR3, IGH/MAF, DS13S319/LAMP1, IGH/BAP, and p53/CEP17. Results: Cytogenetic abnormalities were found in 24 (70.6%) of the 34 MM patients. 10 (29.4%) patients had abnormal metaphases by conventional cytogenetics. Interphase FISH results were abnormal in 21 (61.8%) patients and 11 (32.3%) patients had abnormal interphase FISH but normal metaphases. The evidence of the loss of D13S319 with or without loss of LAMP1 was found in 6 (17.6%) patients, and loss of p53±CEP17 for 2 patients, IGH-BAP for 9 (26.5%) patients, IGH/FGFR3 for 2 patients, and IGH/CCND1 for 7 (20.6%) patients, respectively. However, there were none positive for IGH/MAF. Chromosome 13 abnormalities and IGH rearrangement is correlated with poor clinical outcome. *Conclusions*. Interphase FISH can provide useful information to evaluate the presence of prognostic chromosome abnormalities in addition to metaphase cytogenetics. And it should be used in the routine evaluation of multiple myeloma.

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DIFFERENT PATTERN OF RELAPSE IN MULTIPLE MYELOMA (MM) PATIENTS TREATED WITH NOVEL AGENTS

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Background MM's malignancy of terminally differentiated antibody-secreting plasmacell, homes to and expands in the bone marrow(BM), where it causes a constellation of disease manifestation including BM suppression, osteolytic bone destruction and end organ damage. It's mostly confined to medullary sites and its predominant clinical manifestations are generally related to BM infiltration and destruction of bone. However extramedullary involvement is seen in some patients. During the main course of disease evolution, MM cells depend on BM microenviroment for their growth and survival. Reciprocal interactions between MM cells and BM mediate not only MM cell growth but also protect them against apoptosis and cause bone disease and angiogenesis. Newer therapies, such as thalidomide and bortezomib, capitalize on recent advances in our understanding of biology of MM, including molecular mechanisms by which MM cell-host BM interactions regulate tumor-cell growth, survival and drug resistance in the BM microenviroment. Thalidomide's orally bio-available glutamic acid derivative which inhibits production of TNF-α and angiogenic cytokines such as basic fibroblast growth factor (bFGF) and/or VEGF. Bortezomib's intracellular proteolytic system which regulates degradation of broad spectrum of intracellular proteins, including diverse regulators of cell proliferation (e.g., cyclin-dependent kinase inhibitor proteins) or apoptosis. Aims To describe different pattern of relapse in MM patients treated with thalidomide or bortezomib. *Design and Methods*. We treated, in 48 months, 37 MM patients. M/F was 19/18, median age was 65 (47-90). All patients showed advanced disease (III stage): 15/37(40%) with IgG/k type, 8/37(21%) IgG/l type, 4/37(10%) IgA/K and 5/37(13%) IgA/l type, 1/37(2%) IgD and 3/37(8%) micromolecular type. Patients treated with Thalidomide plus dexamethasone or MP regimen are 14/37(37%), with Bortezomib plus Dexamethasone or Caelyx-Dex regimen are 20/37(54%), with combination Thalidomide and Bortezomib plus dexamethasone or MP regimen are 3/37(8%). Results In our retrospective analysis we observed that 11/20(55%)MM patients treated with Bortezomib presented relapse. 3 of these patients(27%) had relapse with extramedullary but not BM and bone litic lesion involvement. Today 2/3(66%) patients are alive and salvage therapy ongoing. We also evidenced that 5/14(35%) patients treated with thalidomide showed relapse. Two of these patients(40%) had both BM and extramedullary involvement. Today these patients are died. All three patients treated with associated regimen are alive and in complete remission with median follow-up of 19 months (12-24). *Conclusions*. Prognosis and treatment of MM has evolved greatly over the past decade. Development and incorporation of new agents such as immuno-modulators and proteasoma inhibitors into therapy has improved outcomes and is helping patients enjoy longer periods of remission. Their combination are being evaluated to better manage in MM population as our few data confirmed. Despite of these, in follow-up of patients treated with Bortezomib, we suggest to evaluate possibility of extra-medullary disease even if BM biopsy and skeleton radiography are negative. Actually we don't know the pathogenetic mechanism. We speculate that with bortezomib, plasmacellular clone escape from drug control not microenvironment- related. Probably this control hold over in BM and bone compartment where bortezomib continue to inhibit binding of myeloma cells to BM stromal cells and BM triggered angiogenesis.

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MULTIPLE MYELOMA: NEW ATTAINMENTS IN RUSSIAN LABORATORY MEDICINE

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Background. Multiple myeloma (MM) is a group of hematological malignancies characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of monoclonal component (MC) or Bence-Jones protein (free monoclonal kappa or lambda light chains). Cytogenetic and molecular deletion of chromosome 13 is associated with poor prognosis of MM. Last years the appearance of new therapeutic agents provokes revisions in guidelines of laboratory diagnostics of MM. The aim of this study to describe peculiar biological properties of russian patients with MM. *Design and Methods*. 360 patients with median age 63 (44-83) years diagnosed with *de novo* MM were enrolled into this study. Microscopy of bone marrow smears, protein electrophoresis and serum/urine immunofixation (Paragon, Beckman Coulter, USA), serum immunoglobulins (IgA, IgM, IgG) (Immage, BC), serum/urine κ and λ light chains, β-2-microglobulin (Immage, BC),

molecular genetics (FISH) and immunophenotyping (IPT) of plasma cell by multiparametric flow cytometry (Cytomics FC500, BC) using a panel of monoclonal antibodies: CD45-FITC, CD38-FITC, CD138-PE, CD19-PC5, CD20-PE, CD56-PE, CD117-PC5 (BC) were performed. *Results*. MC IgG and κ chain is the most often MC and was identified in 48% cases, and IgG/λ and IgA/λ both 12%, IgA/κ 9%, IgM/κ 2%, IgA 5%, λ 5% and κ 7%. The karyotype was without cytogenetic abnormalities in the most cases (91%). There were detected trisomy 7 (3% of cases), del RB1 (3% of cases), del 14q (3% of cases). The del(13)/-13, t(4;14) and p53 by FISH were not identified in all cases. Immunophenotype profile D38+CD138+CD56+ and/or CD117+ was identified only in 51 cases. All cases with Bence-Jones myeloma demonstrated the absence of CD56 and/or CD117 expression on transformed plasma cells. *Conclusions*. In the era of growing progress in multiple myeloma treatment we need the modern laboratory tests with quality control endorsement. Our report describes peculiar biological properties of MM in the north-west region of Russia with cytogenetic data, flow cytometry phenotype of transformed plasma cells and the type of MC.

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BORTEZOMIB-INDUCED SEVERE HEPATOTOXICITY DURING PROGRESSIVE MULTIPLE MYELOMA: A MEDICAL CHALLENGE TREATED SUCCESSFULLY WITH LENALIDOMIDE

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Background. Multiple myeloma (MM) remains incurable with conventional treatments, with a median survival duration of 3-5 years. Treatment with the proteasome inhibitor bortezomib or immunomodulatory drugs such as thalidomide and lenalidomide represent an important advance that improves outcomes for patients with MM, but their side effects remain a concern. A recent review of PubMed (February 20, 2009) revealed only one patient with MM who developed bortezomib-induced severe recurrent hepatitis (*Rosiñol et al. Arch Intern Med 2005;165: 464*). Aims: To present a second case of bortezomib-induced severe hepatotoxicity during progressive MM successfully rescued with lenalidomide. *Design and Methods*. A 60-year-old man was diagnosed as having stage IIIB IgA-κ MM with a high level of Bence Jones proteinuria (more than 2 g/day). An abdominal subcutaneous fat biopsy detected no amyloid. Electrocardiographic findings showed atrial fibrillation with a rapid ventricular rate, and echocardiographic findings disclosed a left ventricular ejection fraction of 68% without ventricular septal or left ventricular thickness. In addition to intensive supportive care and IV amiodarone, the patient was treated with bortezomib at 1.3 mg/m^2 on days 1, 4, 8, and 11, and dexamethasone 20 mg orally on the day of the first bortezomib injection and on the following day. Scleral icterus with a pattern of cholestatic injury appeared on day 7 (ASAT, 49 U/L; ALAT, 118 U/L; γGT, 217 U/L; Bilirubin, 26 micromol/L). Serology revealed no acute infection with hepatitis A, B, C, or E, cytomegalovirus, Epstein-Barr virus, adenovirus or toxoplasmosis. Tests for primary biliary cirrhosis and autoimmune hepatitis were negative. Caeruloplasmin, urinary copper excretion and alfa1-antitrypsin levels were normal. Ultrasonography of the liver and computed tomography of the abdomen showed moderate hepatomegaly (16 cm) but no bile duct dilation or thrombi in hepatic veins. IV amiodarone was discontinued on day 8; however, on subsequent days liver function continued to deteriorate. This finding suggested that amiodarone was not the cause of hepatic toxicity, and transjugular liver biopsy was performed on day 23. The biopsy showed marked ductopenia as confirmed by the absence of staining with immunohistochemical epithelial markers. At this point bortezomib-induced liver toxicity was strongly suspected, and bortezomib was discontinued. On day 28 the patient experienced symptomatic life-threatening atrial fibrillation refractory to diltiazem, and IV amiodarone was successfully restarted. Simultaneously, progression of MM was noted with worsening renal function, pancytopenia, and increasing serum M protein level (45 g/L) and free kappa-light chain Bence-Jones proteinuria (4.925 g/L). *Results*. Despite limited clinical experience with lenalidomide in severe liver dysfunction, we treated him with lenalidomide (10 mg/day p.o. on days 1-21), in combination with cyclophosphamide and dexamethasone (Morgan GJ et al 2007;37, 268). Treatment with IV amiodarone was continued. Within 1 week of beginning the lenalidomide-based regimen, renal failure resolved and thrombocytopenia improved; thus the dose of lenalidomide was increased to 25 mg/day, and enoxaparin 40 mg once daily was started as prophylaxis for venous thromboembolism. During treatment with the lenalidomide-based regimen, liver function recovered, and his liver function profile had returned to near normal values 50 days after bortezomib had been started. At the time of this writing, 9 months later, the patient has received 8 courses of lenalidomide-based treatment with a very good partial response, and liver function tests remain normal. *Conclusions*.This experience illustrates two important considerations. 1) Regular monitoring of the hepatic function indices during the therapy with bortezomib is therefore indispensable for the early detection of unpredictably severe hepatotoxicity 2) Dosing for lenalidomide in this situation can be difficult, but good clinical results are possible.

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RECOMMENDATIONS FOR USE OF FLC TESTS IN MONOCLONAL GAMMOPATHIES

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Background. Monoclonal gammopathies constitute a group of disorders characterized by the clonal proliferation of plasma cells. The M protein is a tumor marker specific for monoclonal gammopathies because it reflects the clonal production of immunoglobulin. Aims: The objective of this work was an estimate of the use of free light chain tests for diagnosis, screening, identification of clonality, follow up of disease and prognostic evaluation in monoclonal gammopathies. *Design and Methods*. The method of the analysis sums up the results of Guidelines for the diagnosis and management of monoclonal gammopathies. *Results*. According to NACB (National Academy of Clinical Biochemistry) and NCCN (National Comprehensive Cancer Network) recommendations, the tumor markers are: serum and/or urine protein electrophoresis, serume and/or urine immunofixation, serum and/or urine free light chains, serum viscosity and β2-microglobulin. UK and Nordic Guidelines for the diagnosis and management of multiple myeloma state that the "Free light chain assay has also recently been shown to be helpful in monitoring response in the majority of patients with an intact immunoglobulin paraprotein...". Serum free light chain automated immunoassays are more sensitive for the detection of monoclonal light-chain myeloma, nonsecretory myeloma and AL amyloidosis than traditional electophoretic and immunofixation methods. There is not a strong correlation between serum free light chain and measurement of urine free light chain (Bence Jones protein) by 24 h protein electrophoresis. Furthermore, serum FLC ration is an independent risk factor for malignant progression in MGUS patients. *Conclusions*. The determination of serum FLC and serum electrophoresis as first-line tests for investigating possible B-cell disorders gains additional diagnostic information.

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CLINICAL EFFICACY OF VELCADE (BORTEZOMIB) AND DEXAMETHASONE THERAPY IN PATIENTS WITH REFRACTORY OR RELAPSED MULTIPLE MYELOMA

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Background. Recent studies demonstrated that the combination of Bortezomib and several chemotherapeutic agents may have significant activity in multiple myeloma (MM). The most used chemotherapeutic agents are Cyclophosphamide, Thalidomide and Dexamethasone. Aims: In this study we assessed the efficacy and safety of the Bortezomib-Dexamethasone combination for the patients with relapsed/ refractory myeloma. *Design and Methods*. 21 new MM patients were diagnosed and treated in the Hematology Department during a period of two years (2006-2008). The patients who received at least one previous regimen of treatment were enrolled. Bortezomib was given at 1,3 mg/m^2 or D 1, 4, 8, 11 (cycles of 21 days) and Dexamethasone 20 mg/m^2 i.v. or p.o. or D 1, 4, 8, 11(cycles of 21 days). Results: The group was formed out of 14 males and 7 females. The median age of patients was 61 years (range 45-69 years). Median number of previous treatment regimens was two (range 1-4). There was no complete remission obtained. 17 patients obtained partial remission, 3 patients did not respond, and 1 patient died due to the complications not related to the treatment. Median progression-free survival (PFS) was 11.3 months (range 9.3-15.7 months) and median overall survival (OS) from the onset of Velcade-Dexamethasone was 27.6 months (range 20.3-34.2 months).Grade $^3/_4$ toxicities included thrombocytopenia (28.57%), neutropenia (14.28%), peripheral sensory neuropathy (42.85%). There was no treatment-related deaths. *Conclu-*

sions. Velcade-Dexamethasone therapy for patients with relapsed/ refractory MM is highly effective. The side effects such as peripheral neuropathy should be considered. Hematological toxicity did not require treatment disruption. It is necessary to use Velcade in association with other chemotherapeutical regiments like - ciclofosfamide, adriamcin, thalidomide for higher therapeutical efficiency in these patients.

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SURVIVAL PROBABILITIES IN PATIENTS WITH MULTIPLE MYELOMA TREATED AT THE CLINIC OF HEMATOLOGY IN SKOPJE

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Introduction. Multiple myeloma (MM) is a cancer formed by malignant plasma cells. It's an incurable but treatable disease. The treatment of MM has changed considerably over the past decade. Conventional therapy with MP containing agents was the standard treatment option for many years until myeloablative doses of Melphalan supported by an infusion of autologous hematopoetic stem cells were implemented in the treatment protocols. *Aims.* The aim of this study is to present the effects of treatment of MM patients at the Clinic of Hematology in Skopje in the past 15 years. Retrospectively we analyzed data from 112 patients treated with conventional therapy with MP-containing protocols compared to a group of 17 patients treated with high-dose therapy and autologous stem-cell transplantation. *Design and Methods*. 112 patients with MM (M-68; F-44; median age 59 years, range 36-80) were treated with conventional therapy with MP containing protocols, mostly according to VMCP (Vincristine, Melphalan, Cyclophosphamide, Prednisolon) protocol. 17 patients with MM (M-9; F-8; median age 53years, range 43-64) were prepared for treatment with high doses Melphalan and autologous hematopoetic stem cell transplantation. For the induction of remission VAD (Vincristine, Adriamycin, Dexamethason) protocol (4 cycles) was applied. Non-responders (4 patients) received Thalidomide+Dexamethason as a second line therapy for 5 month period and one patient received Bortezomib as a third line therapy. High-dose regimen consisted of Melphalan in doses of 200 mg/m^2. The Kaplan-Meier method was used to estimate survival probabilities. *Results*. Following the conventional therapy remission was obtained in 61% of patients (19% achieved a complete and 42% partial remission after 12 months of treatment). The median survival was 18 months including the patients with an early fatal outcome (25%) who died during the first 2 months after the confirmation of diagnosis. The 5-year survival rate was achieved in 30% of patients. In the high-dose group median survival after transplantation was 56 months, disease-free survival was 22 months; 5-year survival rate was achieved in 50% of patients. *Conclusions*. Our study has shown that the high-dose chemotherapy with autologous stem cell transplantation improves the response rate, event-free survival and overall survival in myeloma over that obtained with conventional chemotherapy.

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PATIENT SUFFERING FROM POEMS SYNDROME TREATED WITH LENALIDOMIDE PLUS DEXAMETHASONE

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Background. POEMS syndrome (Polyneuropathy, Organomegaly, Endocrinopathy, M protein and Skin changes) is also known as osteoclastic myeloma, PEP (polyneuropathy, endocrinopathy and plasma-cell dyscrasia), Crow-Fukase or Takatsuki syndromes. It is a paraneoplastic disorder of unknown etiology although cytokines (VEFG, IL-6, IL-1α) play an important role. No randomized clinical trials are available to provide evidence on the appropriate treatment for this syndrome and the strongest evidence on outcomes come from retrospective analyses. These reports covered alkylators, stem cell transplantation, thalidomide, corticosteroids, radiotherapy and skeletal targeted radiotherapy. In 2005, when asymptomatic and free of organomegaly, a 55-year-old man was diagnosed with chronic myeloproliferative syndrome and essential thrombocythemia in our center. Presence of an IgA monoclonal band was detected. A sensation of fatigue was initially described in the legs during 2006, with progression to the arms. Sensitive-motor polyneuropathy was diagnosed and immunoglobulin-based therapy initiated. The patient was refractory to treatment and respiratory distress was noted. He tested negative for Bcr-abl and Jak2. Amyloidosis was ruled out. Difficulty walking, dyspnea, generalized erythroderma and sclerotic lesions in the hands, without organomegaly, aroused suspicion of POEMS syndrome in 2008. Complementary test confirmed POEMS syndrome with neurological affectation, monoclonal gammopathy and endocrinopathy. *Aims.* We report treatment with lenalidomide (Len) plus dexamethasone (Dex) of a 55-year-old man suffering from POEMS syndrome. *Design and Methods*. Len 25 mg was administered on days 1 to 21 of a 28 days cycle, in combination with low doses of Dex (40 mg/weekly). The patient was closely monitored for efficacy and safety purposes. We present a descriptive analysis of the clinical status of the patient in both pre-treatment and on-treatment periods. Results: Baseline laboratory tests: platelets 976000 mm^3, TSH 13.6 mUI/mL, testosterone 1.51 ng/mL, IgA 1010 mg/mL, light chain lambda 496 mg/dL, VEGF 4339 pg/mL. A agglomerate of lymph nodes was identified by ultrasound in the left inguinal region and the bifurcation of the left common iliac artery. The biopsy of a lymph node showed the plasmocytic form of Castleman disease. Nocturnal respiratory polygraphy showed desaturation and hypoventilation during sleep, with phrenic paresis with severe restrictive respiratory failure. Sclerotic bone lesions were identified in the right femur and in the L5 vertebrae. When Len+dex treatment was applied, low molecular weight heparine (LMWH) was used until the patient was able to walk. A respiratory rehabilitation program was implemented. After 4 cycles of len+dex, substantial clinical improvement was noted: respiratory improvement, walk without cane. LMWH was substituted by ASA 100 mg. Laboratory tests: normalization of hormone levels, platelets 273000, light IgA 788 ng/mL, lambda chain 293 mg/dL, M component not quantified by migration to β region, VEGF 1015 pg/mL. *Conclusions*. Treatment with Len+dex is a feasible and effective option to treat POEMS syndrome. The treatment is well tolerated. More data from prospective were awaited.

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EFFICACY AND SAFETY OF LENALIDOMIDE IN THE TREATMENT OF MULTIPLE MYELOMA WITH RENAL INSUFFICIENCY

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Background and aims. Renal impairment in multiple myeloma (MM) is associated to poor prognosis and worse survival. Standard treatments based on chemotherapy are associated to high toxicity. The apparition of new drugs has improved the evolution of these patients. There is more experience with bortezomib, as its metabolism is independent of renal function. On the other hand, lenalidomide is metabolised by the kidney, so it is necessary to perform dose adjustment. We present four patients treated with lenalidomide. *Design and Methods*. Patient 1: 52-year old man diagnosed of a MM IgG κ IIIA with chains in urine 13 g/24 h. After treatment with one cycle of VBMCP and bortezomib, he developed a tumor lysis syndrome, and renal insufficiency that required dialysis. He continued with bortezomib. After 15 cycles he obtained a PR. For that reason he started lenalidomide (15 mg/3 days a week/21 days) plus dexamethasone, obtaining a CR, without side effects. Patient 2: 66-year old man diagnosed of a MM IgG κ with chains in urine >25 g/24 h, and creatinine 2.2 mg/dL. After treatment with VAD, without obtaining any response, he started lenalidomide (15 mg/48 h/21 days) plus dexamethasone, with a creatinine of 3.1 mg/dL and a creatinine clearance of 25 mL/min. After 4th cycle, the renal function improved, and the dose was increased to 15 mg/day. The treatment was well tolerated, and after 10 cycles he shows an important improvement of his renal function (Creat 1.4 mg/dL), and a PR of the MM. Patient 3: 78-year old man diagnosed of a MM IgG κ IIIB with important elimination of chains in urine, and mild renal insufficiency (Creat: 3.1 mg/dL). He was treated with four cycles of VAD, without obtaining any response, and his renal function decreased. With an elimination of chains in urine of 15 g/24 h, he started bortezomib plus dexamethasone without any response after 3 cycles. For that reason he started lenalidomide (15 mg/48 h/21 days) plus dexamethasone, with a Creat of 6.9 mg/dL and a Creat clearance of 9 mL/min. After 5 cycles, he presents a PR and a significant improvement of the renal function (Creat 4.4 mg/dL). Patient 4: 63-year old man diagnosed of a MM IgA lambda IIIA, with a femur pathologic fracture. He was treated with four cycles of VAD, obtaining a VGPR, and therefore an autologous transplantation was performed, achieving a CR. Years later, he relapsed with an orbitary plasmacytoma and evidence of serum M-protein, so he started bortezomib plus dexamethasone. After 8 cycles, however the plasmacytoma disappeared, M-protein persisted. He also presented diarrhea as a side effect. Months later, his analysis showed renal insufficiency (Creat 2.2 mg/dL; Creat clearance: 32 mL/min) and proteinuria (1 g/24 h), with an M-protein of 2.5 g/L. He

started lenalidomide plus dexamethasone. After two cycles he showed CR. *Summary*. In our experience, lenalidomide in MM patients with renal insufficiency is effective, well tolerated, and with low profile of toxicity.

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ORAL VORINOSTAT COMBINED WITH LENALIDOMIDE AND DEXAMETHASONE IN A PATIENT WITH REFRACTORY END-STAGE MULTIPLE MYELOMA - A CASE STUDY

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Background. While bortezomib, thalidomide, and lenalidomide have provided survival advantages for patients with multiple myeloma (MM), morbidity and mortality associated with MM remains high. Currently, there is a need for new treatment strategies for patients with relapsed and refractory MM who have exhausted current treatment options. Vorinostat is a potent oral inhibitor of Class I and II histone deacetylases and is approved in the US for treatment of cutaneous manifestations of advanced T-cell lymphoma in patients with progressive, persistent or recurrent disease on or following two systemic therapies. Vorinostat synergistically enhances the activity of bortezomib in patients with advanced MM and enhances responses to thalidomide analogs in preclinical studies. *Aims*. We present a case study of a heavily pretreated 73-year-old male patient with end-stage IgA MM who received clinical benefit from combined vorinostat, lenalidomide, and dexamethasone (VLD). *Design and Methods*. The patient was diagnosed with end-stage IgA MM in January 2005. Prior to diagnosis of MM, he had been diagnosed with myeloproliferative disorder (polycythemia vera) and presented at the time of MM diagnosis with myelofibrosis with severe splenomegaly; both of these latter conditions responded to treatment with lenalidomide therapy. Following diagnosis of MM, the patient had failed a bone marrow transplant and numerous previous systemic therapies, such as steroid and biologic agents including 1 year of lenalidomide and dexamethasone combination therapy and >1 year of treatment with bortezomib combined with lenalidomide. In May 2008, >3 years after the initial MM diagnosis, the patient commenced VLD combination therapy. Vorinostat (300 mg, orally) was administered on Days 1-7 and 15-21 of a 28-day cycle, with lenalidomide (10 mg, orally) on Days 1-21 and dexamethasone (40 mg, orally) on Days 1, 8, 15, and 22.

Table 1.

Patient treatment history for MM		
Systemic therapy	Best response	Reason for discontinuation
Thalidomide Dexamethasone	Stable disease	Poor tolerability
Lenalidomide Dexamethasone	Minor response/stable disease with a reduced dose of lenalidomide	Poor tolerability/relapse (reduced dose of lenalidomide)
Bortezomib Lenalidomide Dexamethasone	None	Lack of response
Bortezomib Lenalidomide Liposomal doxorubicin Corticosteroids	Stable disease	Poor tolerability and patient refusal to continue
Vorinostat Lenalidomide Dexamethasone	Very good partial remission	Treatment ongoing

Results. In the second cycle, vorinostat was reduced to 200 mg/day due to development of Grade 3 transient high fevers, which resolved spontaneously. The patient also experienced diarrhea during treatment cycle 1 and the following low-grade adverse events in treatment cycle 2: fevers, weakness, diarrhea, and other gastrointestinal-related events. IgA levels were 2230 mg/dL (cancerous) at diagnosis; VLD therapy reduced IgA levels to 248 mg/dL after one cycle (28 days) and to 194 mg/dL (within normal range) following two treatment cycles (56 days). Other immunoglobulin levels (depressed by the cancerous IgA) increased to normal ranges; IgG increased from 276 mg/dL to 346 mg/dL and 490 mg/dL following treatment cycles 1 and 2, respectively; IgM levels increased from 2 mg/dL to 19 mg/dL and 30 mg/dL after treatment cycles 1 and 2, respectively; M-protein levels in the urine reduced from 3721 mg/24 h to <60 mg/24h after VLD. The patient is currently completing his third treatment cycle and continues to respond to VLD. To date, he has shown a very good partial remission for one treatment cycle (see table). *Conclusions*. For this patient with refractory end-stage MM, oral VLD was well tolerated and conferred substantial clinical benefit, reversing disease progression and normalizing immunoglobulin levels in the blood.

1634

LOW DOSE LENALIDOMIDE WITH LOW DOSE DEXAMETHASONE IN PREVIOUSLY TREATED PATIENTS WITH MULTIPLE MYELOMA OLDER THAN 75 YEARS REQUIRING TREATMENT

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Background. The management of very old patients with active Multiple Myeloma (MM) who have received previous treatments but require further (although in most cases palliative) therapy is very difficult, due to the presence of concomitant diseases, decreased bone marrow reserve, systemic toxicity, relatively decreased renal function and general problems due to old age. Aims. As in this setting the tolerability of standard doses of dexamethasone (DEX), chemotherapy or IMiDs is a concern, we used low doses of lenalidomide and low doses of DEX and we present the preliminary results. *Design and Methods*. Four patients aged over 75 years with pretreated MM and progressive disease were treated with flexible low doses of lenalidomide along with low doses of DEX. Lenalidomide was initially given at a dose of 10 mg daily for 21 days and DEX 20-40 mg (20,20,32 and 40 mg, median 28 mg) for days 1 to 4, every 28 days. The dose of lenalidomide was maintained, when tolerated, up to 6-8 cycles, but moderately increased or reduced according to response and toxicity. Patients received prophylaxis with LMWH and omeprazol. CSF or red cell transfusions were used when needed. Patient risk was stratified following the Salmon and Durie (SD) score and the International Staging System (ISS). Response was assessed with the IMWG criteria. All patients signed an informed consent. Results. Median age was 83 years (range 75 to 90). Previous treatments were cyclophosphamide/prednisone (n=3), melphalan/prednisone (n=1) and DEX (n=1). Three patients had received 1 and another one 2 previous treatment modalities. Median creatinine level was 1.19 mg/dl (0.75 to1.63) and median hemoglobin level 9.8gr/dl (range 9.1 to10.9). 3 pts had IgGα and another IgGα. Median IgG serum level was 3.352 mg/dl (868 to 4990). 3 pts had SD stage IIA and another IA. 3 pts. had IIA and another IA ISS stage. Patients received 2, 6, 6 and 7 cycles, respectively. In one patient treatment was stopped due to toxicity and in another patient lenalidomide was reduced to 5 mg after 2 cycles and maintained for 4 additional cycles. In another one the doses was modified to 20 mg and then to 15 mg. Overall response occurred in all 4 patients (100%): 2 patients achieved Partial Remission (PR) and the remaining 2 achieved significant, but lesser that 50%, reduction of the M-component (Stable Disease), one of them after increasing the dose of lenalidomide. Of note, both PR were achieved after 3 cycles. Expectable and manageable grade II-III bone marrow toxicity occurred in 3 patients. *Conclusions*. Treatment with low doses of lenalidomide and low doses of DEX is an active and tolerable option to be considered in previously treated elderly patients with MM requiring treatment. Low lenalidomide doses can be flexibly modified depending of the quality of the response and toxicity. Longer follow up is needed to evaluate the duration of the response.

1635

ADVANTAGE OF SERUM MEASUREMENTS FREE LIGHT CHAIN IN PLASMA CELLS DISCORDERS

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Background. Accurate detection and quantitation of monoclonal immunoglobulins (Ig) is important for diagnosis and management of monoclonal gammopathies (MG). In particular, monoclonal Ig can be used for screening, monitoring cause and treatment of disease, and for monitoring progression in monoclonal gammopathy of unknown significance (MGUS). *Aims*. The objective of this work was an estimate advantage of serum measurements free light chain (FLCs) tests for diagnosis, screening, identification of clonality, follow up of disease and prognostic evaluation in plasma cells disorders (PCD). *Design and Methods*. The method of the analysis sums up the results of Guidelines for the diagnosis and management of PCD. *Results*. According to Guidelines Work-

ing Group of UK Myeloma Forum and Guidelines on the diagnosis and management of AL amyloidosis state that the "FLC assay has also recently been shown to be helpful in monitoring response in the majority of patients with an intact Ig paraprotein...". Approximately 30% of MG patients (including patients with light chain myeloma (LLMM), primary AL amyloidosis, nonsecretory myeloma (NSMM), and light chain deposition disease (LCDD)) produce FLC as the only monoclonal component, and as many as 96% of patients with intact Ig myeloma produce levels of FLC that can be quantitated in serum. A new, latex-enhanced, immunoassay provides ultrasensitive detection and quantification of FLC in serum (1 mg/L compared with 150-500 mg/L by IFE and 500-2000 mg/L by PE). This assay is able to detect FLC earlier than PE in patients with NSMM and amyloidosis. Serum FLC are also better for monitoring patients with LCDD. *Conclusions*. Serum FLC concentrations increase as urine FLC concentrations increase, but relate better to the changing tumor mass than urine measurements.

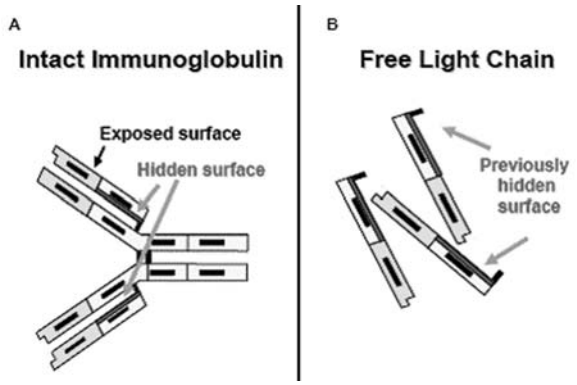

Figure.

1636

SYSTEMIC CAPILLARY LEAK SYNDROME DIAGNOSED BY IMPEDANCE CARDIOGRAPHY IN PATIENT WITH MULTIPLE MYELOMA

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Systemic capillary leak syndrome (SCLS) is rare disorder with a high mortality rate, characterized by rapidly developing diffuse swelling, weight gain and hypotension, hemoconcentration and hypoproteinemia. SCLS are cardinal features of acute lung injury and the acute respiratory distress syndrome , a severe illness associated with mortality of 30-50%. This syndrome is caused by sudden, reversible capillary hyperpermeability with a rapid extravasation of plasma from the intravascular to the intestinal space. Even though SCLS has been suggested to be pathogenic mechanism for the pulmonary toxicity of various drug, such as gemcitabine, retinoids, sirolimus, interferon-α and rare other cytostatic medicines, for example anthracyclin antibiotics. The pathogenesis of SLS is still unclear, but there's recently been a report showing this syndrome in association with monoclonal gammopathy This syndrome can be a fatal disease because cardiovascular collapse can occur in the initial capillary leak phase. This disease could lead to death if the blood pressure is not increased during the initial leak phase. Although theophylline, diuretics, terbutaline, steroids, calcium antagonist Ginkgo biloba extract and plasmapheresis have been suggested as medication ,none of them have been proven to be effective. Considering that this disease is self-limiting, conservative treatment in the acute phase is believed to be very important. We describe a case of SCLS after anthracyclin antibiotics (doxorubicyne) administration in a 71 -years- old male with multiple myeloma IgA kappa stadium III A Durie-Salmon treated a combination of doxorubicyne , vincristine and prednisone (VAD pattern). Two days after chemotherapy (VAD) administration the patient presented worsening dyspnea, diffuse swelling, weight gain and hypotension. Venticular dysfunction, superior vena cava compression or thrombosis or lung infiltrates were excluded by echocardiography and thorax spiral CT scan. The detection of doxorubicin -induced SCLS supports the hypothesis that SCLS could be pathogenic way of doxorubicin pulmonary toxicity. We diagnosed SCLS by means of impedance cardiography (NiccomoTMICG monitor) (ICG). This is a easy to use and safety diagnostic method. Intravenous administration of Furosemid and high dose steroid (Methylperednisoloni) was promptly given , with complete symptom recovery within third days. The next treatment cycle was given on time , without antracyclin antibiotics (doxorubicyne)- VCMP pattern. He wasn't suffer for sever dyspnea , diffuse swelling and hypotension. The impedance cardiography after chemotherapy (52 days) showed normal distribution of fluid between the intravascular space and the interstitial space. Conclucsion: SCLS is rare disorder with a high mortality rate, characterized by rapidly developing diffuse swelling, weight gain and hypotension, hemoconcentration and hypoproteinemia. SCLS are cardinal features of acute lung injury and the acute respiratory distress syndrome , a severe illness associated with mortality of 30-50%. In diagnosis of SCLS the technique was designed of measuring and monitoring the basic haemodynamic parameters in patients by means of impedance cardiography (ICG), also known as 'impedance plethysmography of the chest', 'electrical bioimpedance of the chest' or 'reocardiography'. The method makes it possible to denote stroke volume and cardiac output. It also enables the factors to be assessed that influence the following: preload (measurement of thoracic fluid content), afterload (measurement of systemic vascular resistance), the systemic vascular resistance index, contractibility (measurement of the acceleration index), the velocity index, the pre-ejection period, left ventricular ejection time, systolic time ratio and heart rate. The measurements also provide haemodynamic information during the treatment of patients with cancer to make a swift diagnosis of the cause of complaints such as dyspnoea and hypotonia.

1637

QUANTITATIVE CT PERFUSION MEASUREMENTS IN HODGKIN LYMPHOMA AND COMPARISON WITH 18-FLUORO-DEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY (FDG-PET): PRELIMINARY EXPERIENCE

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Background.Structural CT criteria such as nodal size and appearance have a poor correlation with the vitality of a lymphoma mass, particularly in Hodgkin Lymphoma. However, differences in vascularity between tumors and normal surrounding tissues offer potential for more precise imaging. Many tumor capillary beds have a higher vessel density than normal, with a larger flow of blood per unit volume, or perfusion; moreover, these capillary beds also have increased permeability. CT-perfusion is an exciting CT technology that allows functional evaluation of tissue vascularity. *Aims*. This study investigates the potential for functional CT-perfusion and permeability measurements in distinguishing between active, recurrent disease and residual scar tissue, also in comparison with 18-Fluoro-deoxyglucose positron emission tomography (FDG-PET) that recently become widely used in the management of patients with malignant lymphomas, providing unique metabolic information about tissue vitality and prognosis of patients. *Design and Methods*. 21 patients with Hodgkin lymphoma underwent imaging with both modalities, CT perfusion with 64-detector scanner (VCT, GE Healthcare, contrast material 100 ml, 4 mL/sec) and FDG-PET at staging, early assessment and restaging (n=2), or at early assessment and restaging (n=10) and only at restaging (n=9). All 35 CT perfusion studies were conducted on the most significant lymphoadenopathy and analyzed by using commercially available software (CT Perfusion 3 - Body tumour protocol; GE Healthcare Technologies); blood volume (BV), bloodflow (BF), mean transit time (MTT) and permeability surface (PS) were measured. Results: thirty out of 35 (86%) CT-perfusion and FDG -PET scan resulted concordant. In particular, all examination were positive at staging, while FDG-PET was positive and TC-perfusion negative in a case at early assessment and the two techniques resulted different in four cases at restaging, in which FDG-PET was negative but TC-perfusion was doubt/positive. Conclusions: CT-perfusion like FDG-PET offers the advantage of functional tissue characterization that is largely independent of morphologic criteria. It can represent a potential alternative non-invasive method in evaluating response to treatment in early assessment of Hodgkin lymphoma and in distinguishing between active, recurrent disease and residual scar tissue after therapy. Further and larger studies are needed to confirm these preliminary data and to extend the prognostic value of FDG-PET to TC-perfusion, particularly when used in the early assessment of response to treatment.

1638

CARDIOPULMONARY TOXICITY OF DIFFERENT CHEMO-RADIOTHERAPIC REGIMENS FOR HODKGIN'S DISEASE

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The combination of mediastinal radiotherapy (RT) and polichemotherapy regimens (CT) can produce late toxic pulmonary and cardiac effects which remain often at subclinical level. The majority of studies has been directed to the evaluation of cardiopulmonary function at rest using conventional procedures (spirometry, determination of lung transfer factor for CO, echocardiography) but only minimal research has been directed to evaluate cardiopulmonary function and reserve during exercise. The aim of the present investigation was to compare the late lung and cardiac toxicity of three chemotherapic regimens followed by RT and particularly ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine), VEBEP (vincristine, epi-doxorubicin, cyclophosphamide, etoposide and prednisone) and MOPP-ABVD (mechloretamine, vincristine, procarbazine and prednisone). Therefore we investigated 147 patients suffering from Hodgkin's disease after a follow up of at least 5 years from the completion of CT-RT. Seventy eight have been submitted to ABVD-RT, 36 to VEBEP-RT and 33 to MOPP-ABVD-RT. Patients were submitted to spirometry, determination of lung transfer factor for CO, 2D-Doppler Echocardiography at rest, cardiopulmonary exercise test on cycloergometer and determination of cardiac output by a non invasive method. Patients of the three groups showed tolerance to exercise, and oxygen consumption significantly lower than the predicted values : by this point of view no statistically significant difference were found between the three groups. Patients treated with VEPEB and MOPP-ABVD showed a lower ejection fraction at rest than ABVD patients and moreover patients treated with VEBEP showed a cardiac output per oxygen uptake lower than that observed in the ABVD and MOPP-ABVD groups. These data confirm that the combination of mediastinal radiotherapy and the more common used polichemotherapy regimens produce late pulmonary and cardiac toxic effects which remain at subclinical level. The lower exercise capacity seems to be due to a combination of decreased cardiac performance and impairment of ventilation. The VEBEP regimens seems to be slightly more toxic for the heart probably because of the higher cumulative dose of anthracyclines.

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THE ROLE OF PET-FDG BEFORE AUTOLOGOUS STEM CELL TRANSPLANTATION IN ADVANCED HODGKIN'S LYMPHOMA

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Background. PET-FDG (PET) has assumed in recent years a relevant role in the management of patients with Hodgkin's lymphoma (HL). In advanced stage HL, PET performed after two courses of ABVD chemotherapy has demonstrated a very high negative predictive value (NPV) and positive predictive value (PPV) and is presently considered the most relevant available prognostic factor in correlation with outcome. *Aims*. To investigate the prognostic role of PET performed before autologous stem cell transplantation (ASCT) in resistant or relapsed HL. *Design and Methods*. From May 2005 to November 2008, 19 patients with resistant or relapsed HL underwent a salvage chemotherapy program consisting of 3 or 4 courses of IEV or IGEV chemotherapy with peripheral blood stem cell collection followed by BEAM conditioned ASCT. Seven patients were consolidated with the BEACOPP regimen (2 to 4 courses) before transplantation. Median age was 30 years (22 - 46); 9 were males. At the time of enrolment, B symptoms were present in 1 patient and bulky and/or extranodal disease was recorded in 5 patients; the International Prognostic Score (IPS) was >2 in 6 patients. Nine patients were resistant to first-line chemotherapy, 7 were in first relapse (in 2 cases occurred earlier than 12 months), 3 were in second or subsequent relapse. *Results*. Pre-transplant PET evaluation was negative in 11 cases: of these, 7 are currently in continuous complete remission (CCR) after a median follow-up of 23 months (range 9 - 32), while 4 have relapsed after 5, 12, 20 and 31 months from transplant, respectively. Among the 8 patients autografted after a positive PET, 6 relapsed after a median follow-up of 15 months (range 7 - 28). The NPV of pre-transplant PET was 63.6%; the PPV was 75.0%. The prognostic value of the following parameters at the time of enrolment was evaluated: presence of bulky and/or extranodal disease, number of previous chemotherapy lines, chemoresistance/chemosensitivity to pre-transplant therapy evaluated by CT scan; no statistically significant correlation with the outcome was recorded. *Summary and conclusions*. Our preliminary results show that in resistant or relapsing HL a positive pre-transplant PET predicts progression or relapse in 75% of patients. A negative PET predicts long-term disease control in two thirds of patients. Pre-transplant PET does not have the same high predictive value as interim PET performed during first induction; other prognostic factors need to be considered, although none of those examined showed a statistically significant role. These data need to be further confirmed on a larger number of patients.

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ABVD CAN BE SAFELY ADMINISTERED REGARDLESS OF ABSOLUTE NEUTROPHIL COUNT AND WITHOUT G-CSF SUPPORT IN HODGKIN'S LYMPHOMA PATIENTS

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Background. Dose intensity of chemotherapy is an important factor for complete remission and long-term survival of patients (pts) affected by Hodgkin's lymphoma (HL); it has been recently reported a significant higher incidence of bleomycin-induced lung toxicity and mortality with concomitant use of G-CSF in bleomycin-containing schemes. For these reasons in July 2007 we started to administer ABVD regimen without G-CSF support and regardless of absolute neutrophil count (ANC) before each treatment administration. *Aims*. Here we report the safety outcome of 22 consecutive patients with newly diagnosed HL treated with ABVD regimen at our Institution. *Design and Methods*. Patients received ABVD between July 2007 and February 2009 for stage I-II (17 pts, 77%) and stage III-IV (5 pts, 23%) HL. Median age was 35 years old (range 16-63), ratio M/F = 10/12. A total of 97 cycles were administered so far and three pts are under ABVD treatment at present. *Results.* Mean ABVD dose-intensity was 97.6% and mean cycle duration was 28.6 days (see table); 39% of chemotherapeutic treatment were given with a ANC $<1.0x10^9$/L and 17% with an ANC $<0.5x10^9$/L. Antibacterial prophylaxis was given in five occasions. One grade 3 febrile neutropenia, one grade 3 neurological toxicity and one event of bronchiolitis obliterans organizing pneumonia (BOOP) were observed. No bleomycin-related lung toxicity neither other relevant side effects were recorded. At present, all pts are alive and those who completed treatment are in complete remission (n= 18), at a median follow-up of 12 months (range 6-19) for these latter. One patient with persistent disease after four cycles of ABVD was shifted to MOPP regimen. *Conclusions*. we conclude that ABVD may be safely administered at adequate dose intensity without G-CSF support and regardless of ANC.

	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
N. pts	22	21	21	19	7	7
Mean cycle duration (days)	29.3	29.3	28.0	29.5	28.0	28.0
ABVD dose	100%	100%	100%	100%	100%	100%
Dose intensity	95.5%	95.5%	100%	94.9%	100%	100%

Table 1. Patients and treatment deliveries.

1641

COMBINED VBM (VINBLASTINE, BLEOMYCIN, METHOTREXATE) CHEMOTHERAPY AND INVOLVED FIELD (IF) RADIOTHERAPY (RT) IN EARLY STAGE HODGKIN'S LYMPHOMA (HL): A SINGLE CENTRE EXPERIENCE

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Background. Early stage HL patients (pts) may benefit from a short course of chemotherapy, usually ABVD, combined with IF RT. 18FDG-PET scan in HL performed before therapy is helpful to identify early stage disease pts that can benefit of a more conservative approach. AIMS: To verify efficacy of the four courses of VBM with IF RT on good prognosis early stage HL pts. *Design and Methods*. From May 2001 to February 2008, 37 treatment naïve Hodgkin's lymphoma pts (21 male, 16 female) were treated with four courses of VBM (D1, D8: Vinblastine 6 mg/sm, Bleomycin 10 mg/sm, Methotrexate 30 mg/sm, repeated every 28 days) followed by IF RT. Median age was 37 years (range 16-73). All

pts were staged with CT scan, bone marrow biopsy and blood exams. Histology was classical HL in 28 pts and lymphocyte predominant in 9 pts. Pts were all stage I-IIA (no bulky disease), no more than three sites involved, performance status 0-1 and ESR < 50. IF radiotherapy was performed one month after the last chemotherapy cycle; median dose administered was 30Gy (range 26-30.4) 18FDG-PET were performed at staging to identify a low risk pts group and repeated after chemotherapy and RT. *Results*. Chemotherapy was usually well tolerated. Eight pts experienced G3-4 neutropenia while the main non-haematological toxicities recorded were G3 transaminitis in two pts and G1-2 neurological (constipation/abdominal pain) in 14 pts. With chemotherapy 30 pts achieved a complete remission (CR), 5 pts a partial remission (PR); one patient progressed after chemotherapy and before RT and one patient, not in clinical response after two cycles of VBM, received hybrid salvage chemotherapy. In 5 cases PET was positive at the end of CT, becoming negative after RT in 4 cases. 35/37 (94%) pts completed the whole program, all achieving CR. With a median follow-up of 54 months (range: 6-90), all pts are alive. Two pts relapsed after 8 and 48 months the end of therapy and underwent high dose chemotherapy with autologous stem cell transplant, achieving CR. *Conclusions*. 18FDG-PET scan before treatment effectively helps to identify low risk early stage HL, allowing a more careful selection of pts suitable of less aggressive therapy. In our experience four cycles of VBM chemotherapy combined with IF RT is an effective program. This schedule, without alkylating or anthracycline agents, avoids alopecia and may decrease the risk of hematological and cardiac toxicity.

1642

SUBJECTIVE OUTCOME (QUALITY OF LIFE) IN LONG-TERM SURVIVORS OF HODGKIN´S LYMPHOMA (HL): A CROSS-SECTIONAL STUDY OF PATIENTS TREATED 1995 TO 2003 WITHIN THE GHSG TRIALS IN THE CZECH REPUBLIC

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Background. We investigated the current quality of life (QoL) in long-term survivors of HL treated within the German Hodgkin Study Group (GHSG) trials between 1995 and 2003 in the Czech Republic. Aims: To describe patients (pts) QoL after cure and returning to normal life during the years (yrs) after end of treatment in a cross-sectional design. *Design and Methods*. EORTC QLQ C-30, MFI-20, subjective retrospective evaluation of treatment and a life situation questionnaire (LSQ) wer used for the assessement of the pts' situation after end of treatment. An authorised Czech version of the questionnaires was sent to 172 pts who were disease free and were followed-up for four and more yrs. Results: 142 (82,5%) pts replied. Median age at time of assessment was 33 yrs, median follow-up 5.5 yrs (range 4-12 yrs). Regarding the QoL functional and fatigue scales, pts report a mixed pattern of responses but indicate quite severe limitations in their perceived QoL during later years of follow-up. Emotional functioning and global QoL recovers fully only in 50% of pts 4-12 years after end of treatment and about 25% report constant severe strain. General fatigue remains high with 45% of pts reporting "high fatigue" levels but only 15-30% of pts report high levels of reduced motivation and activity. In physical functioning 70% recover fully and only 6% report very low functioning. In general, women report lower QoL and higher symptom scores over time than men. Study arms and study generations showed no substantial influence on QoL. Compared to the German study population the Czech patients report lower QoL and higher levels of fatigue. *Conclusions*. QoL data from the reintegration process of pts into normal life during the years of follow-up reveal substantial strain and limitations of QoL, particularly in subsets of patients and in specific areas of QoL. Longitudinal QoL assessment within the GHSG trials is ongoing also for Czech pts and will add the QoL course of recovery to the current cross-sectional data. Other factors not yet clearly identified seem to play a more important role for the QoL outcome than e.g. stage and treatment in these patients. Cultural differences in QoL outcome between Czech and German patients were noticed.

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COMPARISON OF EARLY FAVORABLE STAGE HODGKIN'S LYMPHOMA TREATMENTS: A SINGLE INSTITUTION REVIEW

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Background. This study was conducted to compare outcomes of patients receiving combined modality chemotherapy and radiation (CMT) versus other approaches for early stage Hodgkin's lymphoma (HL). *Design and Methods*. A review of patients with non-bulky early stage (IA/IIA) HL treated between 1984 and 2002 was performed to determine the treatment approaches used and the outcomes obtained. Results: There were 173 adult patients with newly diagnosed early stage Hodgkin's lymphoma, with 51% men and 49% women and a median age of 33 years (17-82). Treatment was as follows: extended field radiotherapy alone (EFRT) 49%; chemotherapy alone (CTA) 14%; and CMT 37%. Among CMT patients, 36% received abbreviated ABVD chemotherapy (3-4 cycles) followed by involved-field radiotherapy (IFRT). With a median follow-up of 8.3 years, the estimated 10-year RFS and OS for the entire cohort were 78% and 85% respectively. The 10-year RFS and OS for the various groups were as follows: 69% and 81% for EFRT; 78% and 84% for CTA; and 87% and 89% for CMT. The 10-year RFS was significantly higher ($p<0.05$) among CMT patients. The use of EFRT has diminished from approximately 90% in the 1980's to virtually no use at present, while the use of CTA and CMT has increased significantly ($p<0.01$). *Conclusions*. Early stage Hodgkin's lymphoma treatment has changed dramatically over the past 2 decades, and our results support the superiority and continued use of CMT, specifically abbreviated course chemotherapy and IFRT as an appropriate treatment approach.

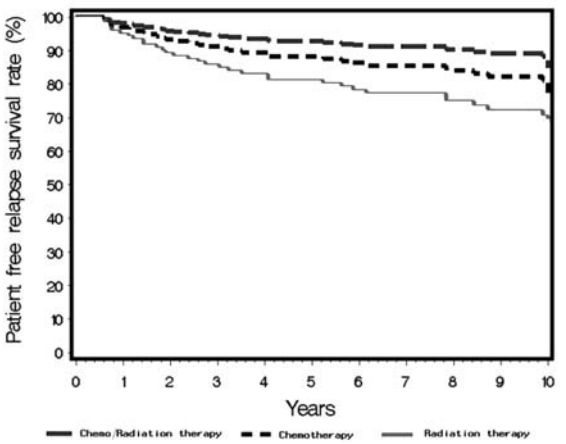

Figure 1. Relapse-Free Survival (RFS).

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DETECTION OF EBV DNA IN PATIENTS WITH HODGKIN LYMPHOMA IN THE CZECH REPUBLIC: FREQUENCY, QUANTITY IN PLASMA, WHOLE BLOOD AND LYMPH NODE

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Background. Presence of latent EBV genome is detected in 30-50% of patients with Hodgkin lymphoma (HL). According to recent works aggressiveness of disease correlates with amount of free EBV DNA present in plasma. Our aim was to investigate the frequency and quantity of EBV-DNA in our patients. *Design and Methods*. In total we have obtained 603 samples of plasma and whole blood from 173 adult patients (161 newly diagnosed, 7 in progression of disease, 5 with relapse of disease) and 22 newly diagnosed paediatric patients with HL. Samples were obtained before start of treatment, after cycles of chemotherapy and

radiotherapy and during outpatient controls. Moreover, in 8 EBV positive patients, we have obtained also samples of lymph node tissue. DNA was extracted using Qiagen Blood Mini Kits (whole blood and plasma) and Roche High Pure PCR Template Prep. Kit (tissue samples). Presents of EBV was tested using real-time PCR technology and samples were normalised to 100 000 human genome equivalents obtained by quantification of albumin gene in the sample (whole blood and tissue) or as copies per 1 mL (plasma). *Results*. EBV was detected in 38 samples of whole blood and 30 of plasma from 13 patients (11 adults) which represents frequency of 6.7% among our patients. Mean of detected quantity was 5400/mL in plasma (600-126600) and 34.9 normalised EBV copies in whole blood (1.1-6815.7). In 10 patients was positive in initial sample only and the disappearance of EBV in peripheral blood correlated with good response to treatment. In the samples from tissues, mean of normalised EBV quantity was $10^9$662 (22-785714), which represents in general the ratio of 1 EBV genome in every cell. *Conclusions*. The observed frequency of EBV positive patients in our cohort is much lower than so far published data. The good correlation between the reaction to treatment and vanishing of detectable EBV confirmed the possibility of using quantitative detection as biological marker of the disease activity in such patients. Moreover the normalization to human genome equivalents showed to be useful in testing of the tissue samples in patients with HL.

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ADVANCED HODGKIN'S DISEASE: PROGNOSTIC FEATURES IN THE EXTREMES

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Upon our previous observation that patients with both clinical stage IIIB and IV Hodgkin's disease (HD) follow an almost identical survival curve, we underwent this study in order to define similarities in their characteristics and determine the parameters which could possibly influence their outcome. In a longer than twenty-five period, out of the total number of observed patients with HD, 172 manifested extremely unfavorable clinical stages at disease presentation. Their actuarial survival is understandably quite poor, reaching 42% at the 10-year mark, and somewhat over 32% at the 20-year observation point. Univariate statistical analyses, performed separately on each stage subgroup, as well as in the subpopulation as a whole, pointed out several parameters that manifested prognostic significance regarding the vital statistics, some marginal and fewer true. Four of these are International Prognostic Index (IPI) parameters, but only one manifests true prognostic significance. Not surprisingly, knowing that the original IPI scoring system had established its validity on patients mostly with early stage disease, some of the prognostic factors incorporated in the IPI, showed no significance at all. Since the whole studied subpopulation already fulfills one criterion among the adverse prognostic factors, it is also understandable that the majority of these patients had an unfavorable IPI score (>=3) also: 70.93% of the subpopulation, mostly attributable to the 93.51% of the stage IV patients. Multivariant statistical analysis left only one of the IPI parameters with significant prognostic influence: abnormal WBC levels (p= 0.020061). However, it also revealed the prognostic significance that the diagnostic and treatment periods carry for the vital statistics of these patients. Development of the disease, the diagnostic period, when the patient is lacking treatment, in a period of more than three months, carries a significant negative prognosis (p= 0.045212). Among the management parameters, meticulous fulfillment of treatment administration in terms of dosage, accounts for significantly more favorable prognosis (p= 0.033629). The analysis shows a x2 value of 12.2728 for three degrees of freedom (p= 0.00651). Although representing an extremely delicate and tenacious population in terms of management, we need to keep in mind that disease characteristics have already classified such patients as prognostically unfavorable. Not being in the position to alter those parameters, we still need to instigate our efforts, skills and knowledge in two domains, in order to reverse the odds and accomplish more favorable prognosis for these patients: quick, precise diagnostic procedures and proper, meticulous, relentless, more aggressive treatment.

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PROGRESSIVE MUSCLE ATROPHY AND WEAKNESS AFTER TREATMENT BY MANTLE FIELD RADIOTHERAPY IN PATIENTS WITH HODGKIN'S LYMPHOMA

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Background. There are still Hodgkin's lymphoma (HL) patients alive who were diagnosed and treated as early as the 1970's or 1980's. With longer follow-up it has become apparent that these patients carry a risk for treatment-related side effects that may not manifest until many years after their initial treatment. Well-known late treatment-related complications are cardiovascular disease and second malignancies. However, patients treated by so-called *mantle* field irradiation can also develop symptomatic muscle atrophy and weakness in the treated area. This can result in neck pain and weakness which may be particularly disabling. These neuromuscular complications as long-term sequelae to local radiotherapy have so far received little attention in the literature. Aims. To describe the damage to the muscles and the pathophysiological mechanism of muscle atrophy and weakness after mantle field irradiation for HL. *Design and Methods*. We investigated 9 patients (ages 33-61 years) treated by mantle field irradiation between 1969 and 1998 (mean follow-up 26,6 years). Irradiation was in most cases delivered in a total dose of 40 Gy and 2 Gy/fraction. The patients are part of a larger cohort of HL survivors who are currently screened at our institution for late sequelae of cancer treatment. They were selected because on clinical examination they manifested weakness of the neck flexors, which was graded 2-4 on the Medical Research Council (MRC) scale (range 0 = no muscle movement to 5 = normal power). We also performed detailed clinical history, dynamometry, ultrasonography of the sternocleidomastoid, biceps and antebrachial flexor muscles and needle electromyography of the affected muscles. Results. All but two patients had neck complaints, mostly pain and muscle weakness, with the two most affected patients presenting a 'dropped head syndrome' (Figure 1).

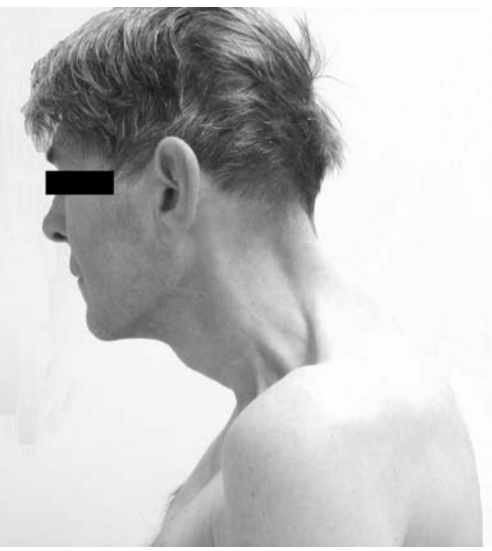

Figure 1.

Dynamometric studies show that there was neck weakness in 7 out of 9 patients, defined as results below the p5 of the normal population. The neck flexors were more often damaged (in 6 patients) than the neck extensors (in 4 patients). On muscle ultrasonography 7 patients met the criteria for abnormal echo-intensity in one or more of the investigated muscles. Abnormal echo intensity of the sternocleidomastoid muscles was seen in only 2 patients. However, the sternocleidomastoid was severely atrophic in 8 of the 9 patients. Needle electromyography of the muscles situated in the radiation field showed mostly myopathic changes, while muscles situated outside the radiation field appeared to have mostly neuropathic damage, suggesting affection of the nerve roots and brachial plexus in the radiation field. *Conclusions*. (i). Patients previously treated by mantle field radiation can develop severe atrophy and weakness of the muscles situated in the radiation field. (ii). Muscles sit-

uated outside the mantle field show mostly neurogenic damage. (iii). The discrepancy between echo intensity and atrophy suggests that muscle damage is most likely caused by an extrinsic factor such as progressive microvascular fibrosis, which is also presumed to cause nerve damage to the radiated field. (iv). Our findings have implications for patient management: both patients and caregivers need to be counseled about the possible neuromuscular sequelae of mantle field radiation.

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AUTOLOGOUS STEM-CELL TRANSPLANTATION IN REFRACTORY OR RELAPSE HODGKIN'S LYMPHOMA: MONOCENTRIC STUDY OF 85 PATIENTS

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Background. Autologous stem-cell transplantation (ASCT) is a common option for first relapse or refractory Hodgkin's lymphoma (HL). However there are few reports with sufficient number of patients and follow-up. The respective place of autologous transplant versus allogeneic transplant is under investigation. *Aims*. To study the efficacy and tolerability of intensive chemotherapy with single or tandem autologous transplant in the setting of HL after failure of first line treatment. *Design and Methods*. We retrospectively analyzed a series of 85 patients with first relapse or refractory HL seen at our institution over a 10-year period, between 1997 and 2007. Patients who relapse were classified in two prognostic groups: poor-risk (two or three risk factors among relapse before 12 months, stage III or IV at relapse, and relapse within previously irradiated sites) and good/intermediate-risk (0 or one risk factor). *Results*. There were 85 patients, with a median age at 29 years. Most patients received first line treatment with ABVD (68%). 42 patients (49%) had primary refractory disease, and 43 patients relapse after first line therapy (51%). Poor-risk and good/intermediate-risk included respectively 20 (48%) and 22 (52%) patients. After high-dose chemotherapy (HDC), 41 patients achieve complete response (CR) or near complete response (nCR), 37 patients were in partial response, stable or progressive disease. 47 patients received single ASCT, 26 patients received tandem ASCT and 11 patients received ASCT and allogenic stem-cell transplantation. The global 5 year overall survival (OS) and disease free survival (DFS) was 76% and 81% respectively. We didn't find any difference on OS and DFS in unvaried analysis for single or tandem ASCT, neither for relapse risk groups. *Conclusions*. We globally find results reported in the literature for global OS and DFS but no difference was shown for prognostic factors or treatment. A recent prospective study of 245 patients with HL has shown the superiority of tandem ASCT versus single ASCT for poor-risk patients without good response to salvage chemotherapy. Despite encouraging results of HL treatment, refractory disease stays a real problem and treatment of these patients need to be improved. Reduced intensity conditioning allogenic stem cell transplantation could be an alternative for young patients relapsing.

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RETROSPECTIVE ANALYSIS OF EARLY STAGE HODGKIN'S LYMPHOMA TREATED WITH CHEMOTHERAPY WITH OR WITHOUT RADIOTHERAPY

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The treatment for Hodgkin's Lymphoma (HL) has evolved over the past several years. Combined modality therapy including ABVD followed by involved field irradiation represents the current standard of care for patients with early stage HL. Long term toxicities are the major concern in survivors of early stage disease. Current approaches attempt to preserve the high remission rate achieved, while reducing treatment-related acute and late toxicities. *Aims*. To compare the outcome of early stage HL treated with chemotherapy with or without radiotherapy, we reviewed clinical features, therapy, and long term outcome of 79 patients treated at our institution from 1999 to 2005. *Design and Methods*. 44 patients (pts) were treated with 4-6 cycles of ABVD plus involved field radiotherapy (CT+RT group) and 35 pts were treated with chemotherapy alone, consisting of 3-6 cycles of the ABVD regimen (CT group). Median pts age was 36 years, 53% were males, 72% presented B symptoms and 75% had clinical stage II. The histology was nodular sclerosis in 82% patients. Patients with bulky disease were excluded from the analysis. The two therapeutic groups were statistically comparable for clinical features and median follow-up (60 and 62 months for the CT and CT + RT group. respectively). Results In the CT+RT group 88% achieved CR and 12% PR after first line therapy whereas in the CT group 83% achieved CR and 17% PR. High dose chemotherapy was employed as salvage therapy for 5 partial responders in the CT and 4 in the CT + RT group. One relapse occurred in the CT+RT group at 12 months after first-line therapy. At a median follow-up of 60 months the disease-free survival was 88% in the CT group and 89% in the CT+RT group (p: n. s.). In the CT+RT group 3 patients (6.8%) developed long term toxicities (one case of Chronic Restrictive Pulmonary Disease, one case of hypothyroidism and one of thyroid cancer at 41, 49 and 53 months of follow-up, respectively) whereas one patient (2.8%) in the CT group developed a late toxic event consisting in cardio-myopathy at 20 months of follow-up (p=ns). *Summary and Conclusions*. Our retrospective analysis shows that a high response rate was achieved both with chemotherapy alone and with chemoradiotherapy; no difference was observed in terms of relapse rate in the two groups. Nevertheless, we observed a higher incidence of long term toxicities in the group treated with the addition of radiotherapy. Multicentric studies and additional cases are required to assess the role of radiotherapy in early stage Hodgkin's lymphoma therapy. A better definition of prognostic factors at presentation and the predictive value of interim fluoro-2-deoxy-D-glucose-positron emission tomography scans may be useful to individualize therapy, improve the remission rate and minimize toxicity.

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OUTCOME OF FEMALE PATIENTS AGED 45 YEARS OR YOUNGER TREATED FOR HODGKIN LYMPHOMA: SINGLE INSTITUTIONAL EXPERIENCE

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Background. In 1998, Hasenclever score identified seven prognostic factors (serum albumin level, hemoglobin level, sex, age, stage IV disease, leukocytosis, lymphocytopenia) that predict the success rate of conventional treatment in patients with locally extensive or advanced stage Hodgkin lymphoma (HL). In particular, younger persons (< 45 years) and women tend to have better outcomes than older individuals and males. *Aims*. To retrospectively evaluate clinical characteristics at diagnosis and outcome of female patients with HL aged 45 years or younger treated in our institution. *Design and Methods*. From January 1991 to August 2008, we diagnosed and treated 121 HL female patients with a median age of 25 years (8-45). Nodular Sclerosis was the most common histological subtype (84%). Forty-nine (49%) patients were in clinical stage (CS) I-IIA including 13 with bulky disease, 62 (51%) were in CS IIB-IV including 20 with bulky disease. According to the GISL criteria, patients were stratified in three prognostic groups: Early Favourable HL (EFHL) (CS I-IIA without risk factors, 18 patients), Early Unfavourable HL (EUHL) (CS I-IIA with bulky disease and/or subdiaphragmatic disease, ESR higher than 40, extranodal involvement, hilar involvement and more than three involved lymph node areas, 41 patients) and Advanced HL (ADHL) (CS IIB-IV, 62 patients). Early HL patients received combined treatment including 4-6 courses of ABVD/ABVD-like regimens and involved field radiotherapy (14,4-36 Gy). ADHL patients were treated with 6 courses of ABVD or COPP/ABV or COPPEBVCAD or BEACOPP, and radiotherapy on residual masses. Overall, 46 patients were submitted to radiotherapy. Refractory/relapsed patients received salvage treatment with 3-6 courses of iphosphamide-based regimens (IGEV, IEP) and ASCT. *Results.* One hundred seventeen patients completed therapy while 4 are receiving treatment today. One hundred eight patients obtained complete remission (91%) and 9 patients were unresponsive (9%). Seven patients (2 with EUHL and 5 with ADHL) relapsed after a median time of 23 months (12-131) from diagnosis. Overall, after a median follow-up of 72 months (5-208) Overall Survival (OS) and Time To Treatment Failure (TTTF) are 97% and 83%, respectively (EFHL: OS and TTTF 100%; EUHL: OS 100%, TTTF 86%; ADHL: OS 95%, TTTF 80%). In the group of the 16 relapsed/refractory patients OS is 79%, and only 3 of them died after salvage treatment because of progressive disease. In regards to the treatment- related secondary cancers we recorded 2 non-Hodgkin lymphomas and 1 breast cancer and all 3 patients are alive so far. *Discussion.* Our results confirm that young female patients affected by HL have an excellent outcome both in early and advanced stages. Considering that

the late treatment-related toxicities represent a major concern for HL survivors, we think that an overtreatment should be avoided especially in early stage HL patients. Therefore, in order to offer individually tailored treatment, prospective studies on PET/CT-guided treatment strategies are underway worldwide

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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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HYPER-IGE (JOB'S) SYNDROME IN AN ADULT PATIENT PREVIOUSLY DIAGNOSED OF LYMPHOCYTE PREDOMINANT HODGKIN'S LYMPHOMA: IMPORTANCE OF DISTINGUISHING SYMPTHOMATIC JOB'S SYNDROME FROM ACTIVE HODGKIN'S LYMPHOMA

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Background. Hyper-Immunoglobulin E, IgE (HIES) or Job's syndrome is a rare primary immunodeficiency disorder characterized by clinical triad of recurrent staphylococcal abscesses, cyst-forming pneumonia and an elevated serum IgE level of > 2000 IU/ml. Most cases are sporadic; however, multiplex families displaying autosomal dominant (AD) and autosomal recessive (AR) inheritance have been described. The etiology of HIES is still unresolved. Therapy for HIES is directed at prevention and management of infections by using sustained systemic antibiotics and antifungals along with topical therapy for eczema and drainage of abscesses. Anti-staphylococcal antibiotic prophylaxis is useful. Interferons, immunoglobulin supplementation, or low-dose cyclosporine A, have been reported to benefit selected patients, but they are not generally indicated. There has been described an increased risk of development of different types of lymphoma in these patient's population. The exact mechanism of this increased risk is not fully understood but some similarities to the pathogenetic model of AIDS-related lymphoma have been established. *Aims*. In the vast majority of cases the Job's syndrome has been diagnosed previously to the lymphoid neoplasia. A high index of suspicion for lymphoma should be given in patients with HIE syndrome who present with lymph node enlargement. We report an adult patient previously diagnosed of lymphocyte predominant Hodgkin's lymphoma successfully treated with immunochemotherapy who developed a sympthomatic HIES mimicking active Hodgkin´s lymphoma. *Case Report*. A 70 years-old male patient was diagnosed of a CD20-positive lymphocyte-predominant Hodgkin´s lymphoma, Ann-Arbor stage II-A, non-bulky, supradiafragmatic. He was treated according to its age and localized stage with 3 cycles of ABVD plus Rituximab (3 doses), achieving a complete remission (CR) confirmed by a negative TAC-PET study. In the follow-up, although radiological studies were normal, the patient developed symptoms and biological signs mimicking active Hodgkin´s disease (malaise with generalized pruritus and a continuosly elevation of reactants of acute phase: ESR>15 mm; Reactive Protein C > 5 mg/l; fibrinogen > 400 mg/dL; blood eosinophilia > 500/mm^3). A complete searching of a systemic disorder and dermatologic and allergologic studies were all negative for a specific process. Because of maintained markedly increased IgE (> 5,000 Ui/mL) with chronic dermatitis, repeated respiratory infections and persistent pruritus, a Hyper-Immunoglobulin E, IgE (HIES) or Job's syndrome was suspected. At the time of this writing the patient has not started any treatment for HIES. *Conclusions.* In order to avoid unnecesary treatments of chemotherapy or radiotherapy in Hodgkin's lymphoma patients in continous CR, HIES should be considered in the differential diagnosis of patients with persistent pruritus and markedly increased IgE.

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CHANGES IN CLINICOPATHOLOGICAL FEATURES IN HODGKIN-LYMPHOMA

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Background. Hodgkin-lymphoma (HL) is an uncommon malignant tumour of the lymphatic system. Nowadays more reports show changing of the disease's epidemiology. *Aims*. Examination the characteristics of our HL patients retrospectively. *Design and Methods*. We examined 439 HL patients, who were treated between 1980.01.01 and 2008.12.31. *Results*. In the first period (1980-89) were 177 patients, 1990-99 (second period) were 147 patients, and between 2000-08 (thrisd period) were 115 patients. We observed a reduced male-to-female ratio (I. period: 1,42, II. period: 1,45, III. period: 1,04). The mean age was 40,1 years, 35,9 years, and 36,8 years (I. v. II. v. III. period). Comparing the distribution of HL cases diagnosed at 3 different time periods, we detected decreased frequency of the mixed cellularity subtype (43.5%, 58.5% v. 42.6% p<0.0098), and an increased frequency of the nodular sclerosis subtype (24.85%, 27,2% *vs.* 34,78% p<0.1734). We diagnosed more early stage patients (33.33%, 30.6% v. 59.12% p<0.0001), than advanced stage (66.67%, 69.38% *vs.* 40% p<0.0001). Five-years overall survival were 68.4%, 73,3%, and 91%. *Conclusions*. The comparsion of HL cases from the same geographic are during different time period provides an opportunity to observe the changing of clinicopathological features of HL.

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AUDIT OF ABVD CHEMOTHERAPY FOR HODGKIN LYMPHOMA PATIENTS REGARDLESS OF THE ABSOLUTE NEUTROPHIL COUNT AND WITHOUT G-CSF; NO TREATMENT DELAYS AND OPTIMAL DOSE INTENSITY

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Background. Historically delivery of the standard Hodgkins lymphoma chemotherapy regimen "ABVD" has been supported with G-CSF and patients experiencing myelosuppression were given chemotherapy treatment delays which affected dose intensity. *Aims.* To review local practice, we investigated whether delivery of ABVD, administered regardless of the absolute neutrophil count, and without routine supportive G-CSF, caused patients to experience treatment delays due to either myelosuppression or sepsis. Dose intensity in the patient cohort was examined

as this has previously been shown to be important in survival. *Design and Methods*. 22 patients were identified and patient notes and pharmacy ("Chemocare") records audited. Dose intensity scores were calculated as per published formulae. *Results*. Of the 22 patients 18 had completed chemotherapy while 4 were on ongoing treatment. The total number of administration days of ABVD was 206 (103 cycles of ABVD). 3 patients had dose omissions of Bleomycin or Vinblastine due to non haematological side effects. 17 patients (77%) experienced at least one episode of grade III or grade IV neutropenia (neutrophils $<1.0x10^9$ L), 12 (55%) experienced at least one episode of grade IV neutropenia (neutrophils $<0.5x10^9$ L) on scheduled administration days . Of 206 administrations 81 (39%) were given with an absolute neutrophil count of $< 1.0x10^9$ L. 3 patients required admission with neutropenic sepsis with only 1 of these patients encountering a subsequent delay in ABVD administration. Of the 18 patients who have completed treatment median dose intensity was 1 (mean 1.0). *Conclusions*. In an unselected population, ABVD administration irrespective of the absolute neutrophil count allowed treatment to be given safely without significant delays caused by neutropenia, thrombocytopenia or sepsis. There is no need for routine growth factor support or dose alteration to maintain dose intensity.

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BCL-6 PREDICTS IMPROVED PROGNOSIS IN HODGKIN LYMPHOMA. TRUE OR FALSE?

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Introduction. In several past years researches in the field of the biology of Hodgkin lymphoma (HL) were very extensive. Several clinical and laboratory feature have been used to compose a prognostic model, the International Prognostic Score (IPS), which is able to predict failure-free survival (FFS) and overall survival (OS). However, this model usually fails to identify patients with very low or very high chances of cure, who might benefit from therapies tailored to their predicted risks. Therefore, attention has turned to the possible identification of biological features that could be added to the IPS in order to better predict clinical outcome. Aim: The aim of this study was to evaluate whether the expressed p53, pRb, bcl-6, EBV and Ki67, combined with IPS, could be important predictive factors for more accurate outcome in patients with HL. *Design and Methods*. Between October 2001 and June 2003, 40 de novo patients with a confirmed diagnosis of Hodgkin lymphoma were studied. Immunhistochemistry was performed on formalin-fixed paraffin-embedded tissue from diagnostic biopsies, with prediluted monoclonal antibodies against p53, pRb, bcl-6, EBV and Ki67. Positively staining H/RS cells were counted and expressed as ratio of total observed H/RS cells. Negative staining was recorded as 0%, and further positivity was graded from 1-100%, depending on the proportion of the positive cells. Results: Group of 40 patients with HL consisted of 60% males and 40% females were analysed, with mean age at the diagnosis 30.2 years (range 15-68 years). A vast majority of patients (85%) had nodular sclerosis histological subtype of HL, 62.2% of the patients were in an advanced clinical stage (III and IV) of HL, and 57.5% had involvement of the mediastinum. Half of the patients had low IPS (0,1,2). Baseline treatment was ABVD while patients with additional risk factors such as large mediastinal mass or three or more involved lymph node areas treated with escalated BEACOPP. Five years OS and FFS were 70.8 and 60.1 months, respectively. In univariate analyses, only bcl-6 positivity (expression bcl-6 > 20%) had a significant effect on OS (77.1 months in bcl-6-positive patients vs 44.4 months in bcl-6-negative patients; $p<0.01$) and FFS (63.9 vs 38.2 months; $p=0.017$). Correlation between the expression of the oncogenes and the response to therapy was not found. As observed previously,the value of IPS was a significant indicator of survival before relapse/death. *Conclusions*. Cell cycle regulatory molecules including p53, pRb, bcl-6, EBV and Ki67, had different levels of expression in patients with HL, but we found that only bcl-6 had influence on the outcomes. Full assessment of the prognostic role of the biological features in patients with HL required studies with larger number of cases and longer observation time.

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BONE NECROSIS IN A PATIENT WITH HODGKIN LYMPHOMA AFTER TREATMENT WITH PEGFILGRASTIM AND SHORT COURSE OF STEROIDS

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Background. Osteonecrosis, also known as aseptic necrosis or ischemic necrosis of the bone is a pathological process that has been associated with numerous conditions and therapeutic interventions. The mechanisms by which this disorder develops are not fully understood. However, compromise of the bone vasculature leading to death of bone appears to be common to most proposed etiologies. The male to female ratio is 8 to 1. Prolonged treatment with glucocorticosteroids is one of the frequently reported causes of this disorder. *Case report*. We present a 28 year old patient diagnosed with nodular sclerosis Hodgkin lymphoma who developed an osteonecrosis immediately after the first cycle of treatment. He had an advanced stage disease with poor prognostic factors (IPI=5). His past history was uneventful and physical examination revealed a massive cervical lymphadenopathy. CT scan showed also bulky mediastinal lymph nodes with evidence of jugular vein thrombosis. Bone marrow (BM) biopsy at presentation showed a normocellular BM with no evidence of malignancy. Enoxaparin and escalated BEACOPP chemotherapy were started. The therapy included 100 mg of oral prednisone per day for 10 days. 48 hours post completion of the first cycle of escalated BEACOPP the patient received a subcutaneous injection of pegylated G-CSF (Pegfilgrastim). The WBC count on the day of Pegfilgrastim treatment was $2,980/mm^3$ the next day the patient developed severe rest groin pain that radiated to the thighs and buttocks. 48 hours after the treatment with Pegfilgrastim his WBC count reached $52,360/mm^3$. The bone X- ray as well as the Technetium-99 bone scan and CT scan showed signs of necrosis of the bone which involved the head of the femur, both femurs and the pelvis. MRI scan showed a hypervascular granulation tissue which is regarded as pathognomonic sign of this disease. A bone biopsy was obtained during a procedure of core decompression and confirmed the diagnosis of bone necrosis. At the same time, a repeated BM biopsy showed a normocellular BM with no evidence of necrosis. A broad workup excluded the presence of hypercoagulable state such as antiphospholipid antibodies, inherited or acquired thrombophilia, sickle cell or Gaucher disease .The patient completed 5 cycles of ABVD and involved field radiotherapy and one year post completion of chemotherapy is in complete remission. He remains with severe pain and there is radiographic evidence of persistent bilateral osteonecrosis of the pelvis and head of the femur. *Conclusions*. Bone pain is a common side effect of treatment with G-CSF; however, we are not aware of any previous reports of bone necrosis due to use of this drug. Our patient received only a very short course of steroids during the first cycle of the treatment. Therefore, it is unlikely that this treatment alone was responsible for the bone necrosis. We assume that the combination of his very high WBC count together with the use of steroids contributed to metabolic, mechanical and ischemic changes that compromised the blood supply to the bone and caused the osteonecrosis in our patient.

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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, regarding chemotherapy and radiotherapy, were available and utilized over different time periods for patients with Hodgkin's disease (HD). Even today, different centers and schools have adopted different treatment modalities as standard. Efforts are directed towards tailoring the optimal treatment method for each patient, or at least 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. Univari-

ant 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 valuable 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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OBSERVATIONS WITH INTERIM PET/CT IN HODGKIN-AND DIFFUSE LARGE B-CELL LYMPHOMAS

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Background. Complete remission and recovery have been achieved in the majority of lymphoma patients owing to development of diagnositcs, modern treatment strategies, and risk-adapted therapy. 18FDG-PET/CT has role in the correct determination of stage, prognostic factors and early recognition of relapsed in Hodgkin's lymphoma (HL) and diffuse large B cell lymphomas (DLBCL). Positive or negative interim PET/CT had stronger connect with prognosis of the disease than prognostic factors, that we knew. *Design and Methods*. We examined 50 patients (32 HL and 18 DLBCL) in the CHEAP (chemotherapy effectiveness assessed by PET) study, after 2. or 3. cycle of the chemotherapy from June 2008. Assessment of the examen based on SUV and we compared this with the pretreatment PET/CT. We detected complete metabolic remission or minimal residual uptake in 80% of the patients. We continued the treatment of these patients. Interim PET/CT was positive in 20% of the cases, we changed the therapy (one patients had high-dose therapy and autolog stem cell transplantation). *Conclusions*. Interim PET/CT is useful for lymphoma patients. But there are some questions that we don't know the answers yet. Shall we do PET/CT after complete the therapy, if interim PET/CT was negative? Shall we change the therapy (cycle, dose or another therapeutic options) if we seen minimal residual uptake on interim PET/CT? Shall we reduced therapy (cycle or dose) if interim PET/CT negative?

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GEMCITABINE (GEMZAR) AS A SALVAGE THERAPY AT RELAPSED AND REFRACTORY HODGKIN' S LYMPHOMA

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Background. Most patients with Hodgkin's lymphoma (HL) can be cured with initial chemotherapy. Effective salvage treatment is needed at about 20-30% patients who had refractory and relapsed disease. The purpose of this study was to analyzed the efficacy and the toxic profile of gemcitabine in patients with relapsed and refractory Hodgkin' s lymphoma. *Design and Methods*. This study included ten patients with Hodgkin' lymphoma diagnosed between January 2001 and April 2007 at the Institute of heamtology CCS, Belgrade. Demographic caracteristics were as follow: male/female ratio was 7:3 (70%:30%); the median age was 43 years (range, 20-76). All patients had advanced disease: 4 pts had CS II B M+, 2 pts had CS III B, 4 pts had CS IV B); International Prognostic Scor < 3 had 4/10 patients. Six patients were refractory to initial therapy. Six patients with mediastinal bulky form of disease were initially treated with BEACOPP regimens and 4 pts received ABVD. Seven patients received involved field radiotherapy. Gemcitabine was administered at a starting dose of 1250 mg/m^2 on das 1, 8 and 15 every 3 weeks in combinations with steroids (dexamethasone). All patients had received at least 2 cycles of gemcitabine (range, 2-6). The median follow-up period was six months. *Results*. Overall response rate was 30% with 1 patient achieving complete remission (CR) and 2 patients partial remission (PR). Hematological toxicity grade 3-4 occurred in 5 patients leading to dose reduction. No other non-hemathological toxicites were observed. Seven patients discontinued treatment because of dissease progression. The median time to treatment failure was 4,5 months. The longest responder has been in CR for over 40 months. *Conclusions*. Gemcitabine is an effective drug with low toxicity profile in patients with refractory and relapsed Hodgkin' s lymphoma. Dose and schedule may be modifided in the future to optimize responses. Further trials using gemcitabine in combination with other chemotherapy are needed.

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THE PROGNOSTIC PROFILE AND THE OUTCOME OF PATIENTS WITH EXTRANODAL HODGKIN'S LYMPHOMA

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Background. Hodgkin's lymphoma (HL), much more than non Hodgkin's Lymphoma (NHL) is predominantly a nodal disease with extranodal involvement being uncommon. Although the treatment of patients (pts) with HL at any stage of presentation is highly successful, a significant minority of pts fails primary therapy. Distinguishing further prognostic factors might contribute to initially better selection of pts for more intensive treatment. *Aims*. To analyze the prognostic value of extranodal involvement in advanced HL pts in order to determine optimal initial prognostic model which could follow more adequate therapeutical modality. *Design and Methods*. In a cohort of 89 pts with advanced HL (CS IIB-IV) treated with ABVD regimen from 1997- 2004, we examined subgroup of 31 pts with extranodal disease for prognostic profile, at diagnosis. The median follow-up was 7 years (yrs). Their significance was evaluated according to the response to treatment and survival period. It was correlated with International Prognostic Score (IPS), bulky mass, >3 sites involvement, ESR>50 as well as molecular parameters Ki67, Bcl2 and BAX. *Results*. The distribution of extranodal disease was: 18 patients had bone marrow infiltration, the spleen in 5 patients, the lungs in 4 patients, the liver in 4 pts. The IPS> 3 had 25 pts and 15 of them had bone marrow infiltration. The EN localisation had adverse effect on OS7y (45% vs 78% for pts without EN, p=0.005) and also on EFS (log rank p<0.05). There was a positive correlation between IPS>3 and EN disease p=.001, as well as bone marrow involvement p=.003. Both pts with EN localization and pts with bone marrow infiltration had high proliferative index (Ki67>50%) p=0.019, p=0.004 respectively. There was no significant correlation with other examined features. Cox's multivariate model did not revealed EN disease as a significant independent prognostic factor (p>0.05). *Conclusions*. Extranodal HL patients with high IPS and high proliferative index are at higher risk of treatment failure and might be eligible for more effective treatment approach.

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FAMILIAL CD3+ T LARGE GRANULAR LYMPHOCYTE (T-LGL) PROLIFERATION PRESENTING WITH CYTOPENIAS: HISTOPATHOLOGICAL, FUNCTIONAL AND MOLECULAR STUDIES

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CD3+ T-LGL proliferations often present with cytopenias (especially neutropenia) and splenomegaly. We report results from detailed analysis of two relatives (father and son) with CD3+ T-LGL proliferation. Case 1 (father): thrombocytopenia, splenomegaly, positive test for anti-nuclear antibodies at the age of 55 (1999). Partial response to corticosteroids. 2002: splenectomy (initial pathology assessment «non-specific findings»), mild persistent neutropenia ever since. Case 2 (son): pancytopenia since the age of 17 (1993). Routine laboratory investigations (including bone marrow aspiration and biopsy): non-diagnostic. 2002: pancytopenia, splenomegaly, splenectomy. Partial recovery of hematologic values, persistent thrombocytopenia since 2003. Strikingly similar results were obtained for both cases on detailed laboratory investigations. (1) Flow cytometry (repeated tests 2003-2006): inverted CD4/CD8 ratio, increased CD3+CD8+CD57+ cells (father>30%, son: 18-22%). (2) Antineutrophil antibodies (GIFT, GAT assays): positive. (3) Molecular testing for elastase mutations: negative. (4) Bone marrow biopsy: 10-15% interstitial T (CD3+CD20-) lymphocytic infiltration (CD8>CD4), increased cellularity, hyperplasia of all series with concomitant dysplastic changes. (4) Spleen biopsy: (i) white pulp: hyperplasia of both the B zone (mantle/ marginal zone, CD20+CD3-) and the T zone (CD20-CD3+, CD4+>CD8+) (ii) red pulp: moderate CD3+CD20- T lymphocytic infiltration (CD8+>CD4+) of the splenic cords and sinusoids. The most important difference between father and son concerned the patterns of T cell receptor β (TRBV) rearrangements. (1) Father: monoclonal TRBV rearrangement amidst a polyclonal background. TRBV gene repertoire: 34 identical TRBV12-3/TRBD2/TRBJ2-6 rearrangements (major subclone) and 16 identical TRBV7-2/TRBD1/TRBJ1-2 rearrangements (minor subclone) from three timepoints over a 6-year period among a total of 147 subcloned sequences. (2) Son: polyclonal TRBV rearrangement. TRBV gene repertoire: heterogeneous. In conclusion, the results from TRBV repertoire analysis support a role for antigen in lymphomagenesis, in the sense that persistent antigenic drive may initially lead to polyclonal proliferation and, under certain circumstances (genetic predisposition?), evolve to clonal disease (T-LGL leukemia). Furthermore, the findings reported here indicate that TCR antigen specificity may determine the clinical presentation by specifically recognizing and destroying distinct hematopoietic lineages.

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FOLLICULAR LYMPHOMA: INCIDENCE, PROGNOSTIC FACTORS AND OVERALL SURVIVAL IN 78 PATIENTS FOLLOWED FOR 7 YEARS(2002-2008) FROM A SINGLE INSTITUTION

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Background. Follicular lymphoma(FL) represents approximately 25% of all adult nonHodgkin's lymhomas in Western countries, usually characterised by an indolent course and a median survival of 10 years. *Aim.* : to identify the prognostic factors that influence overall survival (OS) in patients with follicular lymphomas. *Methods*. We report here our experience with 78 patients treated in our institution since 2002.These 78 patients represent above 2% of the patients with B cell lymhoproliferative disorders seen in our institution.We evaluated the predictive value of pretreatment clinical features:age, sex,Ann Arbor stage,Bsymptoms,ECOG performance status, presence of bulky disease, bone marrow involvement and number of extranodal sites.Patients were evaluated by physical examination,including LDH,beta-2-microglobulin, bone marrow biopsy and radiologic studies(chest x-ray, CT scans).Therapy strategies ranges from single-agent therapy,combination therapy,Interferon,combinations chemotherapy/targeted monoclonal antibody to radioimunotherapy, local radiation therapy. Nine patients with follicular grade 1 lymphoma,Ann Arbor stage I did not require therapy at a median of of 4 years. Fiftyeight patients were BCL2 positive and harbor t(14;18). *Results.*The median age of patients was 62.Men and women are affected equally. According to the WHO classification, 32 % of patients were with grade I FL,24% with grade II FL and 44% of patients were with grade III FL. The survival was 72% at 5 years.Among extranodal sites of disease, bone marrow was the most common in our series .Features associated with shorter survival included advanced stage-especially IV, extranodal and bone marrow involvement,age>60. Serum beta 2 microglobuline was also strongly correlated with survival:76% of those with beta2 microglobuline lower than 3 were alive at 5 years.The most patients had high serum LDH levels, but lower than 1000U/L and a good performance status(0-1).Chemotherapy /targeted monoclonal antibody combination improved responses compared with chemotherapy alone. The best survival was related to the patients treated with Interferferon. There was no correlation between presence of BCL2 and clinical outcome of disease. *Conclusions.* The incidence of follicular lymphoma in our institution is lower that it was reported in literature. Similar results were reported in other institutes from Romania so it seems that the incidence of follicular lymphoma in our country could be lower than Western countries.Presence of t(14;18) is similar to frequency in Western countries but is not of prognostic significance.

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SERUM LEVELS OF TH1 AND TH2 CHEMOKINES IN CUTANEOUS T CELL LYMPHOMAS (CTCL)

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Background and aims. Interferon-inducible protein-10 (IP10)/CXCL10, monokine induced by interferon-α (MIG)/CXCL9, thymus and activation-regulated chemokine (TARC)/CCL17 and CTACK/CCL27, represent Th1 and Th2 chemokines that regulate skin selective T lymphocyte trafficking and recruitment in inflammatory and neoplastic disorders. In Cutaneous T Cell Lymphomas (CTCL), which derive from CD4+ memory Th2 cells, they have been considered critical both for pathogenesis and progression from early to advanced stages, although few and conflicting results are available in literature. The aim of our study was to determine the serum release of these four chemokines in 24 CTCL patients at different stages and to compare their levels with those of a population of healthy donors in order to identify a potential clinical significance; moreover, we intended to assess whether the combination of PUVA and interferon-α2b (PUVA/IFNα2b) in early CTCL affects the balance of serum levels of Th1 and Th2 chemokines and may have clinical importance. *Design and Methods*. Serum samples were collected from 24 CTCL patients at the time of the diagnosis (17 mycosis fungoides/MF-15 early and 2 advanced; 7 Sézary Syndrome) and from 20 healthy donors age- and gender-matched used as controls. In 15 MF patients who obtained a clinical complete remission after PUVA\IFNα2β combination therapy, serum samples were also collected and examined after the end of the treatment. Quantikine kits (R&D Systems, Minneapolis, USA) were used for the ELISA assays, performed according to the manifacturer's instructions, and optical densities were determined using a VICTOR2 Multilabel Counter fluorescence plate reader (Perkin-Elmer, Torrance, CA, USA). *Results*. In all CTCL patients serum CCL27 levels were similar to those of healthy controls, whereas for the other chemokines we observed a statistically significant increase of serum CCL17 and CXCL10 levels in advanced CTCL compared to early MF (6/9 vs 2/15, Fisher's exact test, p=0.021). We also found increased levels of CXCL9 in 5/9 advanced CTCL and in 4/15 early MF (p=NS, between the two groups of CTCL patients). Treatment with PUVA plus interferon-α2b did not substantially change the serum levels of any of these chemokines. *Conclusions*. Although we and others have previously observed enhanced CCL27 expression by keratinocytes in MF cutaneous lesions, suggestive for a possible role of this molecule in lymphocyte skin-homing (1,2), we were not able to confirm an elevation in serum, as indicated by others (2). We therefore propose that CCL27 might be considered a cutaneous, but not a serum marker of disease activity. On the other hand, we found a significant elevation of serum

levels of the other three chemokines (CCL17, CXCL10 and CXCL9) in less than 40% CTCL patients, thus limiting the clinical utility of these proteins as specific disease-markers. However, abnormally high serum levels of CCL17 and CXCL10 tend to be associated to more advanced clinical stages of disease, and they can be retained as potential prognostic markers. Finally, while Interferon-γ and UVB were reported to affect the serum levels of Th1 and Th2 chemokines in CTCL (3-6), in our experience PUVA and interferon-α2b failed to show similar effects, despite achieving complete remission in early MF stages. In this subset of patients, evidence of clinical utility of serum chemokine determination in monitoring the disease during therapy is still lacking.

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MANTLE CELL LYMPHOMA: ONE OR MORE ENTITIES?

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Mantle cell lymphoma (MCL) is pathogenetically linked to the reciprocal translocation t(11;14), and is considered to derive from the neoplastic transformation of the "naïve" lymphocyte residing in the mantle zone (MZ). Nevertheless, the differences between the phenotypic profile of MCL and its proposed normal counterpart (mantle zone lymphocyte) raise significant questions regarding its ontogeny. We present results of a combined morphologic, immunohistochemical and genotypic analysis of 30 MCL cases. The study group consisted of 24 nodal and 6 extranodal MCLs. Morphologically, 17 cases were ascribed to the "common" variety, 5 of which exhibited "monocytoid" and 1 "plasmacytoid" differentiation. The growth pattern was as follows: (1) diffuse: 7/30, (2) mantle zone differentiation (MZDif): 8/30 (3) "vaguely" nodular: 3/30, (4) nodular+diffuse: 10/30, (5) nodular+diffuse+MZDif: 2/30. The immunohistochemical study (on paraffin sections) revealed the following profiles: (1) CD20+, CD79a+, BCL-2+, Cyclin D1+, CD23-, CD10-, BCL-6-, DBA.44-, CD3-: 30/30, (2) CD5+: 28/30, (3) CD43+: 26/30. The cellular proliferation index ranged from 6-96% (median: 20%). Kappa or lambda light chain isotype expression was observed in 10/27 and 17/27 cases, respectively. The breakdown of cases based on the heavy chain isotype was as follows: (1) IgM: 20/28, (2) IgM, D: 7/28, (3) IgG: 1/28. CD27 positivity was observed in 13/25 cases, 8 of which exhibited lambda light chain clonal restriction. Molecular analysis of immunoglobulin heavy chain (IGH) gene rearrangements identified one case carrying rearranged IGHV genes with <98% germline nucleotide identity ("mutated"), 7 cases carrying IGHV genes with 98-99.7% identity ("borderline mutated") and 4 cases with unmutated IGHV genes (100% identity). Six of seven CD27 positive cases with available molecular data were found to carry "borderline mutated" or "unmutated" IGHV genes. In addition, 3/7 IgD+ cases were positive for CD27. In conclusion, the results of this study document the significant phenotypic and genetic heterogeneity of the MCL and suggest the existence of "subtypes" defined on the basis of: (1) the mutational status of IGHV genes ("unmutated" versus "borderline mutated" and rarely "mutated"), (2) the functional profile of the malignant cells ("naïve" versus "activated"/"antigen-experienced"). CD27 positivity in a subset of cases and biased expression of lambda light chain isotype could be construed as evidence for antigen contact and receptor editing, respectively. Finally, in light of the aforementioned results, the mantle zone, "naïve" lymphocyte, cannot be established as the cell of origin of the MCL, at least in a significant proportion of cases.

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PROTEIN KINASE Cβ (PKCB) INHIBITOR ENZASTAURIN HCL (LY 317615) IN MANTLE CELL LYMPHOMA

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Introduction. The protein kinase C (PKC) family of enzymes are serine/threonine kinases essential to the cell signal cascades effecting cellular growth, proliferation and apoptosis. Previous studies in diffuse large B-cell lymphoma suggest that PKC β activity enhances B-cell proliferation and survival. Accordingly PKC β overexpression correlates with poor clinical prognosis in this lymphoma subtype. The pivotal role of PKC β in neoplastic transformation renders it a potential therapeutic target in the therapy of hematologic malignancies. However, the effect of PKC β inhibition in mantle cell lymphoma (MCL) has not yet been reported. *Aims*. To access the effect of PKC ß inhibition in mantle cell lymphoma on cell proliferation, mRNA- and protein expression to define genetic predictors of sensitivity to PKC β inhibition in mantle cell lymphoma. *Design and Methods*. Five MCL cell lines (HBL-2, GRANTA 519, Jeko-1, Z138, Rec-1), two hematological control cell lines (Jurkat, Karpas 422) and patient samples were cultured in the presence of LY 317615 (PKC ß inhibitor). Cell proliferation and viability was assessed by cell counts and WST-1 proliferation assay. Analysis of cell cycle profile and apoptosis was performed by flow cytometry (PI and Annexin V FITC staining). mRNA expression was measured by microarray and real time PCR after 4h and 8h in cell lines. Protein expression was analysed by western blot. *Results*. Treatment of the cell lines with LY 317615 lead to inhibition of cell proliferation and accumulation of cells in the G2, M phase in susceptible cell lines (Hbl-2, Jeko-1), whereas cell cycle profile remained unaltered in the refractory cell lines (Rec-1). Enzastaurin induced CCND1 and BCl2 mRNA downregulation as well as reduced protein levels. A significant downregulation of the global mRNA expression could be observed in susceptible to enzastaurin cell lines (Jeko -1 and Granta 519 ($p<2.2x10^{-16}$) after 8h of treatment, but upregulation in Rec-1 (p=0,0005618). Further mRNA expression analysis are ongoing to determine differentially expressed mRNAs as predictors of enzastaurin sensitivity. *Conclusions*. PKCβ inhibition by LY 317615 inhibits cell growth and induces cell cycle alterations and apoptosis in MCL cell lines. Differences in mRNA expression in susceptible to refractory to enzastaurin cell lines were observed. The characterization of differentially expressed mRNAs as predictors of enzastaurin sensitivity will be useful for a targeted application of combination partners for the treatment of MCL.

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EPIGALLOCATECHIN-3-GALLATE INDUCES GROWTH INHIBITION INDEPENDENT OF THE 67 KDA LAMININ RECEPTOR IN LEUKEMIC CELLS

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Background. Epigallocatechin-3-gallate (EGCg) is the major catechin present in green tea which has attracted much attention because of its various pharmacological effects. Especially, many reports showed that EGCg has anti-tumor properties related to induction growth arrest and/or apoptosis via reactive oxygen species, mitogen-activated protein kinase (MAPK), epidermal growth factor receptor and etc. However, EGCg has been shown to produce hydrogen peroxide rather than induce an EGCg-direct action under certain cell culture conditions. Recently, 67 kDa laminin receptor (67LR) was identified as a cell surface receptor that mediates the direct anti-cancer action of EGCg. The expression level of 67LR strongly correlates with the risk of tumor invasion and metastasis. *Aims*. This study investigated the anti-tumor effects of EGCg against myeloid leukemic cell lines UT-7 and K562, and lymphoid leukemic cell line Raji by examining the relationship between EGCg sensitivity and 67LR expression levels. *Design and Methods*. Each cell was cultured with various EGCg doses (1 nM - 10 μM) for 1 to 10 days to clarify the sensitivity to EGCg. After incubation, the cells were harvested from the culture and the number of viable cells was determined by the trypan blue exclusion method. In the analysis of the expression level of 67LR on the

cell surface and cytosol, each cell was analyzed using a flow cytometer. *Results*. The number of Raji cells decreased in a dose- and time-dependent manner under the treatment with EGCg. A significant growth arrest against Raji cells was observed with the treatment with 1 - 10 μM EGCg. Treatments of 10 μM EGCg induced over 70% growth inhibition at day 5, and treatments of 1 μM EGCg induced 50% growth inhibition in Raji cells at 10 days. On the other hand, UT-7 and K562 cells were resistant to EGCg at several concentrations tested here. However, the expression level of 67LR on the cell surface by a flow cytometric analysis showed the highest expression in UT-7 cells which are most resistant to EGCg-induced growth inhibition (mean fluorescence intensity (MFI): 56.7). Furthermore, no difference was observed in the expression of 67LR on the cell surface among K562 cells and Raji cells (MFI: 3.7, 4.3 respectively). Although Raji cells are most sensitive to EGCg treatments, it presents little 67LR on the cell surface. These results suggest that EGCg-induced growth inhibition is induced independence of the 67LR mediated pathway. *Conclusions*. The growth inhibition against Raji cells was induced at 1 μM EGCg treatments suggesting an adequate concentration for induction the direct effects of EGCg through 67LR. This study implies the possibility that other signaling pathways therefore exist induced by EGCg independent of 67LR in lymphoid leukemic cells.

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FOXP3+ T REGULATORY CELLS EXHIBIT EFFECTOR PHENOTYPE IN PATIENTS WITH B-CELL MALIGNANCIES

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Background. Recent reports have indicated that infiltration of Foxp3+ T-regulatory cells (Tregs) in the tumor area can be associated with better overall survival of patients with B-cell malignancies, which is in contrast to patients with solid tumors. Since the tumor in lymphoma patients is a cell of the immune system it is possible that Treg cells may have a suppressive role in tumor progression. *Aims*. In this study, we have investigated the phenotype and function of Tregs in patients with B-cell malignancies. *Design and Methods*. Peripheral blood was collected from patients with B-cell malignancies and evaluated for the presence of CD3+CD4+Foxp3+ cells. These cells were further characterized by investigating their expression of IL-7R (CD127), IL-2R (CD25), FasL and CD107a, as well as evaluating their biological function *in vitro*. Results Tregs were significantly increased in patients with B-CLL while not in patients with B-cell lymphoma compared to healthy, age-matched controls. However, all patients displayed higher expression of the degranulation marker CD107a, indicating perforin/granzyme release, on their Tregs. No difference in FasL expression was found. Interestingly, patients that did not respond to treatment had fewer effector Tregs than controls. Further, a flow cytometry-based cytotoxic assay indicated that patient Tregs were more efficient in killing autologous B-cells than Tregs from healthy donors. *Conclusions*. Patients with B-cell malignancies have circulating Tregs that exhibit effector phenotype and these cells may participate in the control of tumor cell progression.

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ANALYSIS OF NEOPLASTIC LYMPHOCYTES IN PERIPHERAL BLOOD OF PATIENTS WITH B NON HODGKIN LYMPHOMA WITHOUT LYMPHOCYTOSIS

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Background. Several studies in non Hodgkin lymphomas include peripheral blood involvement as a prognostic factor. Although a high leucocyte count probably reflects peripheral blood involvement, it may also occur without lymphocytosis. In order to detect these cases, we must employ methods more sensitive than morphology. Aim: To study the presence of neoplastic lymphocytes in peripheral blood of patients with B non Hodgkin lymphoma (B-NHL) without lymphocytosis. *Design and Methods*. : We applied a standardized four-color cytometry assay to peripheral blood from 42 patients with B-NHL at diagnosis without lymphocytosis (lymphocytes <4x10^9/L): 8 lymphoplasmacytic lymphoma (LPL), 4 splenic marginal zone lymphoma (SMZL), 1 nodal marginal zone B-cell lymphoma (NMZL), 15 follicular lymphoma (FL), 5 mantle cell lymphoma (MCL), 4 diffuse large B-cell lymphoma (DLBCL), 2 Burkitt lymphoma (BL), 2 MALT lymphoma (ML) and 1 cutaneous B lymphoma (CBL). Samples were stained using a mixture of the following directly conjugated monoclonal antibodies: CD45, CD3, CD4, CD8, CD19, CD5, CD10, λ and κ. A total of 0.5 - 1x10^6 events from each sample were adquired using a BD FACSCalibur flow cytometer with CellQuest software and analyzed with Paint-a-gate software (BD). Results: The median lymphocyte count was 1.9x10^9/L (range, 0.6-4x10^9/L). Light chain restriction and inmunophenotypic aberrations were observed in 18 samples (42.8%) with a median neoplastic lymphocyte count of 0.47x10^9/L (range, 0.01-1.43x10^9/L). Among these altered cases, 8 were predominantly disseminated lymphomas (3 SMZL and 5 LPL), 4 MCL, 4 FL and 2 DLBCL. Peripheral blood involvement by morphology was observed in only 7 samples (16.6%) with a median neoplastic lymhocyte count of 0.56x10^9/L (range, 0.07-1.05x10^9/L). In the other 11 cases (26.2%), the morphologic study was negative and their neoplastic lymphocyte values were lower (median: 0.29x10^9/L, range: 0.01-1.43x10^9/L). *Conclusions*. Flow cytometry allows us to easily identify and quantify circulating neoplastic lymphocytes in an important proportion of B-NHL without lymphocytosis and morphologic evidence. The probably prognostic value of blood involvement analyzed by this methodology should be studied in the future.

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DOWNEXPRESSION OF TCR-ζ CHAIN IN PATIENTS WITH B-CELL NON-HODGKIN'S LYMPHOMAS

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Background. In patients with hematological malignance, cell-mediated immunity is often suppressed, being most profound in those advanced disease. The immune dysfunction, may be due to the disorder of recent thymic output function, the abnormal expression of T cell receptor repertoire and, may in part, due to altered expression of TCR ζ chain, which is an important component of the TCR. *Aims*. To analysis the expression level of signaling transduction factor TCR ζ chain gene in T cells from patients with B-cell non-Hodgkin's lymphomas (B-NHL). *Design and Methods*. Real-time RT-PCR with SYBR Green echnique was used for detecting the expression level of TCR ζ chain gene in peripheral blood mononuclear cells (PBMCs) of 17 patients with B-NHL, and 17 cases healthy adults served as controls. The β2-microglobulin gene (β2M) was used as an endogenous reference. Relative mRNA expression level of TCR ζ chain gene was analyzed by using the 2Δ-Ct-100% method. *Results*. TCR ζ chain gene was expressed in all patients with B-NHL. Compared with healthy control, the relative mRNA expression level of TCR ζ chain gene could be found significantly decreased (p=0.000). All samples from healthy individuals expressed normal level of TCR ζ chain gene, which is 3.26 times higher than that from B-NHL patients. There was no significant correlation between the expression level of TCR ζ chain gene and age in patients with B-NHL. *Conclusions*. The down expression of TCR ζ chain gene could be identified in peripheral blood of patients with B-NHL, which might play a role in cell-mediated immunodeficiency in most of B-NHL patients.

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NEW MOLECULAR MARKERS IN DIAGNOSIS OF SPLENIC LYMPHOMAS

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Background. Since the initial description of splenic malignant lymphomas, an increasing number of publications have dealt with multiple aspects of diagnosis, molecular pathogenesis and treatment. The diagnosis has been until very recently based on the pathological study of the spleen with the conjunction of the clinical features, although the integration of the morphology in bone marrow and peripheral blood with the immunophenotype and molecular characteristics of the tumour makes a more accurate diagnosis now possible. Therefore the classification, the diagnosis and therapeutic management of these patients have been frequently controversial and inadequate. Molecular studies of these lymphomas are starting to reveal new diagnostic and prognostic markers, and identify new potentially useful therapeutic targets. In this context, new cytogenetic and molecular findings were added to existent data (API2-MALT1 - t(11;18)(q21;q21), del 7q, changes in the expression of the p53 protein, over expression of cyclins, etc). *Aims*. The main

objective of this research study was agreeing on the main diagnostic, staging and therapeutic guidelines for patients with splenic lymphomas. *Design and Methods*. With the aim of characterizing these lymphomas more comprehensively, and of identifying new diagnostic and prognostic markers, we performed cDNA microarray expression profiling and tissue microarray (TMA) immunohistochemical studies in a relatively large series of splenic lymphomas. *Results*. Clinical presentation with lymphadenopathies and B-symptoms was mainly associated with the diagnosis of mantle cell lymphoma, whereas splenomegaly was suggestive of marginal zone splenic lymphomas. In our study group 44% patients were diagnosed at an early clinical stage with still conserved hematopoiesis, 32% of them, being diagnosed following a routine hemoleucogramm. Time-to-event analysis, involving Kaplan-Meier, log-rank test, and Cox models, served to identify markers influencing disease-related overall survival (DOS) and progression-free interval (PFI). The estimates of the hazard ratios (HRs) along its 95% confidence interval (95% CI) were computed via the Cox model. Patients were considered to have progressive disease on the basis of progression to a more advanced stage, or the enlargement or new appearance of lymph nodes, development of systemic symptoms, large B-cell lymphoma progression, or death attributable to the lymphoma. Concerning molecular markers we had studied, API2-MALT1 was correlated with prognosis in MALT lymphomas, overexpressions of cyclins was correlated with poor prognosis in mantle cell lymphomas and in some marginal splenic lymphomas and aberrant expression of the p53 protein with lymphoplasmocytoid lymphomas. *Conclusions*. We were trying to create a specific guideline for diagnosis criterias, differential diagnosis, staging, prognostic factors, treatment and response criteria. The guidelines proposed here are intended to contribute to the standardization of the diagnosis and treatment of these patients, and should facilitate the future development of clinical trials that could define more precisely predictive markers for histological progression or lack of response, and evaluate new drugs or treatments.

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HYPERMUTATION OF THE BCL6 GENE IN PRIMARY MEDIASTINAL LYMPHOMA AND OTHER TYPES OF DIFFUSE LARGE B CELL LYMPHOMA

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Beckground. Somatic hypermutation of the BCL6 gene and its expression in lymphoma represent specific marker of the germinal center. Thus, analysis of the BCL6 may aid in explanation of the relationship between primary mediastinal (PMBL) and other types of diffuse large B cell lymphoma (DLBCL). These subgroups of lymphoma arise from different stages of B-cell diferention and differ in their ability to be cured by chemotherapy. *Design and Methods*. Forty (40) patients with NHL were included in the study. Twenty (20) pts with PMBL and 20 pts with other types with DLBCL were analyzed for BCL6 status by immunohistochemistry. All samples were analyzed using an antibody panel characterizing PMBL and DLBCL (anti-cytokeratin 5, anti-CD20, anti - BCL6 antibody, anti - BCL2 antibody, anti - MUM1 antibody). *Results.* The average age of pts with PMBL was 30, 65 years, and average age for pts with DLBCL was 47 years, 18 cases from PMBL group showed BCL6 immunohistochemical expression, and 10 cases (50%) from DLBCL group showed BCL6 immunohistochemical expression. Overall survival(OS) was 46,65 months for pts with PMBL respectively 76,2 months for pts from DLBCL group, Event free survival(EFS) was 46,15 months for pts with PMBL and 76,2 months for pts from DLBCL group, Duration of remission(DoR) was 43,6 months for pts with PMBL and 76,75 months for pts with DLBCL *Conclusions*. The consistent expression of BCL6 protein should be considered of germinal center origin.PMBL originate from an already defined sub-population of B - cells which are different from those leading to other types of DLBCL.

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PROGNOSTIC VALUE OF THE SOLUBLE INTERLEUKIN-2 RECEPTOR IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA: RESULTS FROM A RETROSPECTIVE ANALYSIS

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Background. Interleukin-2 (IL2) is a signalling molecule of the immune system which acts by binding to the interleukin-2 receptor (IL-2R). The IL-2R is a heterotrimeric membrane protein complex consisting of three distinct subunits, the α chain (IL-2Rα, CD25), the β chain (IL-2Rβ, CD122), and the γ chain (IL2-Rγ, CD132). Release of the IL-2Rβ extracellular domain gives rise to the soluble interleukin-2 receptor (sIL-2R). Concentration of the sIL-2R increases under various pathological conditions. Aims: It has been shown that increased concentration of the sIL-2R is a predictive indicator of poor prognosis in patients with diffuse large B-cell lymphoma (DLBCL) treated with chemotherapy alone. The question is whether its predicting value is maintained in the era of rituximab. *Design and Methods*. Pretreatment serum levels of the sIL-2R were measured by enzyme-linked immunosorbent assay (ELISA) in 162 patients with *de novo* DLBCL treated with anthracycline-based chemotherapy alone (N=60) or rituximab combined with chemotherapy (N=102). Univariate progression-free survival (PFS) and overall survival (OS) analyses were performed according to the cut-off serum level set at 116 pmol/l (<116 pmol/l N=92/57% vs. ≥116 pmol/l N=70/43%), and the first-line treatment (chemotherapy alone vs. rituximab combined with chemotherapy). The median follow-up for OS and PFS was 29.7 and 30.4 months, respectively. *Results*. Three-year OS was significantly better in patients with low serum levels of the sIL-2R than in those with high sIL2-R levels, regardless of whether chemotherapy was used alone or in combination with rituximab (OS 84.4% vs. 70%, p=0.005). In the chemotherapy alone group, patients with high serum levels of the sIL-2R had significantly worse three-year OS and PFS when compared with those with low serum levels of the sIL-2R (OS 47.7% vs. 80%, p=0.003, PFS 68.4% vs. 81.5%, p=0.034). But in the rituximab group, no differences related to serum levels of the sIL-2R were shown (sIL-2R ≥116 vs. <116: OS 84.8% vs. 88.5%, p=0.222, PFS 85.1% vs. 87.2%, p=0.765). *Conclusions*. Our results showed that pretreatment serum level of the sIL-2R is significantly related to outcome in DLBCL patients treated with anthracycline -based chemotherapy, but has not maintained its prognostic value in the rituximab era. Significance of the sIL-2R should be re-evaluated in large prospective trials with longer follow-up. It is appropriate to evaluate the significance of the sIL-2R with different cut-off levels.

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TREATMENT OF DIFFUSE LARGE B-CELL LYMPHOMA: CHEMOTHERAPY WITH RITUXIMAB IMPROVED PROGNOSIS IN ALL AGE GROUPS

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Background. Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma, accounting for approximately one third of all new diagnoses. Anthracycline-based combined therapy has been the standard treatment for more than 25 years. The addition of rituximab (R) to chemotherapy (Chemo) has significantly improved outcome in older and low-risk younger DLBCL patients and some data have suggested that the administration of rituximab-supplemented intensive chemotherapy may improve prognosis of poor-risk younger patients. Aims: To prove the efficacy of a treatment for adults with DLBCL in the pre-rituximab and rituximab era. *Design and Methods*. From 1995 to 2007, 290 pts. with newly diagnosed DLBCL were treated with curative intent at the Hemato-oncology Dpt. Olomouc. Patients younger than 65 years were divided into low-risk, intermediate-risk and high-risk group and treated with standard CHOP-like, intensified PACEBO-like and intensive sequential chemotherapy (+/- rituximab). Patients older than 65 years were treated with CHOP-like regimen (+/- rituximab) if they had no

major comorbidity. Results: 3-year overall survival (OS) were 84% in R-Chemo group and 59% in chemotherapy without (p<0.001). 3-year progression-free survival (PFS) were 85% in R-Chemo group and 64% in chemotherapy without (p=0.002). R-Chemo significantly improved OS and PFS in pts. younger and older than 65 years (OS: 90% vs 71%, and 69% vs 37%; p<0.001), (PFS: 87% vs 68%, and 78% vs 53%; p=0.09). R-Chemo also improved 3-year OS in aa-IPI 2-3 in comparison with Chemo (91% vs 45%; p<0.001), the OS in aa-IPI 0-1 wasn´t different (90% vs 89%; p=0,76). In R-Chemo group there were also significantly lower number of relapses/progressions (9% vs 30%; p<0.001) and exitus (13% vs 46%; p<0.001). In multivariate analysis, age, tumor size, lymphocyte count, IPI and therapy with R was identified as independent prognostic factor related to OS. *Conclusions*. Prognostically stratified therapy in younger patients and the addition of rituximab in all age groups markedly improved PFS and OS for DLBCL.

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LIVER BIOPSY DURING SPLENECTOMY REVEALS THAT SPLENIC MARGINAL ZONE LYMPHOMA IS OFTEN AN HEPATO-SPLENIC DISEASE

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Background. Primary hepatic lymphomas are rare while secondary liver involvement by systemic lymphoma is relatively common. However, except for hepatosplenic T-cell lymphoma, the incidence and the clinical relevance of secondary liver involvement by different lymphoma subtypes, that primarily arise in the spleen, have not been so far evaluated. In particular, splenic marginal zone lymphoma (SMZL) is disease usually characterized by isolated splenomegaly and bone marrow infiltration. At now, it is not ascertained the incidence of liver involvement in SMZL, also considering the high seroprevalence of HCV in this lymphoma subtype. AIM To analyze splenectomies performed in pts affected by lymphomas and to evaluate the incidence and features of liver involvement by lymphoma in wedge liver biopsies, when available. *Design and Methods*. We analyzed 45 pts with lymphoma who underwent a laparotomic splenectomy from 2001 to 2008: 19 SMZL, 15 primary splenic DLBCL [12 centroblastic (CB) and 3 T-cell histiocyte rich large B-cell lymphoma (TCHRBCL)], 1 secondary splenic DLBCL (a TCHRBCL case evolution of lymphocyte-predominance HL); 8 classic HL (most secondary), 1 lymphoplasmocytic lymphoma, 1 hepatosplenic T-cell lymphoma. A concurrent wedge biopsy of the liver was available for all pts except for 5 (4 SMZL, 1 DLBCL-CB). *Results*. Liver involvement was detected in 18 pts: 11/15 pts (73%) with SMZL, 4/15 (26%) primary splenic DLBCL (2 CB and 2 TCHRBCL), 3/8 (37%) HL, 1 hepatosplenic T-cell lymphoma. In SMZL a portal pattern of liver involvement was detected in 10/11 pts; in 7 pts it was associated to sinusoidal pattern, and in 2 pts to a nodular/lobular pattern; in a single case the lymphoma infiltrate was confined to hepatic sinusoids. Bile duct lympho-epithelial lesions were detected in 6 cases, cholestatic changes in 5, ductopenia in 2 and hepatitic changes in 4. No case showed progression to DLBCL in hepatic infiltrates. HCV serology was positive in 5/15 (33%) SMZL. Three HCV+ cases showed liver involvement by lymphoma and were HCV-RNA (2a/2c in 2 pts and 1b in 1 patient); in all these cases pattern of liver infiltration was portal; 1 of them has a double pattern of infiltration: portal and sinusoidal. The remaining 8 pts with SMZL involving the liver were HCV-. Phenotype was CD20+, CD79a+, CD5-, CD10-, bcl-6-, cyclin D1- in 15/15, bcl2+ in 14/15 and CD23- in 14/15 SMZL. Among the 12 cases of primary splenic DLBCL-CB, 7 were HCV+; 6 cases showed a germinal center (GC) phenotype, whereas 6 had a non-GC phenotype. Liver involvement was detected in 2 HCV- pts, with a nodular to diffuse pattern. Liver localization was found in 2/3 cases of HCV-TCHRBCL (portal and nodular/lobular pattern). Both DLBCL-CB and TCHRCBCL with liver involvement showed a non-GC phenotype. *Conclusion*s. Our data demonstrate that SMZL is characterized by a high incidence of liver involvement with a frequent portal and sinusoidal pattern. Interestingly, detection of hepatic localization by SMZL seems to be irrespective to HCV status infection and appears as an intrinsic feature of this lymphoma subtype.

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OUTCOME WITH LONG-TERM L-ASPARAGINASE THERAPY OF ADOLESCENTS AND YOUNG ADULTS (AYA) WITH PRECURSOR B AND T LYMPHOBLASTIC LYMPHOMA: PRELIMINARY RESULTS

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Background. Lymphoblastic lymphoma (LBL) is a relatively rare subtype (3-5%) of adult non-Hodgkin's lymphoma. LBL represents a distinctive lymphoma entity with cytological and morphological features similar to those of acute lymphoblastic leukemia (ALL). At present, generally precursor B and T LBL has been treated with intensive ALL -similar chemotherapy. At the same time in a number of shows that high-dose methotrexate and early intensification useful in the treatment of leukemia is not useful in lymphoma (Abromowitch M., ASH 2008). The greatest amount of data obtained in studies in children. Optimal treatment of lymphoma treatment for adolescents and young adults (AYA) with LBL is still not clear. *Aims*. Thus, the purpose of this retrospective study was to investigate treatment outcomes for adolescents and young adults (AYA) with LBL treated with long-term L-asparaginase versus BFM-similar therapy. *Design and Methods*. 18 patients (pts) were enrolled from July 1998 to December 2007. 6 (33%) pts were treated on the protocol NHL-BFM 90 for non-B NHL and 11 (67%) pts to treat pediatric protocols ALL-MB 91 and 2002. In these protocols the pts receive four drug induction with dexametasone 6 mg/m^2 daily for 36 days, daunorubicin 45 mg/m^2 for 2 doses, vincristine 2 mg weekly for 5 doses and and intrathecal (IT) cytarabine and IT methotrexate and IT prednisolone weekly for 6 doses. Consolidation therapy included L-asparaginase in a constant dose of 10000 ME/m^2 weekly for 18 doses and 6-merkaptopurine 50 mg/m^2 (100%) daily and methotrexate 30 mg/m^2 (100%) weekly with weekly doses adjusted according to white blood cell count. Central nervous system (CNS) irradiation is performed only for pts with CNS involvement at diagnosis. Maintenance was carried out up to 24 months. The protocol NHL-BFM 90 called for the purpose of comparison as an effective standard therapy. Results: The patients were male predominent - 14 (78%) pts. 16 (89%) pts have a T-cell immunophenotype. The median age at time of presentation was 21.1 (range 15-42) years. The presenting sites of primary disease included mediastinal mass in 14 (78%) cases. The bone marrow was involved (< 20% blasts) in 8 (44%) pts. CNS involvements were found in 4 (22%) pts. 7 (39%) pts previously received from 1 to 7 (median - 4,5) lines of therapy, such as one with B-NHL: 2 - NHL-BFM 90; 5 - ALL-MB 91/2002. 11 (61%) pts did not have a history of other therapy. 5/7 (71%) pts are in complete remission (CR) on the protocol NHL-BFM 90 vs. 11 (100%) pts - ALL-MB 91/2002. 1/7 (14%) pt had not responded (NHL-BFM 90). 2/11 (18%) pts relapsed after ALL-MB 91/2002. After therapy protocol NHL-BFM 90 relapse was not. 6-years event free survival (6y-EFS) has 71% vs. 67% (median of observation 2.4 years, p>0.05), and 6-years overall survival (6y-OS) has 71 vs. 83% (p>0.05) respectively. *Conclusions*. The results of therapy of 2 protocols for adolescents and AYA with LBL are comparable. Long-term L-asparaginase consolidation is alternative to high-dose methotrexate. Previous failed B-NHL-like therapy is not an absolute disadvantage further treatment.

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FACTORS PREDICTIVE FOR SURVIVAL AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN LYMPHOMA AND MYELOMA. A SINGLE INSTITUTION 11 YEAR EXPERIENCE

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Background. Autologous stem cell transplantation (ASCT) is a generally accepted therapy for multiple myeloma and recurrent lymphoma but subsequent disease progression is a major cause of treatment failure. In a survey of factors predictive for poor outcome, we retrospectively analyzed survival of all patients transplanted at our institution over the period of 11 years. Purpose: To evaluate factors predictive for overall (OS) and progression free survival (PFS) for all patients treated with ASCT at a single institution over 11 years. *Design and Methods*. Between

1997 and 2008 we performed 415 procedures in 406 patients (pts). The indication for ASCT was multiple myeloma responsive to front-line treatment or recurrent/refractory Hodgkin (HL) and non-Hodgkin (NHL) lymphoma after cytoreductive therapy in fit pts. Few very high-risk NHL pts received ASCT in a first remission. The source of stem cells was peripheral blood in 87% and bone marrow or both - in 13% of pts. The median CD34+ cell dose was $2.9x10^6$/kg and the median mononuclear cell (MNC) dose was $1.2x10^8$/kg. A diagnosis was Hodgkin lymphoma (HL) in 45%, diffuse large B-cell lymphoma (DLBCL) in 18%, multiple myeloma (MM) in 16%, mantle cell lymphoma (MCL) in 9%, and other lymphoma - in 12% of pts. Mean pts age was 39 years and male to female ratio was 1.3. The conditioning regimen was BEAM in 58%, Melphalan 200 in 16%, and BuCyV - in 10% of pts. TBI was used in <2% of pts. *Results*. Five year OS for all patients was 61% and PFS was 54%. Treatment related mortality was 3%. In multivariate analysis by Cox model, the following factors were significant predictors for OS: age with 60 years cut-off, quality of remission: first (CR1) or subsequent complete remission and partial remission versus relapsed/refractory disease, and disease diagnosis. Patients younger than 60 years, first or subsequent remission, and diagnosis of HL or MM had a favorable OS. Five year OS for pts of age <60 and ≥60 was 63% and 36%, respectively. Hazard ratio (HR) over 5 years post-ASCT was 35 times higher for relapsed/refractory lymphoma than for pts in CR1 (p<0.001). HR for NHL was 2 times higher than for HL or MM (p=0.001). Factors predictive for PFS were: age, quality of remission, CD34+ cell dose/kg and MNC cell dose/kg. Age older than 60 years, relapsed/refractory disease, lower CD34+ cell/kg and higher MNC/kg dose adversely affected PFS. *Conclusions*. These data indicate acceptable safety and substantial efficacy of ASCT in high-risk lymphoma and myeloma patients. However, more active conditioning regimens and better quality graft preparations are clearly needed. Adverse effect of higher MNC dose on PFS is intriguing and needs a further investigation.

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CLINICAL VALUE OF SERIAL CTNI AND QTC MEASUREMENTS AND ITS CORRELATION WITH DOPPLER ECHOCARDIOGRAPHY IN THE ASSESSMENT OF CARDIOTOXICITY OF PATIENTS TREATED FOR AGGRESSIVE NON HODGKIN LYMPHOMA

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Background. Anthracycline-based chemotherapy is a key point of the treatment of patients with aggressive non-Hodgkin's lymphoma. However, cardiac toxicity remains a key problem in clinical practice. Currently, various methods including electrocardiogram, biochemical markers and Doppler echocardiography have been used to detect anthracycline induced cardiotoxicity. *Aims*. The aim of our study is to determine the clinical need of serial measurement of QTc, serum cardiac troponin I (cTnI) and Doppler echocardiography (conventional and TDI) in detection of early doxorubicin induced cardiac toxicity in patients with aggressive non-Hodgkin's lymphoma. Also, to study the correlation between these various methods of monitoring of cardiac toxicity. *Design and Methods*. Forty-two patients who had a diagnosis of aggressive non-Hodgkin's Lymphoma (NHL), and had been scheduled to receive doxorubicin-based chemotherapy (CHOP) were included in this study (mean age, 46.9±13.01, 26 males and 16 females). In all patients, echocardiography, cTnI, and clinical evaluation were performed before (baseline), after the second, last cycles of chemotherapy, and then 6 months later. *Results*. Serial echocardiographic evaluation of patients treated by doxorubicin-based chemotherapy (CHOP) for aggressive NHL revealed changes in the diastolic LV function early after anthracycline therapy (decrease of E peak velocity and TDI E/A ratio). At the final evaluation, diastolic changes were more pronounced and associated with alteration of the systolic function (LV ejection fraction, FS, and Sa). We observed that TDI could detect indices of diastolic dysfunction prior to alteration in conventional Doppler. cTnI levels measured after the second cycle, and after completion of therapy were significantly higher, compared with those measured at baseline (p<0.001). QTc interval was progressively increased on serial evaluation. cTnI levels and QTc interval measurements were found closely correlated with systolic parameters of both conventional and TDI (EF, FS and Sa) (p<0.05). *Conclusions*. Serial Doppler echocardiographic evaluation and QTc measurment has to be done in patients with aggressive NHL receiving doxorubicin, before, during and even after therapy completion. In our population cTnI was elevated early after therapy, and it can be considered a sensitive and reliable marker of myocardial damage after doxorubicin therapy.

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BEAM OR BUCYE HIGH-DOSE CHEMOTHERAPY FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANTATION IN NON-HODGKIN'S LYMPHOMA PATIENTS

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Background. Effective but less toxic combinations of high-dose regimen is needed considering that expected toxicity of intensive regimens makes almost 50% of all non-Hodgkin' s lymphoma (NHL) patients with old age and poor performance ineligible for autologous stem cell transplantation (ASCT). Aims: The objective of this study was to compare the efficacy and toxicity of two high-dose regimens for ASCT in patients with NHL: BEAM (BCNU, etoposide, cytarabine, and melphalan) and BuCyE (Busulfan, cyclophosphamide, and etoposide). *Design and Methods*. We analysed 65 NHL patients, who underwent high-dose chemotherapy with BEAM (N=43) or BuCyE (N=22), followed by ASCT, at the Asan Medical Center. BEAM was used from February 2002 to October 2005, and BuCyE was used from November 2005 to April 2008. *Results*. Median age was 46 years (range: 15-68), and baseline characteristics, such as gender, International Prognostic Index (IPI), age adjusted IPI, stage and status of disease at ASCT, and median number of infused CD 34+cells/kg were well balanced between groups. The incidence of mucositis, nausea/vomiting, diarrhea and bleeding, and the number of events clinically important infections during ASCT did not differ between groups. Median follow-up for survivors was 49.3 months in the BEAM group and 21.5 months in the BuCyE group. Median overall survival (OS) was 30.6 months (95% confidence interval [CI], 8.19-53.0 months) and 22.6 months (95% CI, 12.1-33.1 months) and median event-free survival (EFS) was 16.1 months (95% CI, 0.0-53.6 months) and 11.2 months (95% CI, 0.0-22.5 months) in the BEAM and BuCyE group, respectively. There were no significant differences in OS (p=0.636) and EFS (p=0.575) between the two groups. *Conclusions*. In our analysis, BuCyE appeared to be not inferior to BEAM for survival. And we found that regimen-related toxicities did not differ significantly between the two groups.

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CONSOLIDATION EFFICACY OF 90Y-IBRITUOMAB TIUXETAN AFTER RESCUE THERAPY IN PATIENTS WITH FOLLICULAR LYMPHOMA

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Background. Therapy with 90Y-Ibrituomab tiuxetan is efficacious and is usually administered after obtaining FDA or EMEA authorization to patients with follicular lymphoma or those in relapse or refractory to rituximab. To date, very few patients have received 90Y-Ibrituomab tiuxetan to consolidate rescue therapy after chemo- or radio- therapy following, in some cases, autologous bone marrow transplantation (ABMT). Aims: To establish the efficacy of 90Y-Ibrituomab tiuxetan consolidation therapy in patients in relapse and its safety in ABMT recipients. *Design and Methods*. We administered 90Y-Ibrituomab tiuxetan to consolidate rescue in relapsed patients with follicular lymphoma after rituximab therapy and after ABMT for a total of 19 patients (13 M, 6F, age range 43-75 years, median age 58 years). All patiets had previously received immunochemotherapy with rituximab and 15 had received at least two lines of chemotherapy. Bone marrow was normal in all except for 1 patient with molecular evidence of involvement. Neutrophil counts were >1500 mmc; platelet counts were >100,000 mmc. Response to therapy was classified as complete response (CR), near complete response (nCR), partial response (PR), no response (NR), disease progression (DP). Response was assessed by clinical examination and a PET-CT scan before and 16 weeks after 90Y-Ibrituomab tiuxetan infusion in order to minimise post-chemotherapy inflammatory changes. *Results*. Before 90Y-Ibrituomab tiuxetan infusion, 10 patients had achieved nCR, 7 PR and 2 NR. At a median follow-up of 13.7 months (range 1-25 months) 18/19 patients survive. One died of disease progression. At 4 months after 90Y-Ibrituomab tiuxetan infusion response in 14 evaluable patients was CR in the 10 (pre-infusion: 7 nCR,3 PR). One relapsed after 24 months. The 5 patients who received 90Y-Ibrituomab tiuxetan infusion after ABMT sustained CR. Two patients achieved PR (Pre-infusion both PR) but disease progressed after 5 and 7 months, respectively. Three patients did not respond (Pre- infusion: 2 NR, 1 PR). In 2 there was rapid DP. Both had a

bulky mass of >5 cm and were in Stage IVB when diagnosed. One died (see above). Safety profile: Three patients developed Grade 3 neutropenia; 14 needed granulocyte growth factor support; 3 developed Grade IV thrombocytopenia, resolved with transfusions. All patients had a good haematological recostitution after 12 weeks. No patient developed severe extra-haematological toxicity or required hospitalization. *Conclusions*. Eighteen of our 19 patients survive with a good quality of life. Patients who were refractory to previous treatments did not respond to 90Y-Ibrituomab tiuxetan infusion either. This approach emerged efficacious and safe as consolidation therapy when these very high risk patients were in nCR or PR before infusion. 90Y-Ibrituomab tiuxetan infusion is also safe after ABMT.

In memory of Prof Antonio Tabilio, close friend and colleague, eminent physician and scientist.

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CEREBROSPINAL FLUID PLEOCYTOSIS AS A DIAGNOSTIC CHALLENGE - DISCRIMINATING REACTIVE FROM MALIGNANT CELLS USING FLOW CYTOMETRY

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The spread of cancer cells into the compartment of the central nervous system (CNS) is a rare complication in solid tumours with an incidence of 5-15% but more common in leukemia and lymphoma with an estimated incidence of up to 20%. Diagnosis of CNS involvement is important for the prognosis and essential for the treatment of choice. Diagnosis of CNS involvement by malignant tumor cells is based upon neuroradiology such as magnetic resonance imaging (MRI) and morphologic examination of the CSF. Especially in patients with diffuse leptomenigeal disease the diagnosis based solely on cytological examination of the CSF is often inadequate. Due to the therapeutic and prognostic importance of CNS involvement for patients with solid tumours as well as lymphoma or leukemia, additional diagnostic methods are required. The aim of this study was to compare morphology and flow cytometry in the diagnosis of lepromeningeals disease in patients with haematological malignancies. Between 1996 and 2008, we performed morphologic and flow cytometric examination of CSF-samples from patients with various malignancies being suspicious of CNS involvement. All patients (n = 126) were treated according to standard protocols. All patients were suspected to suffer from meningeosis carcinomatosa or lymphomatosa most of them were suffering from leukemia or lymphoma. Depending on the suspected diagnosis, a particular marker panel was used. According to the final clinical diagnosis, three groups of patients can be separated: 1.) Patients with inflammatory diseases and non hematopoietic cancer (n = 31), 2.) patients with acute leukemia (25), and 3.) patients with lymphoproliferative disease (n = 71). FACS analysis confirmed the diagnosis in 47/53 (89%) samples. Six samples were diagnosed as non-malignant by FACS analysis (four patients with known diffuse large B-cell lymphoma, two patients with non-hematopoietic cancer). Seventy-four samples were judge morphologically as reactive/normal or non-diagnostic. Using FACS analysis six out of these 74 samples (8%) were categorized as malignant (c-ALL [n=1], B-CLL [n=2], AML [n=2] and DLBCL [n=1]), sixty-eight samples (92%) were classified as non-malignant. Thirty-one patients underwent CSF analysis due to clinical or radiological changes suspicious for meningeosis carcinnomatosa or lymphomatosa but had no evidence of a pre-existing malignant tumor. Twenty-six samples were judged morphologically to be reactive, two were non-diagnostic. FACS analysis confirmed all twenty-eight samples to be reactive. Two samples morphologically obviously stated as leptomeningeal infiltration by carcinoma, exhibited only T-cell marker expression and were found to be reactive by FACS. Eighty-three samples from patients with known lymphoproliferative disorders were analyzed. Fourty-three samples were classified as malignant by FACS, whereas two of the samples had been judged as reactive by morphology. Two samples were non-diagnostic by morphology and could be classified correctly as malignant using FACS. In conclusion, we conducted CSF analysis in patients which did not have known underlying malignancy but had clinical signs and/or imaging findings suspicious for hematopoietic malignancy. We also propose distinct marker panels in order to differentiate between malignant CSF lymphocytosis and reactive B- or T-lymphocytes.

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MINIMAL RESIDUAL DISEASE MONITORING IN MANTLE CELL LYMPHOMA (MCL) USING FIVE COLOUR FLOW CYTOMETRY

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MCL accounts for 5% of Non- Hodgkin's lymphoma. The disease is incurable with conventional chemotherapy; only 8% of patients are alive 10 years after diagnosis with a median survival of 3 to 4 years. Minimal residual disease (MRD) is common and contributes to relapse. MCL cells have a characteristic antigen profile of CD5+, CD20+, FMC7$^+$, CD19$^+$, CD10$^-$, CD23$^-$ with strong light chain expression. Aberrant immunophenotypes can occur, such as lack of CD5 expression and weak expression of CD23. Infiltration of the bone marrow (BM) and spillage into peripheral blood (PB) is common. In Complete Remission (CR) up to 1010 malignant cells can remain in PB or BM. MRD monitoring is a powerful prognostic factor, to predict relapse and in assessment of treatment efficacy. Achievement of MRD negative CR can be the first step to curing many haematological malignancies. *Aims*. To design a five colour flow cytometric panel specific for the diagnosis of MCL and to identify the sensitivity and specificity of the method using dilution studies. *Patients.* PB samples from eight MCL patients were analysed along with a bone marrow (BM) sample from one. PB samples from nine CLL patients and two HCL patients were used as controls. *Design and Methods*. The Beckman Coulter FC500 analyser was used with a two tube, five colour antibody panel (CD5, CD19, CD20, CD3, CD23 and κ and λ). Prism analysis enabled quantification of lymphocytes percentages according to patterns of expression. The percentage of cells positive for each antigen profile were reported for each sample to show that the method is specific. The MCL PB samples were serially diluted with normal PB based on the lymphocyte count of each, up to one mantle cell in 200,000 normal lymphocytes. The percentage of cells positive for the MCL antigens was reported for each of the dilutions. Analysis was to determine whether the results obtained correlate with the dilution factor according to the percentage positive cells obtained from the neat tube. *Results*. MCL patients had an average of 45.2% MCL lymphocytes compared with 3.7% in the non-MCL group and 2.6% in the normal control. CLL patients had an average of 51.7% CLL lymphocytes compared to 2.3% in the non-CLL LPD group and 2.1% in the normal control. Prism analysis enabled us to report the lymphocytes that were CD20$^+$, CD19$^+$, CD5$^+$, CD3$^-$, CD23$^-$ and λ and κ positive as MCL cells. The percentage of cells designated MCL cells did not correlate significantly with those showing clonal light chain expression (r = 0.77). 6 out of the 11 dilution studies produced results that were consistent with the dilution factor. Three samples were shown to be MRD negative by the method and indeed these patients were in confirmed clinical and cytological remission. *Discussion.* Prism analysis did not allow proper integration of the information from 2 tubes to quantify the type of light chain expression on cells classed as MCL cells by the first tube. A seven colour panel could be developed including the antibodies of tube 1 together with κ and λ, to show the specific light chain expression. We underestimated reporting the intensity of antigen expression per cell which may have made the method less robust. In, addition, the dilution with normal blood confounded the MRD issue by including background MCL like cells in healthy samples. Beads are required to obtain absolute counts by flow cytometry and their use may have improved the accuracy of the dilution studies.

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RITUXIMAB PLUS PEGYLATED LIPOSOMAL DOXORUBICIN IN COMBINATION WITH CYCLOPHOSPHAMIDE: A FIRST LINE THERAPEUTIC OPTION FOR VERY ELDERLY OR UNFIT PATIENTS WITH AGGRESSIVE NON HODGKIN LYMPHOMA

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Background. The standard treatment for patients with aggressive B-cell lymphoma, in particular diffuse large-B-cell lymphoma [DLBCL), is cyclophosphamide, doxorubicin, vincristine and prednisone [CHOP) plus rituximab, a chimeric monoclonal antibody against the CD20 antigen. However, some very elderly patients are not fit enough to tolerate CHOP or they have a comorbidity that exclude antracyclin in the regimen. The overall survival in this subset of patients is very poor. Pegylated liposomal doxorubicin is associated with a lower risk of cardiotoxocity than conventional formulations of doxorubicin, allowing the use of higher cumulative doses The aim of this single institution study was

to investigate the outcome of pegylated liposomial plus cyclophosphamide (Cae-CY) and rituximab (R) regimen in patients >/= 75 years old, with diagnosis of DLBCL and cardiological comorbidity. *Design and Methods*. In this study, 22 patients aged over 75 years (median 79, range 75-91 years) with aggressive non-Hodgkin's lymphoma (NHL) (age adjusted International Prognostic Index (IPI): IPI-2 (25%); IPI-3 (35%); IPI-4 (40%)) received pegylated liposomal doxorubicin (25 mg/m^2/day 1), cyclophosphamide (300 mg/m^2 day 1) and rituximab (375 mg/mq^2 day 2), q28. *Results*. 20 patients completed 6 treatment cycles and were evaluable for efficacy and safety. A complete response was achieved in 14 (70%) patients and a partial response in 2 (10%) patients. 4 patients showed stable disease or progressive disease (20%). With a median follow-up of 18 months, the median time to progression was 12 months. The major toxicity was haematologic: grade 2 leukocytopenia occurred in 6 patients, grade 3 thrombocytopenia in 5 patients, but no grade IV toxicity occurred. There were no episodes of clinicaliy significant bleeding. Three patients developed febrile neutropenia. No significant decrease in LVEF or clinical evidence of congestive heart failure was observed during the treatment or in follow-up. Pegylated liposomal doxorubicin plus cyclophosphamide is an effective and weII tolerated regimen for the treatment of aggressive NHL in elderly or unfit patients.

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INHIBITORY EFFECT OF PEGINTERFERON ALFA-2A ON EBV REACTIVATION AND TUMOR RECURRENCE FOLLOWING CHEMOTHERAPY IN PATIENTS WITH EBV-ASSOCIATED EXTRANODAL NK/T CELL LYMPHOMA

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Background. Extranodal NK/T-cell lymphoma, nasal type responds poorly to intensive chemotherapy regimens or has a significant relapse rate in spite of low IPI and loco-regional staging. Poor clinical outcome may result from EBV reactivation during chemotherapy since this unique subtype is closely associated with EBV latency. Some *in vitro* data suggested that various chemotherapy agents, including gemcitabine, doxorubicin, cis-platinum, and 5-fluorouracil, induced lytic EBV gene transcription in latently infected EBV positive cell lines. The presence of the EBV genome within tumor cells raised the possibility of developing therapeutic strategies directed at viral targets. *Aims*. The primary objectives of this prospective, single center, nonrandomized phase II trial are the evaluation of efficacy by determination of progression-free survival after concurrent administration of peginterferon α-2a (PEGASYS®, provided by Roche Korea) and chemoradiotherapy in patients with extranodal NK/T cell lymphoma. In addition, we evaluated the efficacy of peginterferon alfa-2a maintenance therapy for 3 years. *Design and Methods*. All seven patients with newly diagnosed CD56+ EBV+ extranodal NK/T-cell lymphoma were enrolled onto a prospective clinical trial. Step 1; PEGASYS® 180 mcg was administered once weekly on D1, D8 of ProMACE-CytaBOM. 1, 2 cycles. Step 2; PEGASYS® 180 mcg will be administered once weekly on D1, D8, D15, D22 of involved-filed irradiation 3600 cGy (180 cGy x 20, for 4 weeks). Step 3; PEGASYS® 180 mcg will be administered once weekly on D1, D8 of ProMACE-CytaBOM. 3, 4 cycles. Step 4; PEGASYS® 180 mcg will be administered every two weeks after hematologic recovery of high-dose therapy and autologous PBSCT for 3 years. In case of advanced stage and non-nasal lesion, Step 2 was omitted and ProMACE-CytaBOM was extended to 6 cycles. EBV DNA copy based on real-time PCR was monitored. *Results*. Of all 7 patients, 6 patients achieved complete response and 1 patient was dead due to pneumonia during 2nd cycle of chemotherapy. With a median follow-up of 37.5 months (12-44 months), the 3-year PFS were 67%. Of 6 assessable patients, five patients extended to peginterferon α-2a maintenance therapy and four patients showed PFS. Among two relapsed patients, one patient refused a peginterferon alfa-2a maintenance therapy. Titer of EBV DNA copy in whole blood was markedly decreased compared with the titer on initial diagnosis. The main toxicities of peginterferon α-2a were mild flu-like symptoms. *Summary and Conclusions*. This pilot study suggests that peginterferon α-2a may effectively inhibit EBV reactivation and lymphoma relapse. It warrants that larger prospective studies are needed to define the therapeutic role of peginterferon α-2a concurrent administration during induction therapy and maintenance therapy in EBV-associated extranodal NK/T-cell lymphoma.

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INFECTION RISK RELATED VARIABLES IN FIRST LINE CHEMOTHERAPY OF INTERMEDIATE GRADE NON HODGKIN LYMPHOMA IN A SERIES OF 199 PATIENTS

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Background. Lymphoma patients are exposed to infection as a consequence of both the disease itself and the immunosuppression due to the chemotherapy. Evaluating this risk in each patient has been deemed necessary in order to prescribe treatments such as colony-stimulating factors(CSF) or antibiotic prophylaxis. The predictive variables of this risk may depend on the population of patients considered. Aims: To asses clinical and analytical variables at diagnosis in a series of unselected patients with non-Hodgkin lymphoma(NHL), which are related to infection risk in the course of first line chemotherapy. *Design and Methods*. Prospective observational study of all intermediate grade NHL in first line standard chemotherapy at a University Hospital Internal Medicine Lymphoma Unit, from 1996-January until 2007-June.The patients were followed since chemotherapy beginning until three weeks after last cycle. CHOP or alike chemotherapy was administered incorporating Rituximab in B NHL since its approval. Blood counts were scheduled next to expected nadir and until neutrophils count was upper than 500/µL. Filgrastim according to ASCO guidelines and ciprofloxacin prophylaxis was prescribed. The recommendations of IDSA guidelines were followed in febrile neutropenia(FN) and other infectious events. Infectious episodes requiring antimicrobial treatment were considered and classified as microbiologically (MD) or clinically documented(CD) or fever of unknown origin(FUO). We asses the predictive value regarding FN and other infections of the following variables taken at diagnosis: age, sex, B symptoms, performance status(WHO), albumin, β2microglobulin, immunoglobulins, type of lymphoma, bone marrow involvement and IPI. Statistical methods: descriptive, chi2, Student t, Kaplan-Meier survival tables, log rank test, multivariate logistic binary and proportional hazard regression model. *Results*. 199 patients were included, 51% male, 61.6 years(15-88) mean age;24(12%) mantle cell lymphoma, 148(74%) LBCL (10 with AIDS) and 27(14%) peripheral T lymphoma. CSF (filgrastim) was delivered in 62% of patients(12% in primary prophylaxis, secondarily in 47% and as treatment of FN in 3%).We detected 408 episodes of grade IV neutropenia in 141 patients(71%) and 184 infectious events(12 deadly;6%) in 97 (49%), 79 of them FN in 53 patients(27%).FN occurred only in the first cycle in 17% of patients and only later in 72%. MD infections were observed in 51%, CD in 10% and 39% were FUO.Gram negative bacilli were more frequent(26%) than Gram positive cocci(13%) and other agents(12%), but in BSI(n:30) the frequency was similar(14 and 16 respectively).There were no significant difference in the distribution of grade IV neutropenia, FN, type or ethiology of infection among the subtypes of NHL. In the assessment of variables related to the first or any episode of FN were significant: B symptoms (p:0.02), unfavourable IPI (p=0.004) and PS (p=0.0002); in multivariate analysis only PS remained significant in respect to the first episode of FN(HR:1.8, 95%CI:1.3-2.3;p:0.0003E-1)or anyone(OR:1.8; 95%CI:1.3-2.5;p:0.0003E-1.In relation to risk of infection were significant B symptoms (p:0.0001), PS (p<0.0001), unfavourable IPI (p=0.0001) and UNL β2microglobulin (p=0.003); in multivariate analysis only PS remained significant (OR:3.1;95%CI: 1.7-5.5 p:0.0002E-3). *Conclusions*. PS recorded at diagnosis seems a valuable predictor of FN and infectious events throughout the course of first line chemotherapy in our series of intermediate grade NHL patients, probably reflecting both the aggressiveness of the tumor and the patient comorbidity.

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SALVAGE CHEMOTHERAPY WITH NON-PEGYLATED LIPOSOMAL DOXORUBICIN (ADRIAMYCIN), FLUDARABINE, OXALIPLATIN AND CYTARABINE (AFOXA) IN POOR-RISK B CELL NON-HODGKIN'S LYMPHOMA

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The aim of the present phase I/II study was to evaluate the feasibility and toxicity of the combination of non-pegylated liposomal doxoru-

bicin, fludarabine, oxaliplatin and cytarabine (AFOXA) in patients (pts.) with prognostically unfavourable recurrent and refractory B cell non-Hodgkin's-lymphoma (NHL). Between 02/2005 and 01/2009 a total of 25 pts. (12 male, 13 female) with diffuse large B cell lymphoma (n=14), mantle cell lymphoma (n = 6) and follicular lymphoma (n=5) were treated according to the AFOXA protocol. Pts. (median age 59, range 42 - 70) with primary refractory disease (n=19) and second (n=5) or third (n =1) relapse were enrolled. The intensive pretreatment contained a median of 7 (range 1 - 18) cycles of chemotherapy. 21/25 pts. were pretreated with the anti-CD20 monoclonal antibody Rituximab. The AFOXA regimen consisted of non-pegylated liposomal doxorubicin (25 mg/m^2, days 1 + 3), fludarabine (25 mg/m^2, days 1 - 4), oxaliplatin (escalating doses of 100 or 130 mg/m^2, day 5) and cytarabine (escalating doses of 1000 or 1250 or 1500 mg/m^2, day 5). In the phase I part of the study (n=12) the maximal tolerable dose (MTD) was determined for oxaliplatin and cytarabine according to World Health Organization Common Toxicity Criteria (CTC). The primary objective of the subsequent phase II part of the study, which uses the determined MTD, is efficacy. *Results*. In the phase I part we established the MTD for oxaliplatin with 130 mg/m^2 and for cytarabine with 1000 mg/m2. Out of 25 pts.; 16 pts. were treated with the established MTD. 5 pts. (31%) achieved complete remission (CR or CRu) and 2 pts. (13%) partial remission, with an overall response (OR) rate of 44%. A successful peripheral blood stem cell harvest through mobilization with the AFOXA regimen was possible in 7 pts. (44%). *Conclusions*. AFOXA is a feasible salvage protocol for patients with poor-risk recurrent or refractory NHL. The observed toxicity (MTD) seems to be acceptable considering the unfavourable prognosis and intensive pretreatment. The efficacy will be evaluated in the ongoing phase II study.

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NON-HODGKIN LYMPHOMA (NHL) IN ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) IN POST-HAART ERA: ELEVEN YEARS OF EXPERIENCE OF A PORTUGUESE CENTRE

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NHL is a common cause of HIV-related mortality, presenting with an aggressive clinical behaviour and being a therapeutic challenge. The prognosis has improved with the availability of highly active antiretroviral therapy (HAART) and the survival of many of these patients is actually comparable to HIV -negative NHL patients. We evaluated clinical records of patients with HIV-related NHL admitted in our Hospital between January 1997 and July 2008, according to clinical features, treatment, response rate and survival. We diagnosed 60 HIV-related NHL: 48 as systemic NHL, 11 as primary central nervous system lymphoma (PCNSL) and 1 as primary effusion lymphoma. Before diagnosis of lymphoma, 45 of the patients were naïve to HAART (45/58; 78%). The median age of the patients (37M; 11F) with systemic NHL was 43.5 (27-63). It was the first AIDS defining illness in 67% of patients. Regarding WHO classification, 30 (65%) of 46 patients were classified as diffuse large B cell lymphoma (DLBCL) and 9 (19%) as Burkitt/Burkitt-like and the remaining 7 as other histologic subtypes. Severe immunossupression at diagnosis was a common finding (median CD4 cell count of 143/mm^3). Of 46 patients with evaluable plasma viremia at diagnosis, only one had undetectable viral load (<50/mm^3). Extranodal manifestation was present in 93% of the patients (more frequent in bone marrow, gastrointestinal tract, liver and CNS); advanced stage disease with a median of IV (Ann Arbor stage) was observed in 85% and an elevated LDH in 77%. Two patients were treated outside our Hospital, 12 received only palliative treatment and curative chemotherapy (m-BACOD, CHOP, da-EPOCH, Hyper-C-VAD and LMB-96) was initiated in 34 (4 currently on treatment, 20 died of resistance/progression, 8 alive disease-free and 2 relapses). The median overall survival was 180 days (233 days in patients with an intention to cure approach) and 42% were alive at 12 months and also at 4 years. Median age of the patients (10M; 1F) with PCNSL was 34 (23-41). The median CD4 cell count was 13/mm^3. None of them had undetectable viral load. Palliative treatment was adopted in 7 patients while 4 were on an intention to cure basis. Median overall survival was 32 days, with 2 alive and disease free at 41 and 69 months. Although diagnosed in the "post- HAART era", a high proportion of our patients were not receiving HAART in spite of presence of severe immunossupression. A high number of patients, in our series, only received palliative treatment (19/60, 32%). When an intention to cure option was chosen, early mortality was very high, and overall prognosis for HIV-NHL was worse than for HIV-negative patients; however, when complete remission was achieved, the disease had a closer behaviour to HIV-negative population. The results obtained in our population, stress the need to provide an adequate antiretroviral therapy as soon as indicated, thus permitting a curative approach in a higher number of patients with NHL.

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INTEGRATED 18F-FDG PET/CT MAY PREDICT THE OUTCOME OF PATIENTS WITH T-CELL LYMPHOMAS

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18F-fluorodeoxyglucose positron emission tomography (PET) is a highly sensitive method for imaging of aggressive B-cell and Hodgkin's lymphomas. A recently developed integrated PET/computed tomography (CT) system combines a PET camera with a CT scanner in a single session, providing both anatomical and functional imaging at the same position. Achieving PET negativity (complete metabolic response, CMR) after therapy has a strong prognostic impact. Yet the use and predictive power of PET/CT imaging in T-cell lymphomas is still poorly studied. Aim: To evaluate the predictive role of achieving CMR using integrated FDG PET/CT restaging in an unselected population of patients with newly diagnosed T-cell lymphoma. Method: We studied a cohort of 20 patients with T-cell lymphomas who had PET-avid T-cell lymphoma at the time of diagnosis. Histological subtypes were as follows: peripheral T-cell lymphoma, unspecified (PTCL NOS, n=11), anaplastic large cell lymphoma (ALCL, ALK-1-positive, n=1, ALK-1-negative, n=5), enteropathy-associated T-cell lymphoma (EATL, n=2), angioimmunoblastic lymphoma (AIL, n=1), Sézary syndrome (SS, n=1). All biopsies were reviewed by a university centre haematopathologist and the final diagnosis was made according to the WHO classification (2001). The median age at diagnosis was 63 years (30-78), most patients had advanced Ann Arbor stages (15 patients, 75%), IPI score ≥3 had 60% of the patients. All patients were examined with integrated PET/CT (Siemens Biograph Sensation 16) at the time of diagnosis and after first-line therapy. The PET scan was defined as positive if higher than mediastinal or background activity was observed. The CT scans were assessed according to the International Workshop Criteria (Cheson, 1999). Results: After first-line therapy, CMR was achieved in 8 patients (40%), whereas 12 (60%) remained PET-positive. Treatment response (CT) was as follows: complete remission or complete remission unconfirmed (CR/CRu) n=8 (PET-negative n=7, PET- positive n=1), partial remission (PR) n=4 (PET-negative n=1, PET-positive n=3), stable disease (SD) n=3 and progressive disease (PD) n=5 (all SD and PD cases PET-positive). At the time of follow-up, only 1 (12.5%) of the 8 PET-negative patients died and 2 (25%) relapsed or progressed. Of the 12 PET-positive patients, 8 (66.6%) died and 6 (50%) relapsed or progressed. The overall survival (OS) at 2 years reached 46% (95% CI 0.16-0.75); progression-free survival (PFS) was 17%(95% CI 0.00-0.45). While the 2-year OS in the PET-negative subgroup was 67% (95% CI 0.13-1.00), it dropped to 30% (95% CI 0.00-0.62) in the PET-positive subgroup (log rank 0.033). *Summary*. The overall prognosis of patients with T-cell lymphomas is still poor. Our pilot data show that PET positivity after first-line therapy is frequently associated with chemoresistant, rapidly progressive disease and extremely low survival. On the other hand, patients with PET-negative CR have a chance of longer survival. The value of interim PET/CT assessment as a method for early identification of poor responders should be further clarified. Acknowledgements: Supported by the Czech Ministry of Health (IGA NR/9502-3).

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CLINICAL OUTCOME AFTER BEAM AUTOLOGOUS SCT IN RELAPSED B-CELL NHL: EFFECTS OF THE ADDITION OF RITUXIMAB AND/OR ZEVALIN

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Background. Since more than 25 years, autologous stem cell transplantation (ASCT) with BEAM conditioning is a commonly used treatment for patients with relapsed or refractory B-cell non Hodgkin lymphoma (NHL). In recent years, the monoclonal antibody anti-CD20 (rituximab)

and 90-Y labeled anti-CD20 (ibritumomab tiuxetan, Zevalin®) were added to (re)induction therapy and conditioning respectively. *Aims*. Evaluation of clinical outcome of patients with relapsed or refractory B cell NHL, treated with chemotherapy with or without rituximab followed by BEAM with or without Zevalin® and autologous stem cell transplantation (ASCT). *Design and Methods*. In this single center, retrospective analysis we reviewed extensively the clinical records of 200 patients with diffuse large B cell NHL (n=122), folliculair B cell NHL (n=37), transformed B cell (n=31) and other B cell NHL types (n=10), treated with ASCT between November 1984 and January 2009 (Mantle Cell NHL excluded). We defined three groups: patients treated with ASCT preceded by BEAM conditioning (1) without rituximab in reinduction chemotherapy (R- BEAM, n=134), (2) treated with rituximab in reinduction chemotherapy (R+ BEAM, n=48) and (3) treated with rituximab in reinduction and Zevalin-BEAM as conditioning regimen (RZ+ BEAM, n=18). Statistical analysis was performed using SPSS 16.0, Log Rank (Mantel-Cox) test. Event free survival (EFS) is defined as follow-up time in months until disease progression or relapse, and death of any cause. Overall survival (OS) is defined as follow-up time in months until death of any cause. *Results*. The median age was 47 (range 17-64) years and 85 (42.5%) were females. The R- BEAM group differs from the R+ BEAM- and RZ+ BEAM group regarding two variables: the R- BEAM group contains significantly more follicular and less transformed NHL (p=0.03) and there are significantly more patients who received more than 1 treatment before the BEAM therapy (p=0.026) as compared to R+ BEAM and RZ+ BEAM. All other individual parameters, including IPI score, are balanced in the three groups. The maximum follow-up of currently alive patients is 287, 85 and 33 months for patients in the R- BEAM, R+ BEAM and RZ+ BEAM group, respectively. Regarding EFS and OS, there is no significant difference between the R- and R+ BEAM treated group (p=0.303 and 0.657 respectively). Comparing the RZ+ BEAM and R+ BEAM groups, there is also no significant difference in EFS and OS (p=0.079 and 0.212). *Summary and Conclusions*. The addition of rituximab to reinduction chemotherapy did not improve clinical outcome in patients who proceeded to BEAM ASCT, most likely reflecting lymphoma characteristics: patients relapsing after immunochemotherapy. The addition of Zevalin to BEAM in patients who proceeded to BEAM ASCT after rituximab containing reinduction may improve results.

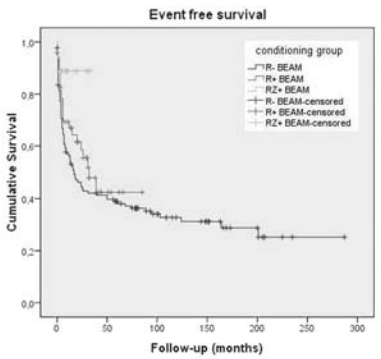

Figure.

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135 CASES OF AIDS-RELATED LYMPHOMAS: RUSSIAN EXPERIENCE

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During 2000-2008 we observed 135 pts. with AIDS-related lymphomas. 96% of them were intravenous drug users; 80% were co-infected with HCV or/and HBV. 106 pts were diagnosed with stage III-IV lymphomas: males - 69, females-37. Median age - 35 yrs (range 22- 68). Median of CD4 counts at presentation was 300 cells/ mL (range 10 - 550); median of viral load - 50000 copies/mL (range 4000 - 7500000). 30% of pts received HAART. Histological and immunohistochemical diagnoses: diffuse large B- cell lymphoma in 60 cases (56%) with primary localization: gastric lymphoma - 3 pts, intestinal - 3, uterine - 2, ovarian -2, thyroid -1, testicular -1. Burkitt lymphoma in 26 cases (24%), plasmoblastic lymphoma - 3 (2,8%), lymphoplasmoblastic lymphoma of oral cavity - 4 (3,7%), Castleman's lymphoma - 1 (0,9%), primary CNS lymphoma - 3 (2,8%), follicular lymphoma - 3 pts over 50 yrs old (2,6%), T-cell lymphoma - 6 (6,6%). 31 pts (29%) died before treatment and in 15 cases (14%) lymphoma was diagnosed initially only at postmortem examination. Remaining 71 pts received chemotherapy: CHOP (6 with DaunoXome), CHOPE, A-B blocks NHL-BFM - 95, A-C blocks BL-2004 (E. Zvonkov et al.) with CNS prophylactics and +\- MabThera. Treatment mortality achieved 26,4% (28 pts) with signs of lymphoma progression at autopsies. Complete remissions were reached in 22 cases out of 71 (31%), overall survival- 58% from 6 to 60 mos of follow-up, disease-free survival (pts in CR followed until relapse) - 85%, 18 pts are at therapy with good response. Post treatment median CD4 count was 200 (range 150-550) and viral load median 20000(range 500-60000). Hodgkin's lymphoma was established in 29 cases: nodular sclerosis in 14 cases (48%), type1 - 34% (10 pts) and type 2 - 13,8% (4 pts); mixed cellularity- 31% (9 pts), lymphoid predominance- 10,5% (3 pts) and lymphoid depletion - 10,5%(3 pts). Abundance of EBV antigens was noted in majority of cases. Males - 15, females -14. Median age 28 yrs (range 23-54). HAART received 10% of pts. Median CD4 count was 550 cells /mL (range 350 -1200), median viral load - 30000 HIV RNA copies/mL (range 1000 -100000). 9 pts (31%) did not receive chemotherapy because of the poorest performance status at admission. 20 pts received chemotherapy: ABVD, BEACOPP2 (escalated), DHAP, DEXA-Beam +\- radiotherapy. Complete remission was achieved in 60% (12 pts), overall survival- 75% and disease-free survival - 100% from 6 to 48 mos. 8 pts are at therapy and at HAART with good response. Post treatment median CD4 count was 700 (ran ge 250- 900), median viral load 12000 (range 500 to 20000). AIDS pts with aggressive lymphomas may receive diagnostic and treatment approaches results of which are comparable with general population. They must have an opportunity to get standard hematological treatment all over the country. Late admittance and low results of overall therapy are caused by patient's personal psychosocial pattern which is worsened by drugs and HCV/HIV cortical damage.

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PULMONARY MARGINAL ZONE B-CELL LYMPHOMA; CLINICAL MANIFESTATION AND TREATMENT OUTCOME

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Background. Pulmonary marginal zone B-cell lymphoma of the MALT type (P-MZL) is a relatively uncommon form of lymphoma. Due to its rarity, the natural history of an optimal treatment modality for this disease has yet to be well established. Aim: we performed a retrospective analysis of the clinical features and treatment outcomes of P-MZL . *Design and Methods*. From 1991 to 2008, a total of 39 patients with biopsy proven P-MZL were analyzed retrospectively. *Results*. The median age of our subjects was 57 (range: 24-79) years. This study involved 20 males (51%) and 19 females (49%). 18 patients (46%) initially were diagnosed without any symptom. Video-assisted thoracic surgery (VATS) was used for diagnosis in 16 patients (41%). The most common site involved was the RUL and RML (8 patients, 20.5%, respectively). 26 patients (67%) were involved single lobe. Two or more lobes involvement was observed in 13 patients (33%). Lung lesions were bilateral in 6 patients (15%). 4 patients had synchronous involvement of extra-pulmonary MZL. Consolidation was the most frequent pattern of parenchymal lesions observed in 27 patients. Overall, 37 of 39 patients were treated with surgery (n=20), chemotherapy (n=14), or radiotherapy (n=3). 31 patients achieved complete or partial remissions. The median time to progression (TTP) was 58 (95% CI: 34.2-82) months. Only one patient expired during follow-up. Synchronous extra-pulmonary MZL was determined to be a poor prognostic factor for TTP. TTP was no difference between operation group and chemotherapy group. *Conclusions*. P-MZL trends to be an indolent disease - characterized by prolonged survival with frequent relapses, similarly to other MALT-type site MZL. To conserve lung function and reduce risk of operation, chemotherapy should be considered for first treatment option of P-MZL.

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SAFETY AND EFFICACY OF R-CHOP14 REGIMEN REDUCING GRANULOCYTE COLONY-STIMULATING FACTORS (G-CSF) VIALS

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Background. Rituximab plus CHOP14 is increasingly used in the treatment of diffuse large B cell lymphoma (DLBCL). Some recent studies lead to the conclusions that intensified R-CHOP could be considered the standard of care for young patients with good prognosis DLBCL and the new standard for elderly patients with DLBCL (MInT trial and RICOVER 60 trial). Dose dense therapy is feasible with GCS-F support which is recommended for10 days. *Design and Methods*. Starting from 2002 we have treated young patients with IPI:0-1 and elderly patients all affected by DLBCL. We prospectively decided to use 7 vials of G-CSF (from +5 to +11) in first ten patients and in absence of infections or delays the following patients were treated with 5 vials (from +7 to +11). Moreover if patients reached a number of leucocytes over 20.000/mm^3 we reduced again the number of vials until three per cycles. We have included 60 pts with DLBCL and 5 with follicular lymphoma grade IIIb, median age was 61 years (range 34-79), 60% had an high-intermediate or high IPI. CHOP was administered every 14 days, preceded on day 1 by rituximab and followed by 7 (in the first ten patients), 5 or 3 days (3 vials in 40% of patients) of G-CSF (filgastrim). Haematological toxicity and feasibility was calculated over 381 cycles administered. *Results*. We have used 1526 GCS-F vials, 5 vials (range 2-7) for cycle and a median of 24 vials (range 10-35) for every pts. The programmed therapy was completed in 62 out 65 pts (96%); three pts swichted to a different scheduling (R-CHOP21). Twelve cycles (3%) have been delayed in 10 pts for severe adverse events. Neutropenia grade 3-4 developed in 2.3% of cycles, febrile episodes in 1.2% of cycles, thrombocytopenia grade 3 or 4 in 1.2% of cycles and hospedalization in 1% of pts. Of the 381 cycles considered, the median nadir of leucocyte was 3850 x10^9/L (range 400-8400), the median nadir of haemoglobin was 11.3 gr/dl (range 5.8-15.7) and the median nadir of platelets was 135000 (range 43000-328000). The complete remission rate was 87% and after a median period of 23 months overall survival was 81%. *Conclusions*. In conclusion in our experience, dose dense chemotherapy (R-CHOP14) supported by G-CSF has been well tolerated by patients, including the elderly. We confirm that the reduction from 10 to 5 or 3 GCS-F vials has not determined an increase of neutropenia, febrile episodes, delays in the treatment and hospedalizations confirming and high rate of response to therapy. Thus R-CHOP14 can be performed with a lower number of G-CSF vials with a clear clinical and biological advantage for the patients and an economical advantage reducing supportive therapy cost.

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IMPORTANCE OF FLOWCYTOMETRIC ANALYSIS OF CEREBROSPINAL FLUID FOR DIAGNOSIS OF CNS LESION IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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Background. Diagnostics of CNS lesion in hematological malignancies is based on flowcytometric, cytological and biochemical examinations of cerebrospinal fluid, imaging methods given their unspecific findings have supportive significance. *Methods and samples*. In years 2003-2007 we examined 257 samples of cerebrospinal fluid from 164 patients with hematological malignancies. These samples were obtained from patients with clinical symptoms, unsymptomatic patients with diagnosis of acute leukemia (preventive examinations) and high-risk patients with non-Hodgkin's lymphoma (NHL). Objectives: Samples of patients with positive flowcytometric findings were analyzed in detail and all methods of cerebrospinal fluid examinations were compared with the results of imaging examinations. *Results*. In 33 examinations of 21 patients (i.e., 7 patients with acute B-lymphoblastic leukemia, 2 patients with acute T-lymphoblastic leukemia, 4 patients with acute myeloid leukemia, 4 patients with diffuse large B cell non-Hodgkin's lymphoma, 1 patient with peripheral T-cell non-Hodgkin's lymphoma, 1 patient with primary lymphoma CNS, 1 patient with mantle cell lymphoma and 1 patient with B-chronic lymphatic leukemia) positive findings were examined by flowcytometric, morfological or biochemical analysis. In 17 cases all methods of analysis were used, in 12 cases (71%) compliance in positivity of cerebrospinal fluid examination was observed. Flowcytometric analysis proved to be a more sensitive method in 3 examinations of 2 patients. In one case in neurologically symptomatic patient with diffuse large B-cell non-Hodgkin´s lymphoma biochemical analysis of cerebrospinal fluid was always positive (3 times), while flowcytometric analysis was positive only in the third examination. Clinical symptoms of CNS afflictions were observed in 13 patients (62%). 12 patients underwent imaging examinations (MRI or CT scan of brain and spinal cord), in 4 cases findings were negative, in 8 cases unspecific positive findings appeared. Overall survival (OS) for patients with CNS hematological malignity remains low, from our sample of 21 patients 19 patients died, median OS from a hematological diagnosis was 15 months. *Conclusions*. Results of our observations unambiguously confirm the necessity of repetitious and complex examinations of all obtained samples of cerebrospinal fluid and imaging examinations especially for patients with clinical suspicion of CNS afflictions.

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THE COMPARISON BETWEEN CONVENTIONAL PROCEDURES AND 18F-FLUORO-DEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY IN RESPONSE ASSESSMENTS OF PATIENTS WITH T-CELL NON-HODGKIN'S LYMPHOMA AFTER TRANSPLANTATION

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Background. Although there had been numerous studies about the usefulness of 18F-fluoro-deoxyglucose positron emission tomography (FDG-PET) for predicting patients' outcomes following autologous stem cell transplantation (ASCT), there had been little reports for T-cell non-Hodgkin's Lymphoma (NHL). Aims: We retrospectively re-evaluated whether post-ASCT PET study had an additional benefit in post-treatment response assessments in T-cell NHLs. *Design and Methods*. Twenty-one patients with T-cell NHLs performed post-ASCT PET study and conventional procedures with computed tomography (CT). The results of post-ASCT PET study were compared with those of CT, and the usefulness of FDG-PET study for response assessments and predicting patients' outcomes, including event-free survival (EFS) and overall survival (OS) were evaluated. *Results*. After the comparisons of response assessments between CT image and PET study, 14 of 21 patients (66.7%) showed concordant results on both CT images and PET study. In these 14 patients, 5 patients showed CR on CT and negative PET result, and 9 patient showed Not-CR on CT and positive PET results (six patients showed matched lesions at both imaging modalities and three patients showed partially matched lesions). 7 of 21 patients (33.3%) showed discordant results. In these 7 patients, 4 patients showed positive PET result which suggested new pathologic lesions which was not reported on CT (two cases of extranodal involvements and two cases of nodal involvements), and 3 patients showed remaining pathologic lesions on CT which was negative PET result. In CT images, 9 patients acquired CR after ASCT. However, 4 of 9 patients (44.4%) showed an early relapse or progression within 4.2 months after ASCT. In patients who did not acquire CR, 3 of 12 patients (25%) have shown disease free status after ASCT with minimum duration of 26.3 months. In PET study, 8 patients acquired negative PET result. However, 2 of 8 patients (25.0%) showed early relapse or progression within 2.0 months after ASCT. In patients who acquired positive PET result, 2 of 13 patients (15.4%) have shown disease free status after ASCT with minimal follow-up of 24.5 months. Overall, there were 7 patients (33.3%) whose response assessments on CT did not correlate with prognosis and 4 patients (19.0%) on PET study. According to CT images alone, the 2-year EFS rate or OS rate in patients who acquired CR did not showed statistical differences compared with patients who did not acquired CR (p>0.05). According to PET results, the 2-year EFS rate was 60.0 ±18.2% in patients with negative PET result and 15.4±10.0% in patients with positive PET result (p=0.026). The 2-year OS rate was 70.0 ±18.2% and 23.1±11.7%, respectively (p=0.062). *Conclusions*. Post-ASCT PET study shows substantial improvement in response assessments, thus in estimating patient's survival after ASCT in T-cell NHLs. Although routine PET study in T-cell NHLs is not recommended, it seems to have a benefit in the prediction of patients' prognosis after ASCT as it does in B-cell NHL.

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INTENSIFIED THERAPY PROGRAM FOLLOWED BY HIGH-DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION AS FIRST-LINE TREATMENT FOR PERIPHERAL T-CELL LYMPHOMA: PRELIMINARY RESULTS OF A PROSPECTIVE STUDY

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Background. Patients (pts) with mature peripheral T-cell lymphoma (PTCL) are known to have a poor prognosis when receiving standard conventional chemotherapy. Therefore, many physicians consider high dose chemotherapy (HDT) followed by autologous stem cell transplantation (ASCT) a standard therapy for these pts. *Aims.* To evaluate the effectiveness and safety of an intensified first-line chemotherapy approach including a conventional MACOP-B regimen followed by alternated IVE/DHAP and HDT-ASCT as consolidation therapy in newly diagnosed pts with PTCL. *Design and Methods*. Between September 2006 and December 2008, 10 adult pts with newly diagnosed PTCL, aged less than 65 years and eligible for intensive treatment, were treated with 12 courses of standard MACOP-B followed by one course of IVE (ifosfamide, etoposide, epirubicin), alternated with one course DHAP (dexamethasone, cytosine-arabinoside, platinum). Stem cells were harvested after IVE and pts who achieved a complete response (CR) were consolidated with a myeloablative HDT-ASCT using BEAM as conditioning chemotherapy regimen. Pts with primary cutaneous, immature lymphoblastic lymphoma or with anaplastic large cell kinase (ALK) positive or negative patients were not included. Histological subtypes were peripheral T-cell lymphoma unspecified (PTCLu) in 9 pts and enteropathy associated T-cell lymphoma (EATL) in the remaining patient. The median age at diagnosis was 46 years (35-64), 7/10 were males, 7/10 pts had advanced (III-IV) stage disease. B symptoms were present in 5/10 pts, 5/10 had high LDH levels, 1/10 a bulky mass and 3/10 presented an extranodal involvement. According to the age-adjusted IPI, 5 pts had an IPI score of 0-1 and 5 a score of 2-3. *Results*. To date, 6/10 pts have completed the full program, while 4 pts are still on therapy. After the MACOP-B regimen, 5/7 pts (71%), achieved a CR/Cru and 2/7 (29%) a partial response (PR). Six pts received the planned intensification therapy; of these, 5 (83%) witnessed a CR/Cru and underwent an ASCT, while 1 (17%) progressed during the IVE regimen. After a median follow-up of 12 months, 2 pts have relapsed at 17 and 13 months from the ASCT and have died of disease progression despite salvage therapy. The patient who progressed during intensification therapy is still alive with lymphoma. There were no treatment-related deaths and all pts experienced mild or moderate toxicities compatible with the planned procedure. The 2-year OS and PFS are 60% and 41%, respectively. *Conclusions*. These preliminary results indicate that an intensified first-line chemotherapy program with HDT-ASCT is effective and safe, and may lead to a potential cure of pts with PTCL. Further prospective studies on larger series of pts are warranted to better define the curative impact of this intensive therapeutic approach for PTCL.

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RADIOIMMUNOTHERAPY IN FOLLICULAR NON-HODGKINS LYMPHOMA: TIME TREATMENT AND PREVIOUS THERAPEUTHIC APPROACHES EFFECTS

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Background. Radioimmunotherapy (RIT) is a novel modality of treatment option for non-Hodgkin lymphoma (NHL). Zevalin radiolabeled with yttrium 90 consists of ibritumomab, a murine anti-CD20 monoclonal antibody, conjugated to the metal chelator tiuxetan for retention of the β emitter (90)Y. Eoncouraging results have been reached until now, even though due to the different settings of patients (pts) that have been treated, they are extremily heterogeneous. Most recent experiences have shown better results when the treatment was started in an early phase of the diesease, where it was chemosensitive and pts were still not pluritreated. Aims: The aim of our study is to verify the RIT therapeutic efficacy, safety and toxicity in pts affected by follicular Non-Hodgkins lymphoma (FL). Pts were stratified according to diagnosis time, disease status and to the quality and quantity of previous therapeuthic approaches received. *Design and Methods*. Since December 2005 to date, 20 pts have been treated with RIT (male/female 10/10; mean age 57 years - range 45-73), who were affected by FL in different phases of the disease: 1 patient at the moment of the diagnosis (I group); 7 pts in partial remission (PR) after first line therapy (II group); 5 relapsed pts after a line of treatment (III group); 4 refractory/relapsed pts after 2 or more lines of treatment (IV group); 3 refractory/relapsed pts after multiple lines of treatment including autologus stem cell transplantation (ASCT) (V group). All pts received the classic scheduled combination of Rituximab 250 mg/mq on days 1 and 8; second Rituximab dose was followed by Zevalin at the dose of 15 MBq/kg (maximum infusion dose was of 1200 MBq). All pts received Cotrimoxazole, Itraconazole and Acyclovir as anti-infective prophylaxis. *Results*. Totally 17 complete hematological remission (85%) were observed and they were distributed as follows: 1 (100%) in the 1st group; 7 (100%) in the 2nd group; 5 (100%) in the 3rd group; 2 (50%) in the 4th group and 2 (66%) in the 5th group. In 8 pts molecular responses were also documented. Out of the 17 pts in complete remission, 6 relapsed between +5 and +20 months from RIT. RIT was well tolerated by all patients and a WHO grade 3/4 hematological toxicity was observed in 8 pts (40%). At the bone marrow aspiration, 1 patient who underwent RIT after post ASCT relapse, showed a secondary myelodysplastic syndrome. *Conclusions*. Treatment with RIT has been, in the whole, well tolerated. Hematologic and molecular responses were satisfactory in terms of long lasting remission. Hematologic toxicity was acceptable. There were no documented infections and no hospitalization was necessary after RIT. Our experience has shown that RIT efficacy was directly proportional to the precociousness and was inversely proportional to the previous therapeuthic approaches received.

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IMMUNOLOGICAL PROFILE LINKED CLINICAL APPEARANCE AND OUTCOME IN ADVANCED STAGE MANTLE CELL LYMPHOMA

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Background. Mantle cell lymphoma (MCL) is uncommon B-cell malignancy with distinct molecular genetics characterized by t(11;14) involving cyclin D1 gene over-expression and typical pathological characteristics. *Aims*. to evaluate immunophenotypic profile and clinical characteristics in with advanced stage MCL as well as their influence on patients overall survival (OS).*Design and Methods*. 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. Cell morphology in PB and BM smears (MGG staining) was analyzed. According to cell morphology, small cell, typical cell and blastoid variant cell categories were determined. *Results*. A total of 50 patients diagnosed between 1996 and 2007 were evaluated. There was 16 patients in IV CS, while 34 patients had leukemic phase at presentation. Among patients, 46 were treated with CHOP, 2 with FND, and 2 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 (74% of patients) and in remainder nodular. Morphological analysis of lymphoma cells showed typical MCL morphology in 36, small cells in 5 and blastoid variant in 9 cases. Patients with blastic MCL variant had higher CD23 expression, compared to typical MCL (19 vs. 5; $p<0.01$), although CD23 Ag was negative (< 30%) in all patients. Hematological parameters showed median Hb 112g/L, Plt $105x10^9$/Ll, WBC $25x10^9$/L. Extranodal involvement (26%) included pleural effusions, bowel, sinus and palpebral infiltration. Among patients, 5 of them achieved complete and 20 partial remission. Median OS was 24 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 ($p< 0.01$), MIPI ($p<0.001$), presence of extranodal localization ($p<0.01$), and diffuse type of BM involvement ($p<0.01$).

Using Cox regression according to OS, MIPI had independent prognostic value (p<0.001). *Conclusions*. In the advanced MCL, had higher MIPI, IPI, diffuse BM infiltration, extranodal disease localization had negative influence on survival. Immunotypization showed elevated, but still negative CD23 expression in blastoid vs. small cell MCL. Accordingly, CD23 could be used as prognostic marker in the blastoid variant as a form of transformed aggressive MCL.

1695

PHASE II PILOT STUDY OF TANDEM CONSOLIDATION USING 90Y-IBRITUMOMAB TIUXETAN (ZEVALIN) AND HIGH-DOSE THERAPY WITH AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN HIGH-RISK PATIENTS IN PRIMARY REMISSION WITH DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Intense consolidation therapy or successive High-dose therapy (HDT) during the early period of diagnosis intended to increase the dose-intensity effects have been performed in aggressive diffuse large B-cell lymphoma (DLBCL), but the range of the effect was thought to be narrow considering the large amount of effort and treatment-related toxicity. Radioimmunotherapy such as Zevalin can overcome the refractoriness of rituximab in low-grade lymphoma and thus a superior response is also expected in diffuse aggressive lymphoma. Tandem stem cell transplantation using HDT is performed in patients with resistant or refractory lymphoma. This offers the chance of a cure in these patients with a poor prognosis, although there are associated risks. In this study, we expect to improve the treatment of diffuse aggressive lymphomas using tandem consolidation therapy consisting of initial consolidation therapy using Zevalin and second consolidation with HDT and PBSC support 3-4 months after Zevalin administration. We expect that Zevalin will have the potential to minimize the tumor burden without cross-resistance to R-CHOP and the risk of HDT-related toxicity and prolong the initial remission durtation. *Aims*. The objective of this study is the evaluation of the efficacy of tandem consoliadation with Zevalin and HDT with autologous PBSCT in high-risk patients in primary remission with DLBCL. *Design and Methods*. Eleven eligible patients (median age 54, 27-75 yr) with high-risk DLBCL (international prognostic index 4/5) were enrolled in this study and received 6-8 cycles of R-CHOP as a front-line chemotherapy. PBSC mobilization was performed after 1-2 course of ICE (ifosfamide, carboplatin, etoposide) or IVAM (ifosfamide, VP-16, ara-C, methotrexate). The administered dose of Zevalin did not exceed the absolute maximum allowable dose of 32.0 mCi (1184 MBq); 0.4 mCi/kg (14.8 MBq/kg) for patients with a normal platelet count, and 0.3 mCi/kg (11.1 MBq/kg) with a platelet count of 100,000 149,000 cells/mm3. The second consolidation (HDT and PBSCT) was performed 3-4 months after 1st Consolidation using Zevalin. Results: All patients received Zevalin therapy but 3 patient did not perform PBSCT as the 2nd consolidation because of PD (n=2) and inevitable use of cryopreserved PBSCs. Among 11 patients (CR=8, PR=3) in primary remission, 6 patients (CR=3, PR=3) had already showed relapse/PD before PBSCT. PBSCT did not affect their aggressive course. Clinical outcome of another 5 CR patients were as follows; DFS (n=2; 23 mo & 33 mo), RIST after relapse (n=2), death due to relapse (n=1). *Conclusions*. In the period of R-CHOP considered as the standard therapy for DLBCL, our pilot study revealed that the 1st consolidation using Zevalin did not provide some beneficial effects on the 2nd consolidation of HDT and PBSCT in primary remission patients with high-risk DLBCL.

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FLOW CYTOMETRY IN THE DIAGNOSIS OF DIFFUSE LARGE B CELL LYMPHOMA - AN ALL WALES LYMPHOMA PANEL EXPERIENCE

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Background. The diagnosis of diffuse Large B Cell Lymphoma (DLBL) is often difficult by flow cytometric analysis. Normal flow cytometric pattern is often seen in large cell lymphoma and Hodgkin lymphoma. Diagnostic correlation with histology by flow cytometry is poorly reported. *Aims*. The aim of the study is to evaluate the efficiency of flow cytometry in the diagnosis of Diffuse Large B cell Lymphoma in comparison with histological diagnosis. *Design and Methods*. The study cohort included all the lymphoid material studied by All Wales Lymphoma Panel (AWLP) between December 2002 and December 2007. It will be distributed around the diagnostic laboratories for further investigations such as flow cytometry, cytogenetics, fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR). We selected samples which underwent flow cytometry as part of the work up from Dec 2002 to December 2007. These were compared with histological and immune histochemistry diagnosis. *Results.* We identified 75 cases of diffuse Large B cell lymphoma diagnosed through All Wales Lymphoma Panel which had flow cytometry as part of the diagnostic work up. Flow cytometry was interpretable in 62 (82%) cases Failed cases (n=13) had no correlation with biopsy type (excision-8, Needle core-4, Resection-1) or site of biopsy (Lymphoid mass-9, tonsil, testes, spleen, and small bowell-1). This was mainly due to poor viability of samples (12 out of 13) and lack of cells (1/13). Failure had no correlation to the Sub type of DLBL (GC-6, Non-GC-6, and Indeterminate-1) There was varying success in identifying the clonality. κ or λ clonal B cells were identified in 42 (56%) cases. Flow cytometry failed identify any clonal B cells in 9 cases. 11 cases showed unusual clonality. Three (4%) cases had no kappa or lambda on the gated B cells while eight (10%) had streaky pattern indicated by taking both κ and λ fluorochromes. There was no correlation with type of DLBL (GC-4, Non-GC-4, and Indeterminate-1). *Summary and Conclusions*. Flow cytometry is successful in diagnosing DLBL in 70% of the incidence and failure was mainly due to poor viability of cells in the sample. Furthermore three patterns of Light chain restriction were identified in this study. We suggest that flow cytometry can be employed in the diagnosis of DBCL but further studies in to different patterns of clonal expression are warranted.

1697

OUTCOMES IN PATIENTS WITH PRIMARY GASTRIC DIFFUSE LARGE B-CELL LYMPHOMA AFTER RITUXIMAB, CYCLOPHOSPHAMIDE, DOXORUBICIN, VINCRISTINE AND PREDNISOLONE (R-CHOP) CHEMOTHERAPY

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Background. Gastrointestinal lymphoma is the most common form of extranodal lymphoma, and the most common involved site is the stomach with histological subtype of diffuse large B-cell lymphoma (DLBCL). Rituximab plus anthracycline-based chemotherapy is the treatment of choice, and the role of radiation therapy and surgery are controversial. *Aims*. The aim of this study was to investigate the patient's outcomes after rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) treatment in primary gastric DLBCL in a single center. *Design and Methods*. We searched AMC Registry for Non-Hodgkin's Lymphoma and found 26 patients with primary DLBCL, who received R-CHOP as first-line chemotherapy. Ten of 26 patients had localized disease. Remaining patients had disseminated disease. R-CHOP was repeated every 21 days in all patients. *Results*. Overall, complete response (CR) was observed in 20 of 26 patients (76.9%). Three-year event free survival (EFS) and overall survival (OS) was 76.5% and 75.0%, respectively. After analyses of 10 patients with localized disease, we found that these patients had received a total 38 cycles, with a median of 3 cycles per patient. Of 10 patients, one patient had 2 cycles of R-CHOP, four had 3 cycles, and one had 4 cycles, all 6 patients above followed by consolidation radiotherapy. Remaining one patient and four patients had 5 cycles and 6 cycles of R-CHOP, respectively. In patients with localized disease, CR was observed in 10 of 10 patients (100%), and both 3-year EFS and OS was 100% (10 of 10 patients). In analyses with 16 patients with disseminated disease, 16 patients had received a total 91 cycles, with a median of 6 cycles per patient. In these patients, two patients had radiation therapy after R-CHOP, one patient had CR before consolidation radiation therapy, and another had partial response before radiation therapy. CR after R-CHOP treatment was observed in 10 of 16 patients (62.5%), partial response in 3 patients, stable disease in 1 patient, and progressive disease in 1 patient. Three-year EFS and OS was 61.1% and 57.8% in patients with disseminated disease. *Conclusions*. R-CHOP regimen showed a promising result in primary gastric DLBCL. Combination with rituximab in CHOP regimen showed excellent prognosis especially in patients with localized disease. In localized disease, CR was 100%, 3-year EFS and OS was 100%. In disseminated disease, CR was 62.5%, 3-year EFS and OS was 61.1% and 57.8%.

1698

CLINICOPATHOLOGIC CHARACTERISTICS OF THE T(14;19)(Q32;Q13)-POSITIV SPLENIC MARGINAL ZONE LYMPHOMA

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Background. Splenic marginal zone lymphoma (SMZL) is well recognized B-cell neoplasm. Which characterized with splenomegaly, lymphocytosis, involvement of bone marrow, sometimes with M-component. Immunologically characterized with typical phenotype of marginal zone. The most frequent findings in SMZL are involvement of chromosomes 1, 3, 7(usually deleted in 7q) and 8. The t(14;19)(q32;q13) is rare cytogenetic abnormality with rearrangement bcl-3 that has been reported in B-cell lymphomas and leukemia. *Aims*.To describe the clinical, morphological, immunophenotypic findings in SMZL associated with t(14;19)(q32;q13). *Design and Methods*. In Hematological Research Centre, Moscow between January 2005 and February 2009 identified three cases SMZL with t(14;19)(q32;q13). In all patients (males with median age 58 years (from 51-67)) debut of lymphoma with B-symptoms, high level of lactate dehydrogenase (LDH), hepatosplenomegaly and only with splenic hilar lymphadenopaty. The median hemoglobin was 110 g/l (range 92-122 g/l). All patients had normal count of leucocytes with an absolute lymphocytosis (median lymphocytes count 72,3 x 10^9 g/l, range 58-83x10^9/L) and thrombocytopenia. Morphological examination of peripheral blood and bone marrow lymphocytes showed that all lymphocytes are atypical with wide cytoplasm, nuclear indentation. In all cases bone marrow involvement was nodular and composed of heterogeneous mixture cells, majority medium sized. The results of immunophenotypic analysis were expression of mature B-cells antigens and absence CD10-, CD23-, CD5-, CD43-, CyclinD1-. In debut two patients were get chemotherapy (CHOP-regimen) However there were progression - enlargement of spleen size and decreased of thrombocytes counts. All patients undergo to splenectomy. Median weight of spleen was 2083 g (range 1800-2850 g). Splenic section generally show massive nodular pattern (involvement of the white and red pulp) associated with diffuse invasion of the sinuses. In all cases discovered high Ki-67. There were occur progression after splenectomy during 3-6 months which is characterized with increase of leucocytes counts (range 45,4 - 101,8x10^9 /l), high level of LDH, appearance of peripheral and visceral lymph nodes. Consideration of increasing leucocytes count and presence of lymphadenopathy in all patients were used alkylating agents (in one patient - chlorambucil and in two - cyclophosphamide). Results: There were normal peripheral blood index and level of LDH, absence of lymphadenopathy in all cases after 4-6 months of treatment with alkylating agents. Median observation was 13+ months (range 15-18 months). All patients were alive. *Conclusions*. t(14;19)(q32;q13)-positive SMZL is distinct variantof SMZL which characterized with high Ki-67, transient progression on chemotherapy and after splenectomy and high efficiency of alkylating agents.

1699

MYELOID PRECURSOR THERAPY IN LYMPHOMA PATIENTS

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Background. Myeloid growth factors help to prevent and cure neutropenic events in malignant lymphoma patients treated by chemotherapeutical regimens. Administering either filgrastim or pegfilgrastim, treatment schedule can be kept well and less dose reductions are needed, which results in better survival rates. *Aims*. The aim of this study was to examine the indications and the outcome of myeloid precursor therapy among our malignant lymphoma patients. *Design and Methods*. Between 2003 and 2007, 249 malignant lymphoma patients received 1655 cycles of different polychemotherapies. Myeloid growth factor therapy was administered in 138 cases by 65 patients, which meant 8.33% of all treatment cycles and 26.1% of all patients, respectively. As for the indications, prevention was more common than intervention (71.7% vs. 28.3%). By preventive usage of growths factors, two third of threatening neutropenic events could be avoided. Side effects were uncommon and mild: grade I-II toxicity was observed in 31% of all treatments. Analyzing the risk factors for febrile neutropenia among patients who received myeloid growth factor therapy compared to those who did not, we found the incidence of comorbidities, hypoalbuminaemia, advanced stage disease and agressive chemotherapies significantly different in the two groups. Interestingly, there was no significant difference between the median age and the incidence of low body surface area. *Conclusions*. Our observations support that myeloid precursor therapy is an effective and safe tool to prevent or treat neutropenia in high-risk malignant lymphoma patients.

1700

IMPROVEMENT OF THE OUTCOME OF DLBC AND FOLLICULAR LYMPHOMAS IN VENEZUELAN PATIENTS TREATED WITH R-CHOP

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A study of patients with lymphoma studied and treated at the Oncology and Hematology Institute (THE LYMPHOMA GROUP) in Caracas, and at other Public and Private Clinics in Venezuela was performed. *Design and Methods*. 1117 patients with lymphoma were studied during the period 1996-2008. The diagnosis of lymphoma was established by lymph node and tissue biopsies (depending of the case), histopathology and inmunohistochemistry studies, and complemented with X rays and TACs. Results: Most of the patients had non-Hodgkin lymphomas (NHL, 727 cases, 65.1%); the Hodgkin lymphomas represented 34.9% (HL, 390 cases), distributed as follows: Nodular Sclerosis (62.6%), Mixed Cellularity (27.7%), Lymphocyte Predominance (4.3%), patients with Lymphocyte Depletion (5.4%). The average age and gender of the patients were Nodular Sclerosis: 32.45 years old (18-65), 129 females and 115 males, Mixed Cellularity: 22.3 years (18-56), 37 females and 72 males, Lymphocyte Predominance 22.6 years old (18-67), 6 females and 11 males, Lymphocyte Depletion 22 years old (20-42) 15 males and 6 females. Stadio I: 5.5%, II: 44%, III: 31.1%, IV: 19.4%. These patients were treated with different protocols according to the year they were treated: MOPP, ABVD, STANDFORD V, HYBRIDO, COPP/ABVD, COPP/EBVD, and since 2004 with BEACOPP. Non Hodgkin Lymphomas (NHL) were distributed as follows: diffuse large B cell (36,4%), follicular (24,1%), MALT lymphoma (10%), Peripheral T cell (5,5%), Mantle Cell lymphoma (3%); 20,8% of the patients had another type of lymphoma such as mycosis fungoides, immunoblastic, anaplastic, Lymphoblastic, Burkitt or Cutaneous B cell lymphoma. Of the Follicular NHL 45% had high FLIPP, while 60% of the DLBC lymphoma had intermediate high IPI score with high risk prognostic factors and only 15% of cases had low risk lymphoma. They were treated with CHOP, CHOP Bleo, MACOB B, ATT, HyperCvad, CHOP MTX and since 2004 with R-CHOP. The R-CHOP as induction in Follicular NHL *de novo* and Rituximab as maintenance produced 46% CR and 56% PR. The CR increased to 77% after two years of Rituximab maintenance during four years observation period. The CR for DLBC lymphoma was 68%. An improvement in the outcome of our lymphoma patients has occurred when we compared the percentage of relapse in different years. *Conclusions*. 1117 Venezuelan patients with lymphoma were studied: 34.9% Hodgkin and 65.1% non Hodgkin lymphomas. The most common subtype of HL was nodular sclerosis (62.6%), being more common in female and young adults. The mixed cellularity HL represented 27.7%, while the lymphocyte predominance and lymphocyte depletion types were only 6,8% and 8,7%, respectively. The most common NHL was the DLBC lymphoma, followed by the follicular lymphoma. The Venezuelan population are integrated by a mixture of different ethnic group: Indians, Caucasians and Blacks but it seems that HL in these patients follows the same pattern reported in other populations. The outcome of Follicular and DLBC lymphoma patients improved using R-CHOP.

1701

A PHASE II TRIAL OF GEMCITABINE, IFOSFAMIDE, DEXAMATHASONE, AND OXALIPLATIN (GIDOX) FOR PATIENTS WITH REFRACTORY OR RELAPSED NON-HODGKIN'S LYMPHOMA

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Background. Gemcitabine combined with cisplatin has been known as an effective regimen for lymphoma treatment in salvage setting. However, this regimen has the modest response with severe nephrotoxcity and neurotoxicity, especially to heavily treated patients. *Aims*. We investigated the response rate and toxicity of gemcitabine, ifosfamide, dexamethasone, and oxaliplatin (GIDOx) for recurrent or refractory aggressive B-cell non-Hodgkin lymphoma (NHL), looking for the more effective and less toxic therapy.*Design and Methods*. Patients with recurrent or refractory diffuse large B-cell NHL or mantle cell lymphoma, measurable disease, and more than one previous chemotherapy regimen were eligible. Treatment consisted of gemcitabine 1000 mg/m^2 intravenously (i.v.) on Days 1 and 8, ifosfamide 2000 mg/m^2 i.v. on Day 1, dexamethasone 40 mg orally on Days 1-4, and oxaliplatin 130mg/m2 i.v. on Day 2, every 21 days. The primary end point was a response after three cycles. Patients could then proceed to stem cell transplantation (SCT) or receive up to six treatment cycles. *Results*. Twenty-seven eligible patients were evaluable for toxicity and response. The median age of the patients was 54 years (range, 18-75 years) and most had diffuse large-cell lymphoma. After 3 cycles, there were 4 complete responses (CR; 15%) and 10 partial responses (PR; 37%). There was an overall response rate (RR) of 52%. The RR after completion of all protocol chemotherapy including SCT was 44% (10 CR, 2 PR). In total 88 cycles of GIDOx, grade 3 and 4 neutropenia occurred in 33% and 16% of cycles, respectively. Grade 3 and 4 thrombocytopenia occurred in 14% and 16% of cycles, respectively. Tow patients (2%) experienced febrile neutropenia. Seven patients (26%) proceeded to SCT. *Conclusions*. GIDOx is an active salvage regimen in aggressive B-cell NHL and can be administered with acceptable toxicity.

1702

TREATMENT WITH ALEMTUZUMAB IN T CUTANEOUS LYMPHOMAS

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Background. Mycosis fungoides (MF) and Sézary syndrome (SS) are the most frequent T cutaneous lymphomas, characterised by acumulation of CD4 + T cells in the skin. The results obtained through classic chemotherapy or new treatment (PUVA therapy, interferons, retinoids, rexinoids) are mediocre. The recent studies propose the treatment with anti-CD 52 monoclonal antibody therapy in these cases. *Design and Methods*. We studied 11 patients with T cutaneous lymphomas (9 cases of mycosis fungoides and 2 of Sézary syndrome), treated with classic chemotherapy (CHOP regimens) and PUVA therapy as first line treatment, obtaining only short and ephemeral remissions. All patients were then treated with Alemtuzumab, 30 mg x 3 / weekly, subcutaneous administration, for 3 months. *Results.* The results were 8 complete remissions and 3 partial remissions ; 1 of the patients with partial remission died because of a complication which had no relationship with the initial disease. Treatments wasn't associated with local adverse reactions or the reactivation of CMV infection. *Conclusions*. The good results obtained recommended this treatment for all patients suffering from T cutaneous lymphomas, refractory or relapsed after the classic therapy.

1703

HIGH AND PROLONGED RESPONSE RATE WITH LOW TOXICITY PROFILE WITH RITUXIMAB IN COMBINATION WITH CHLORAMBUCIL IN UNTREATED FOLLICULAR LYMPHOMA PATIENTS

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Background. The use of Rituximab in combination with chemotherapy it is now standard practise as first-line treatment in patient with follicular lymphoma (FL), because the association has demonstrated significantly improved progression-free survival, complete response rate compared to chemotherapy alone. Despite the progress made in treatment, a great majority of patients still relapse, so treatment options that extend the duration of remission with a low toxicity profile are warranted. *Design and Methods*. Between December 2001 and April 2008 33 patients not eligible for clinical trials were treated at our institution with a combination of Rituximab plus Chlorambucil (Chl). Sixteen were men and the median age at diagnosis was 55 years old. Seventy percent of patients presented grade 1 and 2 FL, while just 5 patients had grade 3 lymphoma. Twenty-one patients had advanced stage of FL and the majorities (82%) were asymptomatic. In terms of prognostic index 19 patients were low risk FLIPI score. The schedule included an induction phase of 4 weekly administrations of monoclonal antibody at standard dose during 6 continuously weeks of Chl at the dose of 6 mg/m^2/daily. After restaging between the 10-12th week from the beginning of treatment, the patients underwent the maintenance phase with Rituximab once a month and 15 day of Chl at the same dosage each month for 4 consecutive months. *Results*. After the induction the ORR was 97% with 11 patients in complete response (CR). At the end of treatment 97% of patients responded and 29 (88%) achieved a CR. One patient showed a stable disease and underwent to intensification and high-dose chemotherapy. In terms of molecular response the 14 patients positive for the BCL-2 rearrangement at diagnosis, after the treatment 9 of the 10 valuable patients were negative. All patients but one concluded the treatment scheduled. One patient discontinued the therapy because of grade 3 hepatic toxicity. None reduced the dosage of Chl during induction (mean dose 10mg/daily), while in the 2nd phase 14 patients reduced the daily dose (mean dose 8,5 mg/daily) mainly at 1st or before the 3rd maintenance because of haematologic toxicity (neutropenia grade 2 6 pts, grade 3 5 pts and platelets <10x^9/L in 3 cases). One case of thorax HZV has been reported. No late toxicity has been observed. With a median observation time from diagnosis of 62 months (range 8-88 months) 24 patients (72%) maintain a CR. Eight patients experienced relapse, included 5 in CR with a median TTF of 25 months (7-37 months). Up to now 2 patients died and just one because of progressive disease. *Conclusions*. Our experience confirmed that the immuno-chemotherapy is able to improve the outcome of follicular lymphoma patients with high and prolonged responses (72% of CR with a median 5 years observation in our study). Despite other chemotherapy regimens, our schedule of Rituximab in combination with Chlorambucil resulted safety and feasible mainly for the patient, who experienced a very low and easy-manageable toxicity profile.

1704

IMPACT OF MODERN CHEMOTHERAPY IN OUTCOME OF NON-HODGKIN LYMPHOMA IN CHILDREN

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Background. The aim of our study was to evaluate the epidemiologic and pathological features of non-Hodgkin lymphoma(NHL) in children, as well as comparative treatment results according to the type of the therapeutic regimes employed : modern protocols-type BFM versus classical regimes.*Design and Methods*. A retrospective review of 135 patients (pts) treated and followed-up in the Institute of Oncology- pediatric oncology department, Bucharest between 1990 -2005 was performed. Eligibility criteria required: pathologic diagnosis of the subtype of non-Hodgkin lymphoma, staging according to the international accepted standards, chemotherapy +/-radiotherapy depending on the stage and histological type and follow-up of at least 3 years after the completion of therapy. Until 1995 the chemotherapy consisted of protocols type CHOP, COPP, COP, CVP; starting with 1996, more aggressive regimes-type BFM, LSA2-L2 were applied. Results: The data we obtained showed a predominance in males (65%) and in the 10-14 year old group

(36%). Most of the pts were diagnosed in stage III -IV (89%). The distribution of the histological types: NHL type B (39.6%), NHL nonB (45.5%), anaplastic NHL with large cells (5.9%). NHL low-grade (8.9%). An 100% survival rate was registered in NHL with large cells and NHL with low-grade, irrespective of stage. The curable stages (I, II) survival rate was of 100% in NHL type B and of 80% in NHL non-B. In advanced stages the survival rates were higher in stage III of NHL type B (65%) versus type non-B (44%) and in stage IV of NHL non-B (33%) versus type B (18%). Global survival was of maximum 100% in NHL low-grade and of minimum 45% in NHL nonB. Modern aggressive protocols type BFM induced higher survival rates in comparison with classical regimes : 68% versus 48%. *Conclusions*. The most frequent histological types were B and non-B NHL, 91% of cases were diagnosed in advanced stages, and significant efficiency of modern aggressive protocols was registered in our study.

1705

EFFICACY AND SAFETY OF 90Y-IBRITUMOMAB TIUXETAN (ZEVALIN) IN MABTERA/INMUNOTHERAPY PRETREATED NON FOLLICULAR B CELL LYMPHOMA PATIENTS

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Background. 90Y-ibritumomab tiuxetan (Zevalin) was EMEA approved for mabtera pretreated, refractory or relapsed, Folicular Lymphoma patients. However there are currently several clinical trials evaluating radioimmunotherapy in other subtypes of lymphoma and clinical situations. *Aims*. We present observations from our clinical experience with a retrospective study to evaluate safety/effective of Zevalin as salvage or consolidation in 14 mabtera/inmunotherapy pretreated non follicular B cell lymphoma patients. *Design and Methods*. Between october/2007-december/2008, 14 mabtera pretreated B cell NHL patients received Zevalin. Five patients as consolidation after mabtera+chemotherapy (1 or 2 lines), 9 patients as salvage therapy (2-5 previous lines), of those, 2 received Zevalin with BEAM, followed by autologous SCT, and 7 in monotherapy. Zevalin dose was platelet count adapted, over $150x10^9$/L received 0.4 mCi/kg zevalin, 0.3 mCi/kg, if less. For each patient, clinical examinations and imaging studies (18F-FDG PET/CT before therapy and at 12 wk after RIT) were used to document response to therapy using the revised International Workshop Criteria (IWC). Primary endpoints were overall response rate (ORR), CR rate and primary safety variable were the incidence of hematological or critical toxicities. *Results*. Patients main characteristics at diagnosis (n=14): median age (range) was 75 years (36-88); 79% were olders than 60 years. Male/female: 10/4. Ann arbor stage classification: I:0/II:3/III:4/IV:7; 79% were advanced stage. "B" symptoms: n/y 12/2. Bone marrow involvement n/y: 4/14 (28.5%). Bulky disease none. ECOG over 2 (43%). Only 2 patient present a IPI 0-1. Histological subtypes: mantle 3 (2 variant blastoid), marginal 1. Large B cell 5, ot hers 5. Average previous lines was 2.7. ORR was 64%, CR was 50%: 4 of 7 patients how achieved CR, received RIT as consolidation and 3 as salvage therapy. 5 patients received zevalin as consolidation therapy. Present day all are alive, 4 in CR. 9 patients received zevalin as salvage therapy after 2-5 chemotherapy lines. 3 are still in CR. 2 patients are alive on treatment; 4 patients have died (Z-BEAM-TASPE:1) all cases with NHL progression. The median PFS of the 14 patients who received RTI was 6 months with follow-up time of 12.0 months (range 4-26 months). All the patients presented hematologic toxicity with grade 3/4 of neutropenia, thrombocytopenia or anemia in 8 (57%), 8 (57%) and 3 (14%) respectively. We have observed in 4 patients a diagnosis of second neoplasia after RIT: 3 skin cancers and one patient with bladder cancer. *Conclusions*. RIT with 90Y-Ibritumomab Tiuxetan is a safe and effective approach for patients affected by NHL and heavily pretreated with Rituximab + chemotherapy.

1706

CLINICOBIOLOGICAL FEATURES, OUTCOME AND PROGNOSTIC FACTORS OF 104 PATIENTS WITH EARLY-STAGE (ANN ARBOR I-II) DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL): EXPERIENCE IN A SINGLE INSTITUTION

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Background. DLBCL is the most frequent non Hodgkin lymphoma. It presents as a localized disease in 30% of cases. *Aims*. To analyze the main clinicobiologic features, response to treatment and outcome of patients with early stage DLBCL. *Design and Methods*. Patients with Ann Arbor stage I-II DLBCL diagnosed in a single institution during a 17-year period were identified in a data base. Clinicobiological features, treatment, response and survival were analyzed. Multivariate analysis of the variables predicting response was performed using a logistic regression model. Survival curves were constructed by Kaplan Meier´s method. Multivariate analysis for survival was performed using the Cox model. *Results*. One hundred and four patients were identified. Forty-six (44%) were males, median age was 56 years. The characteristics of the disease at diagnosis were as follows: stage I, 44 patients (42%); bulky disease, 40 (38%); primarily extranodal disease, 45 (43%); ECOG 0-1, 69 (67%); B symptoms, 16 (15%); elevated LDH, 91 (88%) and elevated β2 microglobulin, 41/80 (51%). IPI subgroups were: low risk, 49 (47%); low-intermediate risk, 37 (36%) and high-intermediate risk 18 (17%). One hundred patients (96%) were treated with CHOP/CHOP-like chemotherapy, 33 (32%) of them with rituximab and 61 (58%) followed by extended field radiotherapy. Eighty-six patients (82%) achieved complete remission (CR), 9 (9%) partial remission, and 9 (9%) failed. Five year event free survival (EFS) and overall survival (OS) were 68% and 71% respectively. Thirty-three patients died during follow-up, most of them due to disease progression. Three patients died due to neoplasms in CR: gastric adenocarcinoma, pancreatic adenocarcinoma and acute myeloblastic leukemia. A multivariate analysis was performed, including the main significant variables identified in the univariate analysis (age < 60 years, bulky disease, performance status, IPI, β2 microglobulin, rituximab and complementary radiotherapy). Bulky disease (p=0.04) and elevated β2 microglobulin (p=0.001) had prognostic value to predict poor response. Age > 60 years (p=0.04) and elevated β2 microglobulin (p=0.035) significantly predicted poor EFS. IPI (p=0.007) and β2 microglobulin (p=0.0086) were the most important factors predicting OS. No differences were found in terms of response, OS and EFS regarding treatment with rituximab. *Summary*. Early-stage DLBCL presented as an extranodal disease in almost half of the patients. Almost half of the patients presented bulky disease. Elevated β2 microglobulin predicted poor outcome either for response as for OS and EFS. The exact rule of treatment with rituximab in this subset of patients has yet to be determined in a prospective clinical trial.

1707

PRIMARY CNS LYMPHOMA - THE OUTCOME AFTER THE THERAPY WITH DEANGELIS COMBINED MODALITY TREATMENT - SERBIAN LYMPHOMA STUDY GROUP EXPERIENCE

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Background. Primary central nervous system lymphomas (PCNSL) are aggressive malignancies, having one of the worst prognoses among lymphomas. The best and generally accepted treatment modality for PCNSL has not yet been identified. AIM. This study was performed to assesses the therapeutic outcome and late side effects of combined modality treatment (CMT) presented by DeAngelis et al. We tried to determine the possible prognostic value of clinical and laboratory parameters in patients (pts) with PCNSL. *Design and Methods*. 16 pts diagnosed with PCNSL between 1994 and 2008 received CMT. The diagnosis was obtained by biopsy of the tumor, all the pts had diffuse large B-lymphoma. The CMT was consisted of methotrexate (Mtx) 1g/m2 i.v. at day 1 and 8, Mtx i.t. 12mgx6, 40 Gy whole brain radiotherapy + 14,4 Gy to the tumor site, and 2 courses of Ara-Cx2d i.v. Before the start of treatment CT scans and bone marrow biopsy were performed to exclude any other lymphoma involvement. In all pts on the admission, complete laboratory work up (including LDH) and ECOG performance status were determined. *Results*. The median age was 53 (range 27-66), 11 pts were male and 5 female. In the whole group nine pts (56%) achieved remission: complete remission (CR)-3 pts (19%) and partial remission (PR) had 6 pts (37%). Seven pts (44%) didn't respond to therapy and died within 6 months after the diagnosis was established. Median overal survival for the whole group of pts was 9 months and by now 22 months in patients who responded to treatment (6 pts are still alive). One pt died in first relapse occurred 4 months after completing CMT, before starting "selvage" therapy. Two patients in first relapse (first after 17 months, the second after 27 months) were retreated with CMT without radiotherapy and achieved remissions lasting 38 and 7 months, both of them died within 17 months after second relapse and were treated with cyclophosphamide, Mtx and procarbazine combination. The elevated

LDH level was present in half of pts both in responders and non-responders group. Eight pts had ECOG ≥3 (three pts older than 60 yrs) and only 2 of them responded to CMT. In the CMT responders group, two patients developed severely symptomatic and one mildly symptomatic leukoencephalopathy. *Conclusion*. According to our experience, treatment with combined regimen led to a marked prolongation of survival in patients who responded to therapy. Having in mind that the risk of late side effects exists and that the response rate was 56%, further investigations seems to be necessary for finding appropriate, more efficious and less toxic treatment for PCNSL. According to our experience, high ECOG and older age may be predictive for poor prognosis of these patients.

1708

GOOD OUTCOME OF PRIMARY MEDIASTINAL B CELL LYMPHOMA (PMBCL) IN PATIENTS TREATED WITH RITUXIMAB-CHOP PLUS RADIOTHERAPY

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Background. The optimal treatment of PMBCL is not well-defined today. In retrospective studies third generation chemotherapy regimens, namely MACOP-B or VACOP-B, plus radiotherapy (RT) achieved better results in comparison with conventional CHOP. Moreover, the impact of the addition of rituximab (R) to CHOP in the treatment of PMBCL is still debated. *Aims*. To retrospectively evaluate clinical characteristics at diagnosis and outcome of patients with PMBCL treated with R-CHOP in our institution. *Design and Methods*. Between 3/2003 and 8/2008, 19 untreated patients with PMBCL have been admitted in our institution. Disease evaluation was performed with whole-body computed tomography at diagnosis, after 3-4 courses of chemotherapy and at the conclusion of treatment. 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 sequential chemotherapy (HDSC) followed by autologous stem cell transplantation (ASCT). The planned treatment consisted of 6 courses of R-CHOP21 in aaIPI=0 or 8 courses of R-CHOP14 in age-adjusted-IPI (aaIPI) >0, plus involved field (IF) RT (25-36 Gy). Final restaging included PET/CT (4 months after RT or 2 months after ASCT) in all complete responder patients. Patients received G-CSF or peg-G-CSF in order to maintain dose-intensity of planned treatment, and trimetoprim-sulfametaxazole, acyclovir and nistatine as infections prophylaxis. Retrospectively, we evaluated the response rate, overall survival (OS) and disease-free survival (DFS). *Results*. Four patients were male and 15 female, with a median age of 32 years (15-54). Stage II was present in 15 patients and stage III-IV in 4. Fourteen patients had B symptoms; aaIPI was 0 in 3 cases and 1-2 in 16 cases. Bulky disease was reported in 16 cases. Today 18 patients completed treatment and are evaluable. At early restaging, 17 patients (94%) showed at least a PR. After completion of R-CHOP plus IFRT, 14 (82%) of the 17 PR patients obtained a CR, 2 (12%) remained in PR, and 1 patient (6%) had PD. Three of the 4 patients who received HDSC plus ASCT (1 after first restaging and 3 after treatment conclusion) obtained CR, while 1 patient died 20 months after initial diagnosis because of PD. After a median follow-up of 35 months (6-70), OS is 92%. We observed no relapse so far. Consequently, DFS of the 17 patients who achieved CR is 100%. All patients maintained planned dose-intensity. No cases of serious infections were recorded. In summary, the addition of rituximab to CHOP plus IFRT seems to be feasible, safe and effective in inducing durable CR in a high proportion of our small cohort of patients with PMBCL. Large, prospective studies are needed to further confirm our encouraging results.

1709

A PHASE II STUDY OF GEMCITABINE IN PATIENTS WITH ADVANCED STAGE MARGINAL ZONE B-CELL LYMPHOMA

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Background. Therapeutic approaches for marginal zone B-cell lymphoma (MZL) are still evolving. Localized MZL responds favorably to local treatment such as surgery and/or local radiation therapy. However, MZL presents as a disseminated disease in one-third of the cases at the diagnosis. Moreover, relapses involving distant sites after local therapy have been reported. Therefore, the search for effective forms of systemic therapy is an important issue. *Aims*. We conducted this multi-center, phase II trial to investigate the efficacy and safety of gemcitabine single chemotherapy in patients with stage III/IV MZBCL. *Design and Methods*. Patients received gemcitabine 1250 mg/m^2 on day1 and 8 each cycle. The treatment was repeated every 3 weeks and continued for 6 cycles until disease progression, withdrawal due to toxicity, or withdrawal of consent. *Results*. Between Sep 2006 and Sep2008, a total of 16 patients were enrolled with informed consent at this trial from 6 institutes in Korea. Among these patients, 4 patients were dropped out without evaluation. The median age of the evaluated 12 (9 males, 3 females) patients is 62 (range 25-73) years. Seven patients (58%) had extranodal sites involvement. All of patients had received previous treatment about MZL. The patients received a total of 69 cycles of gemcitabine chemotherapy (range 3 - 6 [median 6] cycles/person). There were 2 PR (17%), 9 SD (75%), and 1 PD (8%). There were 8/69 cycles (12%) of grade 3/4 neutropenia. Non-hematologic toxicities were mild and tolerable. There were 5 cycles (8%) of delayed chemotherapy (median 1 week) because of neutropenia. Dose reduction was needed in 12 cycles. But, there was no treatment-related death. The median TTP was 10.2 months (95% CI, 5.3-15.1). As the response rate in stage I did not justify progressing to stage II (≥8/15), this study had to be discontinued in accordance with the established protocols. *Conclusions*. Gemcitabine as a single agent, in this dosage and schedule, has minimal clinical activity in relapsed or refractory MZL

1710

DOSE-ADJUSTED R-EDOCH-14 SUPPORTED BY PEGFILGRASTRIM OR FILGRASTRIM IN PATIENTS WITH HIGH-RISK DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL): FEASIBILITY AND PRELIMINARY EFFICACY

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Background. Poor prognosis DLBCL represents 50% of all DLBCL with overall cure rates ranging from 50-60% with modern dose-dense immunochemotherapy regimens such as R-CHOP14. Using an alternative strategy, as infusional and dose-adjusted R-EPOCH, we have shown an 83% of complete responses (CR), with an estimated 5-year overall survival (OS) rate of 75% (*García-Suárez et al. Br J Haematol 2007, 136:276*). Despite this improvement in outcome, the search for new treatment strategies should continue. Therefore, compared with prior R-EPOCH we decided to investigate whether the introduction of dexamethasone (40 mg IV on days 1-5) in place of prednisone (based upon data which demonstrated that the former was associated with enhanced CNS penetration) and the reduction of treatment intervals from 3 to 2 weeks would be feasible and might improve the outcome in this group of patients. *Aims*. To evaluate the feasibility and clinical activity of DA-R-EDOCH-14 as upfront therapy in patients newly diagnosed of poor prognosis DLBCL. *Design and Methods*. Herein,8 consecutive patients (median age 61 years; range 46-73) of an ongoing observational prospective study in newly diagnosed patients with DLBCL and an age-adjusted IPI ≥2 are reported (aaIPI=2 in 25% and aaIPI=3 in 75%). Other patient´s characteristics were: stage IV (100%), high LDH (100%), > 1 extranodal site involvement (50%), ECOG ≥2 (87.5%), bone marrow involvement (25%), and bulky disease (25%). Dose adjusted R-EDOCH-14 was administered every 14 days if feasible for 6-8 cycles, supported by pegfilgrastrim on day 5 in a single per cycle sc dose of 6 mg or filgrastrim with a 6-day schedule from day 6 to 11 at a dosage of 5 µg/kg/d. The type of G-CSF administered was at the discretion of the treating physician (6 patients received pegfilgrastrim and 2 patients filgrastrim). Complete blood counts were monitored at least twice weekly. Toxicity was calculated over 53 cycles administered; feasibilitiy was calculated over 44, since the first cycle in each patient was not susceptible to delay or dose-reduction. *Results*. Of the 44 cycles considered, 31 (70.5%) were delivered on time. Four (50%) patients required dose escalations to achieve an ANC nadir, with 30% of cycles having at least 120% of the entry-dose level. The average RDI was 110% for etoposide, doxorubicin and cyclophosphamide. Grade 4 neutropenia, thrombocytopenia and anaemia were recorded in 70%, 7.6% and 22% of cycles and occurred in 100%, 22% and 44% of the patients, respectively; The median duration of grade 4 neutropenia was 2 days (range: 0-5).The inci-

dence of grade 4 neutropenia in the pegfilgrastrim and filgrastrim cycles was 73% and 66%, respectively. In addition, the mean duration of grade 4 neutropenia was also similar with both G-CSF formulations (2.1 and 2.3 days, respectively). ANC nadir was invariably observed around day 10 to12 of treatment. Only 9 cycles (17% of total) were complicated by neutropenic fever occurring in 4 patients (50% of patients). Severe non-haematological toxicities were registered in 8 cycles (15% of cycles), involving 4 patients (50% of patients). There were no toxicity-related deaths. Out of 7 evaluated patients for efficacy after 10 months follow-up (range: 5-24+), 6 patients (85.7%) achieved CR. *Conclusions*. This ongoing study supports that dose adjusted R-EDOCH-14 is a feasible regimen with no increased toxicity compared with the 3-weekly dose-adjusted R-EPOCH standard regimen. Despite the small number of patients and cycles, our data suggest a comparable neutrophil support with pegfilgrastrim or a 6-day filgrastrim schedule. The CR rate seems very promising even our cohort of patients represent a group with particularly unfavourable outcome.

1711

FALSE POSITIVE PET FINDINGS IN LYMPHOMA MANAGEMENT

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Background. Positron Emission Tomography (PET) imaging has become a useful tool in assessing lymphoma at diagnosis, during and after therapy. Standard uptake value (SUV) measurement is thought to improve accuracy, even though there is no clear cutting value to differentiate malignant from non-malignant lesions. PET's strongest point is a high negative predictive value; thus a negative PET scan at the end of therapy predicts a very low risk for relapse. Unfortunately positive predictive value is not so high and false positive results remain a major drawback of the technique. At our institution we perform a PET scan at the end of therapy in those patients with lymphoma known to be consistently PET positive (Hodgkin and aggressive non-Hodgkin lymphoma). Additionally a PET scan is obtained in patients with doubtful lymphoma locations at initial staging.

Table 1

	Demographics	Histology at diagnosis	Time from end of therapy to PET imaging	SUV Max	Location	Biopsy result	Follow up
# 1	Female 39 yr	DLBCL	3 weeks	14,8	Retroperitoneum	Fibrosis	63 months
# 2	Female 63 yr	DLBCL	7 weeks	5,36	Lung and hiliar nodes	Mycobacterial infection (culture)	3 months
# 3	Female 30	Hodgkin	10 months	2,69	Mediastinum	Fibrosis	10 months
# 4	Female 72 yr	Non malignant	Initial diagnostic workup	11,1	Tonsil	Tonsil inflammation	8 months
# 5	Female 31 yr	DLBCL	6 weeks	5,2	Illium bone	Bone remodelling	15 months

Case series. We present a series of five patients where PET scans have been considered positive for lymphoma considering SUV values as well as clinical data. In all cases final diagnosis was a nonmalignant process. Biopsy procedures were performed in all cases but one, where microbiological cultures settled final diagnosis. Follow up has been very close and no lymphoma relapse has been detected. Results are detailed in Table 1. *Conclusions*. Despite using SUV values, false positive results are not uncommon in PET imaging for the management of lymphoma. There are cases in which fibrosis, inflammation or infection can resemble malignancy. In this scenario, a new biopsy is essential, thus arising the problem of access to residual nodes or lesions. Although PET improves accuracy in lymphoma management, its use can launch new issues such as the need for biopsy in PET positive images.

1712

CHOLESTEROL AND APOLIPOPROTEINS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES: A PRELIMINARY REPORT

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Background. Abnormal plasma lipid levels was observed at diagnosis in patients with various types of cancer, including hematological malignancies. Relationship between low baseline level of cholesterol and poor prognosis in cancer patients is well-established. There are few data regarding possible links between plasma cholesterol or apolipoproteins levels and pathophysiological consequences of tumor growth such as inflammation or activation of coagulation system in patients with hematological malignancies. *Aims*. The aim of the study was to assess plasma cholesterol and apolipoproteins levels and their relationships with laboratory parameters of tumor burden, inflammation status and coagulation/fibrinolysis status in men with newly diagnosed hematological malignancies. *Design and Methods*. The study population included 80 men. Hematological group consisted of 33 de novo diagnosed patients (mean age: 53,85±13,18). There were 15 patients with non-Hodgkin lymphoma (NHL) in stage IV and 1 patient with Hodgkin's lymphoma (HL) in stage II acc. to Ann Arbor Staging System, 15 patients with chronic lymphocytic leukemia (CLL) in stage II (1 patient), III (4 patients) and IV (10 patients) acc. to Rai Staging System, 2 patients with multiple myeloma (MM) in stage II and III of disease acc. to International Staging System. Control group consisted of 47 aged-matched healthy men (mean age: 49,06±10,00]). Nephrotic syndrome, severe renal failure (GFR<40 mL/min/1,73 m^2), hypo- or hyperthyroidism, familial hypercholesterolemia and use of any medication that could affect lipid metabolism were the exclusion criteria of the patients and controls. The following parameters were determined in fasting blood samples obtained from all eligible hematological and control subjects: total cholesterol (TC), low-density lipoprotein-cholesterol (LDL), high-density lipoprotein-cholesterol (HDL-C) and its subfractions (HDL2 and HDL3), triglycerides (TG), apolipoproteins A1 and B (ApoA1, ApoB). Additionally, laboratory parameters reflecting tumor burden (activity of lactate dehydrogenase [LDH]), inflammation status (C-reactive protein [CRP]) and coagulation/fibrinolysis status (D-dimer and fibrinogen) were performed in every hematological patient. In hematological group the blood samples were taken prior to the initiation of chemotherapy. RESULTS: Statistically significant differences between the groups included reduced TC (155,61±43,42 mg/dl in hematological group vs 171,77±27,98 mg/dl in controls; p=0,048), HDL-C (32,57±11,57 mg/dl vs 43,73±8,88 mg/dl; p<0,001), HDL3 (25,89±9,56 mg/dl vs 34,87±7,97 mg/dl; p<0,001), ApoA1 (0,95±0,31 g/l vs 1,44±0,27 g/l; p<0,001) and ApoB (0,98±0,35 g/l vs 1,14±0,27 g/l; p=0,033) levels. ApoA1/ApoB ratio was higher in hematological than in control group (1,20±0,85 vs 0,80±0,18; p=0,003). Plasma levels of LDL, HDL2 and triglycerides did not differed between the groups (p>0,05). There were negative relations between ApoA1 level and LDH activity (r=-0,51; p=0,08) and ApoA1 and CRP levels (r=-0,52; p=0,015). TG correlated with D-dimer and fibrinogen levels (respectively: r=0,38; p=0,031 and r=0,35; p=0,047). *Conclusions*. The study confirms that hematological malignancies are associated with alterations in plasma total cholesterol, its HDL fractions and apolipoproteins levels. Relationships between ApoA1 or triglycerides levels and parameters of tumor burden, inflammation or coagulation/fibrinolysis status require further investigations.

1713

INCIDENCE AND SURVIVAL OF PRIMARY EXTRANODAL LYMPHOMA IN ZARAGOZA (SPAIN)

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Background. There are a few studies in order to estimate the differences in incidence and survival of primary extranodal Lymphoma (PEL) compared with nodal. In Zaragoza (Spain) a population-based Cancer Registry (ZPCR) that includes all non-haematological and haematological (HMs) incident cases, is conducted since 1960. *Aims*. To review all cases diagnosed of lymphoma during the period 1992-2004. 2. To estimate the incidence of PEL in the ZPCR registered cases during this period. 3.

To calculate the survival of PEL compared with nodal lymphoma. *Design and Methods*. We had reviewed lymphoma cases occurred in patients residing in Zaragoza(Spain) during the period 1992-2004 that selected from ZPCR. All cases were reviewed in order to confirm the primary location and the morphology classification according to REAL classification. The population at risk was 9.255.818 person-years. The crude (CIR) and age standardized incidence rate (ASR) were calculated, using the European population as standard. Kaplan-Maier method was applied to calculate median survival time and their 95% confidence intervals, as well as 5-year survival. The end of follow-up was 31 Dec 2007 and log-Rank was used to compare survival curves. Results. Among all 4,570 HMs, a total of 1,987 (43.4%) were NHL (CIR: 19x105 person-year), 331 (16.6%) of them were classified as PEL, yielding an ASR of 2.7x105 person-year (males: 183 cases (55.2%), mean age: 59.2 years, ASR: 3.1x105 person-year and females 148 cases (44.7%), mean age: 66.8 years ASR: 2.4x105 person-year. According to topography the most frequent sites were digestive tract (50.4%), skin (19.8%), gland tissue (10.0%), oral cavity-pharynx (7.9%), lung (2.9%), CNS (2.4%), orbit (2.1%) and others (4.5%). The median survival for PEL was 6.61 years (95%CI: 3.7-9.5) and for nodal Lymphomas 5.01 years (95%CI: 4.1-5.9). The 5-year relative survival was 53.5% and 50% respectively. There are not significant differences between extranodal/nodal groups, different locations and gender. The differences in survival were related to histological subtypes. Theses results were similar to those found in the EU, according EUROCARE data. *Conclusions*. The occurrence of PEL according to gender and mean age is similar to nodal NHL. No significant differences in survival were observed between nodal and extranodal NHL, probably the survival is more conditioned by the histology than the location or gender.

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1714

HEPATITIS C INFECTION AND NON HODGKIN LYMPHOMA IN 37 EGYPTIAN PATIENTS: A SINGLE INSTITUTE EXPERIENCE

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Background. Hepatitis C virus (HCV) infection has recently been recognized as a potential cause of developing B-cell lymphoproliferative disorders. Several studies have reported a significantly higher prevalence of HCV in patients with NHL (i.e. 20% in Italy, 6.4% in other European countries,14% in Japan and 11% in USA). HCV is endemic in EGYPT, according to the WHO (*Z. Mesban & A.Wakil; HCV in Egypt _2003*), HCV has been declared a global health problem with approximately 3% of the world's population infected with HCV- Egypt is considered to contain the highest prevalence of HCV in the world, the national prevalence rate of HCV antibodies positivity was estimated by the Egyptian Ministry of Health & Population (MOHP) in 1999 to be 18.9%, with genotype 4 representing over 90% of the cases in Egypt. Aim: Defining the epidemiological aspect of HCV infection in newly diagnosed untreated NHL Egyptian patients with special emphasis on the relationship between HCV infection & NHL, raising the possibility of combining antiviral therapy (Interferon/ribavirin) with lymphoma treatment protocols in HCV +ve NHL patients to achieve better therapeutic outcomes. *Design and Methods*. Sera collected from patients recently diagnosed with NHL presented to our institute between December 2008 through February 2009, 37 patients were screened for the presence of HCV antibodies with commercially available serologic tests. Reverse transcription-PCR was carried out in 6 cases for quantitation of HCV-RNA. Results: Thirty seven patients were included in our study; 24 Males and 13 Females with a mean age of 42 (range 18-65).Twenty six patients (70.3%) were high grade NHL and 11(29.7%) low grade NHL. HCV positive patients were 15(40.5%) and 22(59.5%)were HCV negative. Quantitative RT-PCR was performed for 6 HCV positive patients out of 15 and they were all in the low to moderate viraemia range (1x104_5x105 IU/mL). *Conclusions*. In our study inspite of the limited number of patients results suggest the presence of a correlation between HCV & NHL, but we are still screening patients coming to our institute to establish a statistically significant correlation.

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RITUXIMAB AND MALABSORPTION SYNDROME INTESTINAL IN PATIENTS WITH NON-HODGKIN LYMPHOMA. ONE COMPLICATION NOT PREVIOUSLY DESCRIBED

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Background. The combination with R to induction chemotherapy improves the overall survival (OS) in patients with non-Hodgkin lymphoma of B lineage (LNHB) and their use in the maintenance treatment of patients with follicular lymphoma improves progression-free survival (PFS). We have observed the occurrence of diarrhea after treatment with rituximab in patients Aims: Assess the development of intestinal malabsorption syndrome in patients treated with R and analyze factors that promote. *Design and Methods*. We reviewed the medical records of 5 patients with NHL treated with R subsequently developed malabsorption syndrome consisting of chronic diarrhea esteatorreica, significant weight loss, abdominal distension, borborigmos, edema and muscle atrophy *Results*. Of the 5 patients (3M and 2H) who developed intestinal malabsorption syndrome, two were follicular lymphoma who were still in remission maintenance therapy with R: 375 mg/m2 dose, 4 doses every 6 months for 2 years. The other three patients (2 NHLDLC and 1 orbital MALT NHL) had received rituximab as part of chemotherapy protocol for remission induction. Three had received FluCy-R diagnosis as the induction protocol. The other two received R-CHOP14 diagnosis. In addition, all were in remission of lymphoproliferative disease. Four patients were ex-smokers and an active smoker. The analytical parameters: hypoalbuminemia, hypoproteinemia, hypocalcemia, hypocholesterolemia, deficiency of folic acid / B12, fat-soluble vitamins and prealbumin. In the radiological study with barium shows acceleration of intestinal transit.Antitransglutaminasa antibodies and antigliadin antiendomysium antibodies unchanged. Parasites in stool and repeatedly negative stool.CMV antigenemia negative. Endoscopic and pathological studies rule out inflammatory bowel disease, colitis and CMV lymphomatous infiltration *Conclusions*. In our series there is a plausible time sequence between the administration of R and the subsequent development of intestinal malabsorption syndrome. Discontinuation of treatment with R produced a significant symptomatic improvement in patients. The use of fludarabine regimens and dose density (R-CHOP14) as part of the induction scheme could encourage the development of malabsorption syndrome in patients treated with R. However, further studies are needed to confirm these findings

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POSITRON EMISSION TOMOGRAPHY FINDINGS IN PATIENTS WITH LYMPHOMA-ASSOCIATED HEMOPHAGOCYTIC SYNDROME

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Introduction. Haemophagocytic syndrome (HPS) is a clinicopathological entity characterized by systemic proliferation of histiocytes, fever, cytopenia, liver dysfunction, hepatosplenomegaly and often coagulopathy. It is often associated with infection, autoimmune diseases and haematological malignancies, particularly non-Hodgkin's lymphoma. Lymphomas manifesting initially with HPS often pose a diagnostic challenge as the majority of cases have no significant lymphadenopathy for early histological diagnosis. There is paucity of data on specific features of Positron Emission Tomography (PET) in patients with lymphoma-associated HPS (LHPS). *Case Report*. Herein, we describe 3 cases of LHPS and their characteristic PET scan features. These 3 patients had pyrexia of unknown origin and pancytopenia. Extensive workup did not reveal any infection or autoimmune aetiology. The Computed Tomography (CT) findings of all 3 patients were largely unremarkable for significant disease. Their PET scans however showed extensive and diffuse fluorodeoxyglucose (FDG) uptakes in bone marrow of the axial skeleton with little involvement in lymph nodes. Bone marrow biopsies showed evidence of haemophagocytosis in all 3 cases, but only 2 of the marrow trephines confirmed the diagnosis of large B cell lymphoma and peripheral T-cell lymphoma (unspecified). The third case of anaplastic large cell lymphoma was diagnosed by biopsy of a FDG-avid supraclavicular node. *Discussion*. Patients with LHPS often present with fever of unknown origin, associated with cytopenia and liver dysfunction. Lymphadenopathy may be absent or minimal. Histological diagnosis of the lymphoma is often delayed due to its atypical presentation. Bone marrow biopsy is required to confirm the presence of haemophagocytosis

and to detect any lymphomatous infiltration. CT scan may not be sufficient for appreciation of the full extent of the disease. Our 3 cases illustrate the usefulness of PET scan which shows distinctive features of extensive lymphomatous involvement in LHPS, which may otherwise be missed on conventional CT scan.

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THE EVALUATION OF EFFICACY AND RELIABILITY OF BIOMED-2 CLONALITY ASSAY IN DIFFERENTIAL DIAGNOSIS OF LYMPHOPROLIFERATIVE DISEASES IN 37 CASES: SINGLE CENTER EXPERIENCE

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Background. The differential diagnosis of lymphoproliferative disorders has been based on immuno-histochemical findings in specimens. Recent studies have indicated requirement of further analysis, especially for unique cases. Molecular techniques such as clonality assay have been implemented into the diagnosis and follow-up of lymphoid malignancies. AIMS:The aim of this study was retrospective evaluation of 37 cases that were diagnosed as lymphoproliferative disease and assessed for T-and/or B-cell clonality using BIOMED-2. *Design and Methods*. 37 cases were evaluated by Pathology and Medical Biology Departments of our institution between October 2007 and January 2009. The surgically excised samples were sent directly to the Pathology unit and after primary evaluation; samples of cases which need to have molecular confirmation for diagnosis were transferred to Medical Biology unit for clonality assay by using BIOMED-2 primers for either B- or T-cell clonality. Clonality assay was performed according to kit manual and instructions provided by kit. Of 37 patients, 14 were female and 23 were male. The median age was 34.44 years. The source of samples were as follows; 2 blood aspirations, 3 bone marrow aspirations, 1 cerebrospinal fluid, 4 fresh tissue samples (3 lymph nodes, 1 gastroendoscopic biopsy) and 27 paraffin-embedded tissue blocks (13 lymph nodes, 4 bone marrows, 3 gastroendoscopic biopsy, 1 skin, 1 bladder wall, 1 parotis, 1 tonsil, 1 retro-orbital mass, 1 brain biopsy). 20 samples were evaluated for T-cell clonality, 15 for B-cell clonality and 2 for both T-cell and B-cell clonality. *Results* Results are provided in Table 1.

Table 1. The results and the diagnosis of the cases.

Clonality assay	Pre-diagnosis	Confirmation
B-cell	Marginal zone lymphoma	5/7
	Diffuse large B-cell lymphoma	2/2
	Lymphomatoid granulomatosis	2/2
	Follicular lymphoma	1/1
	Nodular lymphocyte predominant Hodgkin lymphoma	0/1
	B-cell acute lymphoblastic leukemia lymphoma	1/1
	B-cell leukemia lymphoma	1/1
T-cell	Peripheral T-cell lymphoma (PTCL)	4/9
	NK/T-cell lymphoma	0/1
	T-cell acute lymphoblastic leukemia lymphoma	1/1
	T-cell chronic lymphocytic leukemia	1/1
	Lymph node infiltration of Mycosis Fungoides	1/1
	Hodgkin lymphoma	1/1
	Dermatopathic lymphadenopathy	1/1
	Reactive lymphoid infiltrate	1/1
	Kikuchi lymphadenopathy	2/2
	Infiltration of CSF by PTCL	0/1
	Hypereosinophilic syndrome ± PTCL	0/1
B- and T-cell	PTCL	0/1
	T-cell rich B-cell lymphoma	1/1

The diagnosis was changed after clonality assessment in 20% of cases by B-cell analysis, in 35% of cases by T-cell analysis, overall 30% of all cases studied. *Conclusions*. Our results have shown that clonality assay by BIOMED-2 multiplex PCR tubes provide a powerful strategy for the differential diagnosis of T- and B-cell malignancies and is essential for the diagnosis of certain cases.

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CLINICAL PROFILE OF PATIENTS WITH PRIMARY GASTRIC LYMPHOMA, AND EVALUATION THE R-CHOP REGIMEN. A RETROSPECTIVE STUDY

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Background. In recent years, it has been reported that addition of rituximab of chemotherapy has improved outcomes in several types of non Hodgkin lymphoma (NHL) without significant addition of toxicity. There are many controversies regarding the treatment for primary gastric lymphoma (PGL). The aim was to analyze the clinico-pathologic characteristics of patients with PGL and evaluate the treatment of this disease. *Design and Methods*. This retrospective study enrolled 15 patients with PGL, diagnosed between January 2005 and September 2008. All patients clinical, biochemical, radiological and endoscopic features were evaluated. The histological diagnosis was established by endoscopic biopsies. All patients were treated with chemotherapy with or without surgery, but none of them have received radiotherapy. Results. The mean age was 42 years (range 19 - 68 years), with male predominance. The most common symptom was abdominal pain in 50%, followed by abdominal mass and weakness. Duration of symptoms varied from 15 days to six months. Histological subtypes were Diffuse Large B Cell (DLBC) in 46%, marginal zone B cell NHL of Mucosa Associated Lymphoid Tissue (MALT) in 46% and B small cell in 8%. *Helicobacter Pylori* infection was seen in the majority of MALT type NHL. The surgery was performed to five patients with stag IE PGL. All patients received chemotherapy whose the gold standard was CHOP which is associated to Rituximab. No major toxicities related, response was evaluated by repeat endoscopy after completion of 4 and 6 or 8 cycles. Patients with MALT as histopathology received HP treatment. Fourteen patiets are alive with a median foolow up 30 months (range 04- 54 mo). *Conclusions*. These preliminary data indicate that the R-CHOP regimen was well tolerated and may be effective producing complete remission.

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TREATMENT OF PERIPHERAL T-CELL LYMPHOMA UNSPECIFIED SINGLE CENTRE EXPIRIENCE

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Background. The optimal treatment strategy for patients with Peripheral T-cell Lymphoma-Unspecified (PTCL-U) remains controversial. *Design and Methods*. Herein we have retrospectively analyzed 24 cases fulfilling the criteria defined by the WHO classification. All patients were diagnosed and treated at Clinic of Hematology CC Nis, from January 1991 until December 2008, with median follow up of 15 months (range 3-96). Majority of patients 20 (83,3%) received yclophosphamide/doxorubicine/vincristine/prednisone therapy (CHOP). Alemtuzumab combined with CHOP was administered in one case 1 (4, 16%) and high dose therapy regimen "LALA 94" used in acute lymphoblastic leukemia was given in another case (4, 16%). Dose intensified doxorubicin, cyclophosphamide, vindesine, bleomycine and prednisone (ACVBP) plus intensified consolidation was given to 3 patients (12,5%). *Results*. During the first 24 months of follow up, cumulative probability survival was 54, 3%. Factors significantly associated with reduced survival in multivariate analysis were elevated LDH (p=0,027) and complete response vs. no response vs. partial response to therapy (p=0, 0016). There was no difference in remission induction between CHOP and ACVBP groups (2=0.34). Prognostic model PIT was successful in identifying risk group patients (p= 0,030), while simplified two-class PIT seemed to be superior over simplified two class IPI. (p=0, 003179 versus p=0, 017) There was no difference in overall survival among patients treated with CHOP alone and those receiving more aggressive regimens (p= 0,651). Possible explanation might be in high treatment associated mortality given the fact that more aggressive approach was chosen for patients with unfavorable prognosis. *Conclusions*. Aggressive chemotherapy still remains controversial in high risk PTCL-U.

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HEPATOSPLENIC γδ T-CELL LYMPHOMA IN A HIV-INFECTED PATIENT: REPORT OF AN EXCEPTIONAL ASSOCIATION WITH EMPHASIS ON CYTOMORPHOLOGICAL FEATURES

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Background. Non-Hodgkin lymphoma (NHL) is the most common AIDS-defining malignancy and most cases are high-grade, aggressive subtypes. Lymphomas occurring more frequently are Burkitt-type, diffuse large B-cell, primary effusion, and plasmablastic lymphomas. T-cell lymphoma comprises only 3% of all AIDS lymphoma, and we have not specifically found, in the revised bibliography, the appearance of hepatosplenic T-cell lymphoma in AIDS patients. *Aims*. We describe a case of hepatosplenic γδ T-cell lymphoma in a HIV-infected patient, an extremely exceptional association. *Design and Methods.* After 13 years from diagnosis of HIV infection, a 37-year-old patient with an irregular compliance to antiretroviral therapy was studied because of fever, dyspnea, abdominal pain, marked hepatosplenomegaly, and a blood count showing WBC $0.5x10^9$/L, haemoglobin 70 g/L, and platelet $78x10^9$/L. At this moment, CD4+ T-cell were $41/mm^3$ and plasma viral load 497 copies/mL. Bone marrow biopsy showed a medium-sized, CD3+ lymphoid cell infiltration with a predominantly intrasinusoidal pattern. With the suspect of hepatoesplenic T-cell lymphoma, splenectomy was performed and pathological analysis showed distended and infiltrated sinusoids by a monotonous population of atypical, CD3+ neoplastic lymphoid cells. A diagnosis of hepatosplenic T-cell lymphoma, stage IVB, was established. Early at postsurgery period, leukocytosis (WBC $24,7x10^9$/L) with a significant lymphocytosis ($19.8x10^9$/L) was detected in blood count, with atypical lymphoid cells at light microscopy (small to medium-sized with non-granular basophilic cytoplasm, irregular nuclei, and a visible, mostly sole, nucleolus). Phenotypic analysis showed T-cells 95,2% and B-cells 3,3%, and two types of T-cells were detected: a majority malignant subset (75%) positive for CD3, CD2, CD7, CD56, CD16, and TCRγδ (negative for CD4, CD8, CD5, CD1a, CD57, CD25, CD11c, and TCRγδ), and a minority subpopulation (18,3%) of normal T-cells (CD3+CD4+ 4,3% and CD3+CD8+ 14,2%). An initial good response to chemotherapy (CHOP) was obtained but, as expected, the course was aggressive and relapse occurred five months later, again as leukemic-phase disease. The patient died after six months from diagnosis. Figures showing morphology and immunophenotypical analysis of peripheral disease expression, and bone marrow and splenic pathological features, are presented. *Conclusions*. The WHO classification of tumours of lymphoid tissues does not mention the blood as site of involvement of hepatosplenic T-cell lymphoma, not even as unusual. In addition, hepatosplenic T-cell lymphoma has not been reported among the rare cases of peripheral T-cell lymphoma occurring in HIV-infected patients. As cited by other authors, we must be awarded that in the antiretroviral therapy era, non-AIDS-defining malignancies account for more morbidity and mortality than AIDS-defining malignancies. For the best of our knowledge, this is the first reported case of a hepatosplenic T-cell lymphoma in a HIV-infected patient.

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PEGFILGRASTIM TO SUPPORT R-CHOP Q14 CHEMOTHERAPY IN THE PATIENTS (PTS) WITH AGGRESSIVE B-CELL NON-HODGKIN'S LYMPHOMA (NHL)

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Background. CHOP chemotherapy administered every 21 days has been the standard regimen for treatment of aggressive NHL for many years. Recent studies have shown improvements in both complete remission and survival following addition of Rituximab to CHOP 21 (*Coffier et al., NEJM 2002*) and following reduction of the cycle length of standard 21 day CHOP to 14 days (*Pfreundschuh et al., Blood 2004*). Previous studies with CHOP 14 have demonstrated the need of filgrastim to allow the administration of chemotherapy at planned dose and on time in this setting. *Design and Methods*. This study evaluates the feasibility of the combination of CHOP plus Rituximab (375 mg/mq) repeated every 2 weeks (CHOP-R 14) supported with a single administration pegfilgrastim (6 mg, on day 2) up to six cycles in pts with aggressive B-cell NHL. *Results*. Up today 21 pts have been enrolled. Patients' characteristics are: median age 65.5 years (46-73), sex 9M/12F, ECOG PS 0(16), 1(4), 2(1), B symptoms 39%, stage I(1), II(7), III(6), IV(7), extranodal location 5 (24%). Overall 95% of pts received full dose chemotherapy on schedule for all planned cycles (range 4-7). Chemotherapy was delayed and dose reduced in 10% and 5% of pts respectively. Main grade 3-4 adverse events per pts were: neutropenia 29%, febrile neutropenia 10%, thrombocytopenia 10%, anemia 24%. One pts died because of treatment related sepsis. Responses at the end of treatment (21 evaluable pts) were: complete 62%, partial 33%. Up today the median follow up is 12,6 months, median PFS is 25,6 months and OS have not been reached. *Conclusions*. These results indicate that the delivery on schedule of dose-dense CHOP-R 14 to pts with previously untreated aggressive B-cell NHL is safe and active with once per cycle pegfilgrastim support.

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PROGNOSTIC PROFILE IN THE PATIENTS WITH BALT (BRONCHUS- ASSOCIATED LYMPHOID TISSUE) LYMPHOMA

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Background. Bronchus-associated lymphoid tissue (BALT) lymphoma is a rare subtype of low grade B cell marginal zone non Hodgkin's lymphoma. BALT represents 10% of all MALT lymphoma, 3-4% of all extranodal lymphomas and 0,5-1% of all malignans of the lung. The purpose of this study was to analyzed the prognostic profile in the patients with BALT lymphoma. *Design and Methods*. This study included seven patients who had BALT lymphoma diagnosed between January 2001 and April 2008 at the Institute of hematology CCS, Belgrade. Demographic characteristics were as follow: male/female ratio was 2:5 (28,57%:71,43%); the median age was 64 years (range, 37-73). On presentation, five patients (71,43%) had nonspecific respiratory symptoms and all of them had B symptoms. Patients were seronegative for human imunodeficiency viruses (HIV and hepatotropic viruses HCV and HBsAg). Three patients had Sjogren's syndrome, reumatoid arthritis and pulmonary tuberculosis, respectively. The diagnosis was based on open lung at one and transbronchial biopsy at 6 pts. Patohistological findings suggested lymphoma of marginal zone B cell lymphoma, and immunohistological profile confirmed the diagnosis: CD20+/CD10'/CD5'/CyclinD1'/ CD23'/IgM' with Ki-67< 20%. According to the Ferraro staging system, four patients (57,14%) had localized disease (stage I E-II E) and three had stage III E. Bulky tumor mass had two patients. Four patients (57,14%) had ECOG performance status (PS) 0 and three patients had a PS 1. Six patients (71,43%) received chemotherapy consisting of chlorambucil alone (doses was 10 mg p.o. during ten days monthly up to 6 cycles). One patient underwent surgical resection, followed with chlorambucil. Results: A complete response with initial therapy was achieved in two patients (28,57%) and a partial response was obtained in five patients. All the patients were alive during the median follow-up period of 39 months (range 6-82 months). During the follow up period two patients relapsed into the other extranodal localization, but achived stable PR after CHOP regimen and they are regularlly followed up. *Conclusions*. BALT lymphoma tends to be localised disease at the time of diagnosis, responds well to surgical treatment or mono chemotherapy with chlorambucil and has a favourable prognosis.

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PRIMARY T-CELL ANAPLASTIC LYMPHOMA ASSOCIATED TO A BREAST IMPLANT: CASE REPORT

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Background. Breast Non-Hodgkin lymphomas are rare, with approximately 0.04-0.5% of all malignant breast tumors, and the vast majority are B-cell lymphomas. In contrast, T-cell phenotype has been rarely reported and some of these lymphomas are associated to breast implants. The neoplastic cells with T-cell phenotype are $CD30^+$, express cytotoxic granule-associated proteins, EMA and clusterin, and are anaplastic lymphoma kinase-1-negative. *Design and Methods*. We present a case of a woman who recently changed silicone breast prostheses, with a fast growing mass along one implant. *Results*. A 56-year-old white female patient had a recent history of plastic surgery to change old breast

implants, with a normal mammogram one month before the procedure. Nearly two months after the surgery, she noticed a firm nodule in right breast, located at superior limit of the implant. The MRI described a lobulated nodule with 36 x 33 x 24 mm, with early peripheral enhancement, classified as BIRADS V. A right mastectomy with sentinel lymph-node mapping was carried out. The cryosection suggested sarcoma histology. Further pathological analysis (H&E stain) showed undifferentiated carcinoma, with negative axilar lymph node, and the immunohistochemical staining was performed. The neoplastic cells express CD30, CD45, granzyme B, clusterin, CD43 (weak) and TIA-1. They do not express ALK, CD3, CD2, CD5, CD7, CD20, CD15, ER/PR, AE1/AE3, OSCAR, p63, keratin 903, S-100 or EMA. The morpoholgy and immunoperoxidase stains support a diagnosis of anaplastic large cell lymphoma, T-cell phenotype, ALK negative. A postoperative chest and abdominal scan was performed and showed no other sites of disease. She had no laboratory abnormalities and her HIV and HTLV testing were negative. *Conclusions*. This case is unique for its unusual presentation; similar cases have been reported in the literature. Although an increased risk of breast lymphomas in patients with silicone prostheses has been speculated, no studies have been conducted so far.

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PEDIATRIC EXTRANODAL NK/T-CELL LYMPHOMA, NASAL TYPE

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Background. Extranodal, nasal-type NK/T-cell lymphoma (ENK/TL) cells express a cytotoxic T-cell or NK-cell phenotype, including CD56 and TIA-1. Nasal-type NK/T-cell lymphomas arise from extranasal sites such as the skin and are often associated with Epstein-Barr virus (EBV) infection. Asian adults are affected most commonly. Very few pediatric cases are reported in the literature, of which only 5 presented with cutaneous involvement. In the current study, we report a case of primary orbital NK/T-cell lymphoma, nasal type with successful chemotherapy treatment. *Aims.* Estimate clinical, morphological and immunological features of pediatric ENK/TL. *Results*. A 13-year-old European boy presented with a 1-month history of right-side exophthalmos in September 2002. His physical examination was significant for right ophtalmoptosis, ophthalmoxerosis, decrement in visual acuity. A computed tomography (CT) scan of the head revealed tumor mass with homogenous structure, sharp contours in a cavity of right orbita (65x23x35mm). Tumor dislocated bulb of right eye, pushed aside optic nerve and bulbar muscles. The tumor was gallium-avid. A CT scan of the left orbita, chest and abdomen revealed no tumor mass. The total serum lactate dehydrogenase activity was normal as a standard peripheral blood test and cerebral fluid. A bone marrow aspiration showed normal cellularity and no tumor involvement was detected by light microscopy. A biopsy of the right orbital tumor mass demonstrated a circumscribed mass of intermediate-sized lymphoid cells, located near the vessels. Tumor cells by immunohistochemical staining for CD57, CD45 and TIA-1 were positive, for CD2, CD3, CD5, CD7, CD10, CD19, neuroblastoma antigens were negative. The stain for EBV latent membrane protein was strongly positive. Flow cytometric analysis demonstrated intense expression of CD56 and weak expression of CD45. The tumor cells were negative for CD4, TCRγ/δ, TCRγ/δ. The patient received treatment on the non-B-NHL-BFM-90 Programme. After 1 phase of I protocol, CT scan revealed a significant decrease in tumor size: 33x10x20mm. ophtalmoptosis decreased, sense of vision improved. After an initial partial response the treatment was continued on HR1-ALL-BFM90M Protocol. After the first course the tumor size was 14x13x70mm., eyesight recovered, ophtalmoptosis disappeared. After HR2 and HR3 courses - 8x4x6mm; therefore the patient was treated with fractioned involved field radiation (36 Gy total). One month after radiation therapy the tumor size was 7x3x6mm, which was estimated as a fibrosis. The last CT scan was on June 2008, the size of fibrosis mass was 7x4x6mm. So at the present moment our patient is a 19-year-old boy, who is alive in a first common remission of extranodal NK-cell lymphoma, nasal type.

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IS OBESITY A RISK FACTOR FOR NONHODGKIN MALIGNANT LYMPHOMAS?

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Background. Sedentary life and hypercaloric diet is favoring the weight gain and obesity. In present, there are discussions regarding the pathways among which obesity is involved in oncogenesis. *Aims*. Our aim was to study if there is any correlation between the body mass index (BMI) and non-Hodgkin malignant lymphomas. *Design and Methods*. We have studied a group formed by all the 56 patients with non-Hodgkin malignant lymphomas who were in the evidence of the Hematology Department from the County Clinical Hospital from Sibiu in 2008 (group A) and a group of 64 consecutive patients with non-malignant diseases, who were hospitalized in the Second Medical Department of the same hospital in the first half of February 2008 (group B). At the two studied groups, we have analyzed: age, height, weight, BMI, blood pressure or the presence of arterial hypertension diagnosis, glycemia or the diabetes mellitus diagnosis, triglycerides, cholesterol levels or the diagnosis of dyslipidemia. We have compared the results of the two studied groups and we have statistically analyzed them using the t Student test. *Results*. The average age of the patients from group A was 64.82±11.04 years, which did not differ significantly from the one in group B. The BMI was significantly higher in group A (26.29±3.5 kg/m^2), comparing with group B (23.82±2.75 kg/m^2) (p=0.032). Arterial hypertension was significantly more frequent in group A comparing with group B (p=0.041). The number of components of the metabolic syndrome was significantly higher in group A comparing with group B (p<0.0001). There were no significant differences between the two groups regarding the levels of glicaemia, cholesterol or triglycerides. *Summary*. In our group of patients with non-Hodgkin malignant lymphomas, BMI, arterial hypertension and the number of components of the metabolic syndrome were significantly higher comparing with the ones of the patients with non-malignant diseases, fact which sustains the idea that overweight and obesity could constitute a risk factor for non-Hodgkin malignant lymphomas. It will be useful to investigate, by multicentric studies, on a higher number of patients, this association, and the possible role of weight loss on the risk and evolution of malignant hemopathies.

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EFFICACY AND TOLERABILITY OF LIPOSOMAL DOXORUBICINE IN CARDIOPATHIC PATIENT WITH NON HODGKIN LYMPHOMA

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Background. R-CHOP protocol is the gold standard treatment for Non Hodgkin's Lymphoma and doxorubicine is the drug with the higher antitumoural activity. However, disease (cardiotoxicity, drug-resistence, etc.) and patient characteristics (advanced age, comorbidity) limit the use of that protocol. In frail patients the use of liposomal anthracycline instead of classic one may reduce cardiotoxicity even though manteining the same antitumoral efficacy (i.e. R-COMP regimen). *Aims*. We investigated the use of liposomal doxorubicin in patients affected by NHL with associated cardiopathy. *Design and Methods*. From 2003 to 2008, in our Haematology Unit ASL NA-1 we treated 24 patients affected by NHL. All patients were affected by heart diseases (heart failure, atrial fibrillation, etc.). All the patients underwent echocardiographic evalutation of left ventricular ejection fraction (LVEF%) before starting treatment, during treatment and 3, 6, 12, 18, 24 months after the end of therapy. Patients' characteristics were: 16 male and 8 female; median age 69 years (range: 66-80); istology: 14 Diffuse Large B Cell Lymphoma (DLBCL), 4 cutaneous NHL; 3 Mantle Cell Lymphoma (MCL) and 4 Follicular (G3) Lymphoma. At diagnosis LVEF was less than 50% (35-45%) in all patients. *Results*. One month after the last cycle all patients were subjected to a disease re-staging: 85% of patients were in CR, 12% in PR, 3% in progression disease or resistance. Nobody had a reduction of LVEF; interestingly, 2 out of 24 patients showed an improvement of LVEF. Overall survival (+24 months) was 85%. *Conclusions*. We confirm the efficacy of R-COMP regimen in the treatment of NHL. Moreover, the use of liposomal anthracycline allows the treatment of cardiopathic patients with aggressive lymphoma. Further studies are warranted to confirm the safety and effectiveness of polichemotherapy with liposomal anthracycline in NHL frail patients.

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AUTOIMMUNE HEMOLYTIC ANEMIA AS A RISK FACTOR OF POOR OUTCOME IN PATIENTS WITH SPLENIC MARGINAL ZONE LYMPHOMA

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Background. Splenic marginal zone lymphoma is a rare disease, accounting for 1% of all lymphomas. The main disease features are splenomegaly, lymphocytosis and cytopenias often related to hypersplenism. Autoimmune phenomena are present in 9 to 20% of patients. SMZL generally has an indolent clinical course with a median survival of 10 years, and a 5-year survival rate of 70%. *Design and Methods*. We reviewed our single center experience of 13 patients with SMZL, where long term (at least three years) follow-up data are available. Results: Based on the prognostic model developed by Integruppo Italiano Linfomi 31% (4/13) of our patients had good, 38% (5/13) had intermediate and 31% (4/13) had a poor prognosis. The presence of two out of three prognostic factors (anemia, elevated LDH, low serum albumin) assignes the patient into the high risk category. In patients with anemia and an elevated LDH due to hemolysis, the outcome seems to be especially poor. Three out of 13 (23%) cases were complicated by autoimmune hemolytic anemia. All patients with AIHA died 7-28 months after the diagnosis. The mean follow-up time of those nine patients (69%) who are still alive is longer than 5 years (42-106 months). SMZL patients with AIHA had significantly (p<0.001) worse survival than those without AIHA. *Conclusions*. The main finding of our study is that the presence of AIHA is an adverse prognostic factor in SMZL.

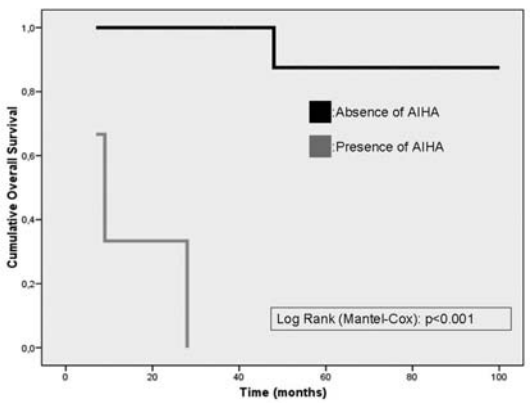

Figure 1. Presence of AIHA and survival in our patients.

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CORRELATION OF PRETREATMENT VALUES OF S - VEGF AND PLATELET LEVELS IN DIFFUSE LARGE B CELL LYMPHOMA PATIENTS

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Background. Level of S - VEGF above median value is an early poor prognostic indicator for CRR and OS in diffuse large B cell lymphoma patients (*Pedersen et. al. 2005*). Platelets are the main source of VEGF in cancer patients with *dynamic equilibrium* of VEGF amount in platelets and circulation (*Gunsilius et al. 2000*). Platelet level above normal is accompanied with high pretreatment values of S - VEGF but without any influence on lymphoma patients survival (*Salven et. al., 2000*). *Aims*. To define possible correlation between pretreatment values of S - VEGF with platelet levels and relations to response rate and overall survival. *Design and Methods*. Serum samples of 50 DLBCL patients were collected at out institution between February 2006. and July 2007. and tested by ELISA S - VEGF Amersham Biotrak. Patients were under surveillance for a study since August 2008.g. Two S - VEGF categories were established: under or above median m S - VEGF - cat 1. and cat 2., with correspondent values of platelets below and above normal level (450 000). Groups were tested for complete response rate (CRR) and median overall survival (OS). *Results* - Patients were divided according to VEGF categories and categories with normal and elevated platelets (Tables 1 and 2).

Table 1. VEGF category range number pts.

cat 1. < 111,68 pg/ml	15,0 - 109,8 pg/mL	25
cat 2. > 111,68 pg/mL	111,3 - 1009,5 pg/mL	25

Table 2. Platelet category range number pts.

platelet level < 450 000	105 000 - 435 000	35
platelet level > 450 000	453 000 - 726 000	11

***NA = 4.**

Relations between s - VEGF categories and platelets levels below and above normal were:

Table 3. Category platelet level < 450 000 platelet level > 450 000.

cat 1. < 111,68 pg/ml	21 (84%)	2 (8%) *
cat 2. > 111,68 pg/ml	14 (70%)	9 (36%) **

*** NA = 2 (8%) ** NA = 2 ((%), (Fisher Exact test: p = 0,035)**

Two separate categories of patients were formed by s - VEGF and platelets levels. Those categories were compared on complete response (CR) vs. no complete response (no CR) (Table 4)

Table 4. Category CR no CR.

cat 1. <111,68 pg/mL and platelet level < 450 000	17 (80,95%)	4 (19,05%)
cat 2. > 111,68 pg/mL and platelet level >450 000	5 (35,71%)	9 (64,29%)

(Fisher Exact test: p = 0,019)

and by median overall survival (mOS) (Table 5).

Table 5. Category m OS 95%CI number pts.

cat 1. <111,68 pg/mL and platelet level < 450 000	inf * inf-inf	21
cat 2. >111,68 pg/mL and platelet level > 450 000	13 8-inf	9

(inf * - not reached)

(Log-Rank test: X -2 = 4,7; df = 1; p = 0,031)

Conclusions. Patients with low serum level of VEGF and normal number of platelets and high serum level of VEGF and elevated number of platelets are two distinct categories of diffuse large B cell lymphoma patients. There is statistically better complete response rate (CRR) and longer median overall survival (m OS) in category of patients with low serum VEGF level and normal number of platelets.

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EVIDENCE OF GVL EFFECT IN NK/T CELL/NASAL TYPE LYMPHOMA: A CASE REPORT

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Mature T/natural killer lymphoma/leukemias (T/NK L) represent 5-15% of all non-Hodgkin lymphoma. Prognosis is poor, with 5-year survival less than 30% (except for anaplastic lymphoma) and there is no consensus about therapy. Autologous stem cell transplantation (ASCT) might be an option in these patients. Conventional allogeneic stem cell transplant might be considered in some high-risk patients. We report a 60 years old man who came to our observation on February 2007 complaining of fever, nasal obstruction and multiple subcutaneous nodular lesions; head CT scan revealed a large rynopharingeal mass, involving ethmoidal and maxillar sinus; biopsy of the mass showed findings of extranodal T cell/NK nasal type lymphoma. Total body CT scan and bone marrow biopsy were normal; EBV was negative. Patient received 3 courses of CHOP, with Campath 1H at day 1 (C-CHOP), with a partial response. Second line therapy was Hyper-Cydam was stopped because of a second lymphoma progression; patie nt underwent allogeneic transplant from compatible, conditioning regimen was Thiotepa, Fludarabine and Cytoxan; GVHD prophylaxis was made with Cyclosporine and Methotrexate; patient received Peripheral Blood Stem Cells. During the aplastic phase there was CMV reactivation; engraftment was rapid and complete. No acute GVHD was present when the patient was discharged. One month after transplant all cutaneous lesions had disappeared, rinopharingeal mass persisted, albeit slightly reduced; chimerism showed a full donor pattern. Cyclosporine was quickly reduced and patient developed a grade III acute GVHD, (skin rash and diarrhea) requiring steroids. A CTscan performed 8 weeks, after GVHD onset, showed a consistent reduction of rynopharingeal mass. We observed several flares of intestinal GVHD, requiring steroids; the last CT scan, 7months after transplant, documented complete disappearance of rynopharingeal mass, without other signs of Lymphoma. Up to now the patient is alive and i n Continuous CR, with sporadic intestinal GVH flares, responding to intermittent steroid administration..Our experience shows that even in a patient with extranodal disease, refractory to 3 lines of chemotherapy, RIC transplantation achieved a dramatic response and the rapid withdrawal of immunosuppression after transplant was followed by complete disappearance of lymphoma, contemporaneous with the acute GVH onset, strongly arguing for a potent GVL effect also in this particular setting of patients.

1730

EFFICACY AND SAFETY OF INTRATHECAL LIPOSOMAL CYTARABINE IN COMBINATION WITH METHOTREXATE AND STEROIDS FOR THE TREATMENT AND PROPHYLAXIS OF LEUKEMIC AND LYMPHOMATOUS MENINGITIS: A SINGLE INSTITUTION EXPERIENCE.

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Background. Intrathecal (IT) chemotherapy is an important component of the prophylaxis or treatment of hematologic malignancies in the central nervous system (CNS), especially in patients with acute lymphoblastic leukemia and aggressive lymphomas. The three commonest IT drugs are methotrexate, cytosine arabinoside (Ara-C), and corticosteroids. Liposomal cytarabine (Depocyte) is a sustained-release formulation of cytarabine developed for intrathecal administration, ensuring prolonged cytotoxic drug concentrations of cytarabine in cerebrospinal fluid. Liposomal cytarabine permits to decrease frequency of lumbar punctures, without loss of efficacy, because intrathecal levels of the drug remain cytotoxic for up to 14 days. *Aims*. With the aim to evaluate the safety and efficacy of treatment and prophylaxis schedule with methotrexate, liposomal cytarabine and methyl-prednisolone. *Design and Methods*. From October 2007, 6 patients affected by aggressive lymphomas (HG NHL) (5 cases) and acute lymphoblastic leukemia (ALL) (1 case) underwent CNS prophylaxis with methotrexate 12 mg, liposomal cytarabine 50 mg and methyl-prednisolone 40 mg IT every 15 days in our Institution. Moreover 2 patients (HG NHL 1 pt and ALL 1 pt) have been treated with same schedule for neoplastic meningitis. The clinical and hematological features of the patients were: 6 males, 2 females; median age 60 yrs (range: 32-73). *Results*. After a median follow-up of 9 a total of 80 lumbar punctures have been administered. In any case a III-IV WHO grade toxicity has been observed. All 6 patients undergone prophilaxis did not show the occurrence of neoplastic meningitis. The remaining 2 patients treated for neoplastic meningitis showed a complete clearance of blasts after a median of 3 lumbar punctures and are still in remission. *Conclusions*. In conclusion, although the relatively low number of cases, simultaneous IT administration of methotrexate, Liposomal cytarabine and corticosteroids was well tolerated and appeared to be particularly effective in the prophylaxis for aggressive lymphomas patients and acute lymphoblastic leukemia patients, who achieved long term remission. Certainly, confirmation in a larger cohort of patients is now warranted

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PRIMARY B-CELL CUTANEOUS LYMPHOMA - A SINGLE INSTITUTION EXPERIENCE

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Background. Primary cutaneous B cell lymphomas (PCBCL) represent a rare form of non-Hodgkin's lymphoma (NHL). The new WHO-EORTC classification for cutaneous lymphomas distinguishes 3 main types of PCBCL: primary cutaneous marginal zone B-cell lymphoma (PCMZL), primary cutaneous follicle center lymphoma (PCFCL), and primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL,LT). PCBCL have good overall prognosis with a high response rate to therapy but with almost half of cases relapsing al least once. PCDLBCL,LT has poorer prognosis than the other two types of PCBCL. There is no standard treatment but several studies have shown long term survival with surgery, radiation, chemotherapy and/or immunotherapy. Most institutions use radiation therapy as the main modality after excisional biopsy. *Aims*. To analyze the clinical features and therapy outcome in patients from our Hematology Department. *Design and Methods*. we diagnozed 5 cases during the last 5 years (3 males, 2 female) with ages ranging from 24 - 79 (median 61 years); 1 case was PCMZL, 3 cases with PCFCL and 1 case of PCDLBCL with multifocal, extended cutaneous and subcutaneous involvement. Initial evaluation was made by physical examination, blood counts, biochemistry, chest radiograph, computed tomographic scan of chest and abdomen, bone marrow biopsy. Neither of the cases had bone marrow involvement; in the case of diffuse B cell cutaneous lymphoma, we noted moderate splenomegaly preceding the NHL in the context of chronic C virus hepatitis. The follow-up ranged from 4 to 62 month (median 8 month). Results: The initial therapy consisted in excision and local radiotherapy (20Gy) in 1 case of marginal zone lymphoma (PCMZL) and 1 case of follicular lymphoma (PCFCL) with single lesion, limited disease. In 1 case of limited PCFCL, only excision was performed. All cases are in complete remision after a median follow-up of 6 month. The third case of follicular cutaneous lymphoma was diagnosed 2 years after the onset of the first lesion. At diagnosis, he had several lesions limited to the left supraclavicular area so that the initial treatment consisted in 4 ×CHOP chemotherapy followed by complete remision. He relapsed after 1,5 years at the same site but also with regional lymphadenopathy and received 6 R-CHOP courses with the achievement of a second remision (still lasting after 18 month). The case with diffuse lymphoma, aged 79, presented at diagnosis with extended cutaneous involvement and subcutaneous extension of the lower anterior thoracic region and of the upper anterior abdomen. After 4 CEOP courses we observed the remision of cutaneous lesions and of subcutaneous infiltrates. *Summary*. PCBCL are rare lymphomas. Generally, at onset the disease is limited with single lesions; excision +/- local radiotherapy is followed by remission. In the case of extended, multifocal disease we reported relapse in the first 2 years involving also the regional lymphnodes with good response to immuno-chemotherapy. According to the WHO-EORTC classification which divides PCBCL into two main groups, with PCDLBCL,LT having poor prognosis, we treated our case with systemic chemotherapy despite the advanced age - with favorable outcome.

1732

AN UNUSUAL CASE OF PRIMARY RENAL LYMPHOMA DISSEMINATING TO BONES

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Background. Diffuse large B cell lymphoma (DLBCL) represents approximately 30% of all non-Hodgkin's lymphoma (NHL) and is characterized by a relatively frequent extranodal involvement. A simultaneous multifocal extranodal involvement is quite rare. Most of these cases arise in the gastrointestinal tract (stomach and/or bowel), with or without other extranodal disease localizations such as lung or bone. A renal involvement can be demonstrated during widespread diffusion of advanced-stage NHL. Among all cases of NHL with a renal involvement at diagnosis, only a minority has a renal localized origin without distant lymph nodes and meets the criteria for primary renal lymphoma. This is a very rare entity, often with an aggressive clinical course and a rapid subsequent dissemination. *Results*. We described here an unusual case of extranodal DLBCL diagnosed as a renal mass associated with multiple osteolytic lesions. A 50-year-old man presented with a 3-month history of left flank pain and an episode of gross hematuria. Computed tomography (CT) of the abdomen revealed a large tumor mass of the left kidney, without locoregional or distant lymph node involvement. A radical nephrectomy was performed under a preliminary diagnosis of renal cell carcinoma, but histological examination of the kidney revealed a diffuse large B cell NHL. Clinical staging was then completed by a CT scan of thorax and neck and bone marrow biopsy, with negative results. Because of the occurrence of right knee pain, a magnetic resonance imaging (MRI) was also performed, showing a large osteolytic lesion of femoral epiphysis. A US-guided fine needle aspiration biopsy showed a NHL infiltration. A subsequent total body fluorodeoxyglucose-positron emission tomography (FDG-PET) confirmed multiple bone lesions of right femur, ischium, and jaw. Considering the high risk presentation of this case (advanced stage with multiple extranodal involvement and abnormal LDH), we choose an intensified immunochemotherapy with the R-CHOP 14 scheme. The patient received also bisphosphonate infusions, with great clinical benefit. A complete restaging after the fourth cycle showed a good partial response (FDG-PET was slightly positive only at right knee). The patient underwent other four cycle of R-CHOP 14, achieving a final complete haematological remission. *Conclusions*. This is a case of aggressive B cell NHL probably arising in the kidney (principal mass detected at diagnosis) and rapidly spreading to bones. Also in this poor-risk presentation, an intensified immunochemotherapy allowed a complete clinical remission.

1733

CENTRAL NERVOUS SYSTEM MANIFESTATION OF PRIMARY EFFUSION LYMPHOMA IN A HIV-NEGATIVE PATIENT

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Background. Primary effusion lymphoma (PEL) is a rare neoplasm of large B cells that usually presents as a serous effusion without detectable tumor mass. It is associated with Human Herpes Virus (HHV)-8 infection and occurs virtually exclusively in the setting of immunodeficiency. PEL accounts for 4% of all HIV-associated Non-Hodgkin lymphomas and for less than 1% of all NHL in HIV negative patients. Establishing the diagnosis of PEL requires HHV 8 presence. *Case report*. A 67 year-old Caucasian woman presented with a two months history of dyspnoea, fever and cough with bloody expectoration. CT scans revealed a pulmonary mass, massive pleural effusion, and a pararenal mass on the left side. The histological and cytological workup of effusion and solid pulmonary mass showed pleomorph cells expressing leucocyte antigen (LCA) but no cytoceratins. Immunocytochemistry was strongly positive for CD45 and VS38, slightly positive for CD138, and negative for CD38. B or T cell- markers, markers for a lymphatic source of the tissue (incl. CD79a, CD30, CD43, CD2), light chain restriction, or clonal IgH/ TCR γ rearrangement were not detected. The staining for HHV 8 was strongly positive. Laboratory workup revealed a leucocytosis, lymphopenia with a shifted CD4/CD8 ratio, and a hypercortisolism. Based on the morphology, pattern of surface markers and HHV8 positivity the diagnosis of a primary effusion lymphoma (PEL) was established. Screening for human immunodeficiency virus (HIV) was negative. After 7 cycles of chemotherapy according to the CHOP-14 regimen a partial remission was achieved. However after three month a massive progression of disease required salvage chemotherapy. ICE regimen (ifosphamide carboplatin, etoposide) was used for stem cell mobilization and followed by one course high dose chemotherapy according to BEAM regimen (BCNU, etoposide, cytarabinoside, melphalan) and autologous stem-cell transplantation. The therapy was well tolerated and the CT scan showed a partial remission. Two month later the patient noticed a progressive bilateral visual loss. Cranial CT scan showed bilateral occipital contrast enhancing lesions. Chest CT scans revealed a massive progression of both thoracic tumor mass and effusion. Extended blood and cerebrospinal fluid (CSF) workup including microbiological and serological investigations was unremarkable. Since cerebral manifestation of PEL has never been proven an opportunistic infection of the central nervous system (CNS) was taken into consideration. When the patient's condition deteriorated to blindness and severe dementia a stereotactic brain biopsy confirmed a CNS manifestation of PEL. The patient refused any further treatment and died six weeks later. *Conclusions*. In summary, we here report the first histologically confirmed CNS manifestation of PEL in a HIV negative patient. A biopsy-proven CNS manifestation has not been reported so far. We emphasize, that normal CSF studies do not exclude CNS involvement and suggest that in PEL patients presenting with symptoms or signs suggestive for a CNS dysfunction, cerebral manifestation of PEL should be taken into consideration.

1734

TREATMENT OF DEEP VENOUS THROMBOSIS WITH LOW MOLECULAR WEIGHT HEPARIN IN PEDIATRIC CANCER PATIENTS:SAFETY AND EFFICACY DATA

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Background. There are very little data regarding treatment of venous thromboembolism in children undergoing chemotherapy. *Aims*. We designed this study to unify treatment of venous thromboembolism in oncology pediatric patients at our department. At the same time we wanted to evaluate safety and efficacy of the newly designed treatment schedule. *Design and Methods*. Data from the pediatric oncology patients with deep venous thrombosis (DVT) treated at the Dept.of Pediatric Oncology, Brno, were collected prospectively over a 2-year period (January 1, 2006 - December 31, 2007). All patients received low molecular weight heparin (LMWH) at the intitial dose of 1.2 -1.5 mg /kg body weight twice daily subcutaneously (s.c.) for the first 7 - 10 days. Afterwards, the dose was lowered to 1.5 mg / kg s.c. once daily. We kept this dose unchanged for a minimum of 3 months.For the first 6 weeks of treatment the platelets were maintained ≥20x10^9/l with no LMWH withdrawal. For the rest of the treatment, LMWH was interrupted once platelets reached 20x10x10^9/l. *Results.* A total of 33 patients were followed for a median of 6 months. DVT was symptomatic in 15/33 patients (46%) and asymptomatic in 18/33(54%) patients. Complete thrombus resolution occurred in 22/33(67%) patients, partial or no recanalization was achieved in 11/33(33%) patients. Eight patients (8/33;24%) were diagnosed with posthrombotic syndrome (PTS). The risk of PTS was significantly higher for patients with symptomatic DVT compared to those with asymptomatic DVT . Neither patency rates nor the risk of PTS showed a positive correlation with the achievement of therapeutic anti Xa activity. Thrombocytopenia <20x10^9/l occurred at least once during LMWH treatment in 30/33(91%) patients. None of the patients experienced severe bleeding whereas mild bleeding episodes were observed in 5/33(15%)patients. *Conclusions*. Our treatment schedule has proved to be both safe and reasonably efficient in treating of DVT in children undergoing chemotherapy. Further studies on larger patients groups are warranted.

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INTRODUCING A USEFUL MONOCLONAL ANTIBODY FOR THROMBOLYTIC THERAPY

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Background. Human plasminogen is a single-chain glycoprotein of 92 kDa, consisting of 791 amino acid residues and contains five kringles. Several monoclonal antibodies (mAb) against specific parts of the human plasminogen molecule have been developed. *Design and Methods*. In this

study, we investigated the effects of an anti-human plasminogen monoclonal antibody, A1D12 on Glu-plasminogen activation in presence of u-PA, t-PA and streptokinase. The kinetic activation of Glu-plasminogen by it`s activators (u-PA, streptokinase and t-PA) in presence of A1D12 following the effects of the antibody and its comparative ligands on conformational changes of two plasminogen forms by fluorescence and circular dichroism spectroscopy were investigated. Enhancing of β-structure percentage of Glu-plasminogen in presence of A1D12 according to CD-spectra study was coinciding with higher fluorescence intensity in Glu-plasminogen-FITC than Lys-plasminogen-FITC by A1D12 inducing. *Results*. A high similarity between EACA and A1D12 in inducing of Glu-plasminogen conformational changes was concluded. The kinetic analysis of the time course of Glu-plasminogen activation at varying concentrations of S-2251 showed that the A1D12 increased catalytic efficiency of Glu-plasminogen activation by all plasminogen activators without changing in KM values significantly. Thermal inactivation studies also confirmed the conformational change results. We suggested that the binding of A1D12 from F(ab) region to N-terminal epitope of Glu-plasminogen following conformational changes to a more open structure enhanced catalytic efficiency of activation reactions. *Conclusions*. It may be useful to apply clinically monoclonal antibody A1D12 for the therapy of some thromboembolic events by humanizing the F(ab) region of the antibody.

1736

ORAL ANTICOAGULANT THERAPY WITH ACENOCUMAROL IN ELDERLY PATIENTS OLDER THAN 85 YEARS: COMPARATIVE ANALYSIS WITH ANTICOAGULATED PATIENTS BETWEEN 60 AND 70 YEARS OF AGE

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Background. It has been postulated that oral anticoagulant therapy (OAT) in elderly patients is associated with a higher hemorrhagic risk due to three main reasons: the concurrence of another diseases, possible pharmacological interactions and a poor ambulatory control (mostly secondary to a dependence on other persons and a poor understanding of treatment). *Aims*. To analyse OAT in a wide group of patients older than 85, and to compare this series with a control group from 60 to 70 years of age. *Design and Methods*. We have retrospectively studied a group of 58 patients who began OAT over 85 years old (median 88.6 years, range 86-95) and a group of 91 patients that began the treatment when they were between 60 and 70 years of age (median 63.3 years, range 60-70), the latter group randomly chosen from our database. Taking into account that there were not carriers of prosthetic heart valves in the group of patients older than 85, we only included patients with indications to maintain INR between 2.0 to 3.0 in both groups. The most frequent indications in the group over 85 years were isolated atrial fibrillation (75%) or associated with ischemic ictus (9%) or coronary cardiopathy (8%); in the 60-70 years old group, isolated atrial fibrillation accounted for 57% of patients, and 10% associated with ictus, being miocardiopathy the third main indication (9%). As valuable variables, we included in both groups the time of follow-up under OAT (F, in months), the number of analytic measurements in the same time (NAM), the NAM/months of follow-up ratio, the median INR value, the acenocumarol weekly dosage (total mg/week), the percentage of INR values within therapeutic range, and the presence of hemorrhagic or thrombotic events during OAT. The statistical analysis included Student´s t and Jonckhere-Terpstra´s tests.

Table 1

Variable	Group 60-70 years (n= 91)	Group > 85 years (n=58)	p value
Patients with isolated atrial fibrillation (%)	57	71	0.09
Follow-up (F, months)	24.7 ± 12.2	21.6 ± 12.4	0.14
NAM / F – Ratio	1.08 ± 0.23	1.09 ± 0.22	0.78
Median INR value	2.57 ± 0.34	2.48 ± 0.35	0.12
Weekly average dosage (mg)	17.07 ± 7.65	12.47 ± 4.89	<0.001
INR within therapeutic range (%)	56 ± 15	48 ± 17	0.003
Major hemorrhagic events	1	3	0.16
Thrombotic events	None	None	0.30

Results. Main findings are expressed in the following table (percentage or median and standard deviation). *Conclusions*. In our experience, anticoagulated patients older than 85 years of age do not need analytic controls more frequently than those between 60 and 70, although the percentage of INR values within therapeutic range is significantly lower in these elderly patients. As described by other authors, required dosage of acenocumarol to obtain a correct anticoagulation level is lower in patients older than 85. In this preliminary study we find that incidence of hemorrhagic or thrombotic events is similar in both groups, but further studies with a larger number of patients are required to confirm these findings.

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PREVALENCE OF ANTIPHOSPHOLIPID ANTIBODIES IN PATIENTS WITH ANTI-THYROID ANTIBODIES

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Background. Anti-thyroid antibodies are pathogenic in the development of autoimmune thyroid disease. Antiphospholipid antibodies (APLA) are a family of immunoglobulins that may be responsible for venous and arterial thromboses. *Aims.* We have studied the prevalence of antiphospholipid antibodies in patients with autoimmune thyroid disease. Method 70 patients (50 women and 20 men) with antithyroid antibodies (anti-TG, anti-TPO) were examined for antiphospholipid antibodies: ACA (IgG,IgM), β2GPI (IgG, IgM), and anti-Prothrombin. The median age was 50,5 years(31-70) and only 5 of them (7,4%) had a history of atrial fibrillation. Also these patients were immunologically tested (CRP,RA-test,C3,C4 and ANA). The anti-TG, anti-TPO and thyroid hormones T3,T4.FT3,FT4 were measured with ADVIA Centaur XP Immunoassay of Siemens. ACA (IgG,IgM), β2GPI (IgG, IgM), and anti-Prothrombin were measured with Elisa (Genesis Biodiagnostics). All the immunological tests were performed with Nephelometry (plasma protein determination) in BN Prospec analyzer of Dade Behring. ANA were detected with indirect immunefluorescence. *Result*s (Table 1) 18,5% of patients with thyroid antibodies had also antiphospholipid antibodies, 2 of them IgG subtype of ACA , 4 IgM subtype of ACA and 1 both IgG and IgM. In addition 1 had IgG subtype of β-2GPI and 2 IgM subtype of β-2GPI .2 patients had anti-prothrombine antibodies. One of the patients had clinical evidence of antiphospholidid syndrome. All measurements of APLA were found to be borderline or slightly elevated. 21 patients (30%) demonstrated high CRP levels and only 5 (7,1%) low C3 concentration. ANA were found slightly positive in 8 patients(11,4%) with no evidence of a clinical syndrome. *Conclusions.* Our study proves an increased prevalence of antiphospholipid antibodies in patients with thyroid antibodies of 18,5%.We have to prolong the follow up of these patients in order to determine the clinical significance of APLA while in cases of strongly elevated APLA we have to check the patients for thrombophilia in order to prevent a thrombotic event.

Table 1.

Anti-TG and anti-TPO increased		n: 70	%	
ACA increased	IgG	3	4,2	Total 18,5 %
	IgM	5	7,1	
β-2GPI increased	IgG	1	1,4	
	IgM	2	2,8	
anti-Prothrombin increased		2	2,8	
CRP >3 mg/l		21	30	
RA-test >15IU/ml		0	0	
C3 <0,9g/l		5	7,1	
C4 <0,1g/l		0	0	
ANA		8	11,4	

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ECONOMIC ASSESSMENT OF SIDE EFFECTS OF NEW ANTICOAGULANTS

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Background. Long term anticoagulation is currently made with vitamin K antagonists (VKA). When patients on treatment with VKA require to be reversed, vitamin K is frequently given. In a few hours coagulation tests become normalized. If immediate correction is required for critical bleeding or urgent surgery, prothrombin complex concentrated (PCC) administration is usually needed. In the near future, probably the new oral anticoagulants, direct thrombin inhibitors or factor Xa inhibitors, will replace the VKA. These drugs do not have a specific antidote, but their short half-life (dabigatran 12-14 hours; rivaroxaban 7-11 hours) suggest that only in cases of extreme emergency its effect will need to be immediately reversed. Although there is little evidence about, it seems that the most effective treatment would be to administer recombinant activated factor VII (rVIIa). Based on data from studies with ximelagatran, we believe that major bleeding episodes in anticoagulated population with new drugs will not differ significantly from the current incidence. We assume that the indication to administrate rVIIa in our area will be close enough to the current PCC use. *Aims*. We try to assess the economic impact that this change in strategy will lead, entailing the use of rVIIa rather than PCC. *Design and Methods*. Our hospital has a reference population of approximately 1300000 inhabitants. We directly control about 3500 patients on VKA treatment and about 2500 are being managed in primary care. We have compiled all patients on treatment with VKA that required administration of PCC in 2008 because of critic bleeding or the need of urgent invasive procedures. *Results.* During this year, 39 patients on VKA received PCC: 20 because of urgent surgery, 15 because of critical spontaneous bleeding and 4 because of major bleeding after trauma. 22 (56.4%) patients were taking acenocumarol and 17 (43.6%) warfarin; 11 (28.2%) patients had an excessive level of anticoagulation and 28 (71.8%) were in the therapeutic range. The cost of the medium dose of PCC used in our patients (1500 F IX IU) is 579 , and the cost of the recommended rVIIa dose (90 mg/kg) calculated for a standard weight is 3225 . This will r epresent an increase of 2646 per patient treated. Given the fact that in our area about 6000 patients are being anticoagulated, this means that the expected change in anticoagulant drug treatment will cause an increase of 17,199 per 1,000 patients per year invested in immediate reversion strategies. Conclusions: The recommendation of administrate rFVIIa in patients treated with new anticoagulant drugs that need to be immediately reversed may cause an increase in costs of 17,199/1000 patients treated per year.

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THE IMPACT OF THE GENETIC POLYMORPHISM G455 ON FIBRINOGEN B-CHAIN GENE IN PATIENTS WITH CORONARY ARTERY DISEASE AND HEALTHY INDIVIDUALS: EFFECTS ON INFLAMMATORY AND THROMBOTIC PROCESS

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Background. The genetic polymorphism G455A on fibrinogen b-chain gene has been associated with the risk of coronary artery disease (CAD). However, it is not clear if it is associate with inflammatory and thrombotic process in patients with coronary artery disease. In the present study we examined the impact of this polymorphism on low-grade inflammation and thrombotic process in patients with CAD and healthy individuals. *Design and Methods*. The study population consisted of 191 CAD patients and 52 healthy individuals (controls). The G455A polymorphism was estimated by PCR and suitable restriction enzymes. Serum levels of C-reactive protein (CRP) and fibrinogen were measured by nephelometric methods. In addition, D-dimers levels were measure by standard coagulometry techniques. Results: The genotypes contribution was GG: 48.2%, AG: 40.3%, AA: 11.5% for CAD and GG: 50.0%, AG: 34.6%, AA: 15.3% for healthy individuals. Fibrinogen levels were much higher in CAD than controls for the G455A polymorphism (448.1±130.3 vs 382.5±103.8 mg/dl, $p<0.001$). Among patients with CAD AA patients had significantly higher levels of fibrinogen only than GA patients (AA: 517.5±144.0, GA: 434.0±132.2, GG: 443.0±121.0 mg/dl $p<0.05$ for AA vs GA). In controls, there was no difference across the genotypes (GG: 335.1±174.6, GA: 448.3±581.1, AA: 278.6±105.2 mg/dl, p=NS for all). For AA and GG patients with CAD there were significant higher levels of fibrinogen than AA and GG controls (517.5±144.0 vs 278.6±105.2 mg/dl, $p<0.01$ and 443.0±121.0 vs 335.1±174.6 mg/dl, $p<0.05$ respectively). Interestingly, AG controls had significant higher fibrinogen levels than CAD (448.3±581.1 vs 433.7±132.2 mg/dl, $p<0.05$). Patients with CAD had significantly higher levels of D-dimers than controls for the G455A polymorphism (566.2±664.1 vs 357.8±333.1 mg/L, $p<0.01$). Coronary artery disease patients with AA and GG genotypes had significantly higher levels of D-dimers than controls, while there was no difference for AG genotype (551.7±321.5 vs 278.7±105.2 $p<0.05$, 616.4±817.4 vs 335.1±174.6 $p<0.05$ and 511.4±526.7 vs 448.3±581.1 mg/L p=NS). Despite the fact that CRP levels were higher in CAD than controls for the G455A polymorphism, this difference was not significant (2.8±4.0 vs 2.4±3.0 mg/L, p=NS). *Conclusions*. The genetic polymorphism G455A on fibrinogen b-chain gene strongly affects fibrinogen levels and merely thrombotic process in patients with CAD. However, it does not have an impact on inflammatory process. These findings indicate that mechanisms independently to inflammation, but associated to thrombosis may be involved in the expression of this polymorphism.

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THE IMPACT OF THE GENETIC POLYMORPHISM G58A OF FIBRINOGEN A-CHAIN GENE ON LOW-GRADE INFLAMMATORY STATUS IN PATIENTS WITH CORONARY ARTERY DISEASE

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Background. Fibrinogen levels are associated with the presence of coronary artery disease (CAD) and inflammation is an important component of coronary atherosclerosis. The impact of a genetic polymorphism on fibrinogen a-chain gene, the G58A on low-grade inflammation in patients with CAD remains unknown. In the present study we examined the potential effect of this polymorphism on low-grade inflammatory state in patients with CAD. *Design and Methods*. The study population consisted of 179 subjects angiographically documented for CAD. The G58A polymorphism was detected by PCR and suitable restriction enzymes. Serum levels of C-reactive protein (CRP) and fibrinogen were measured by immunonephelometric methods. In addition, levels of sCD40-ligand (cd40L) were determined by enzyme-linked immunosorbent assay (Elisa). *Results.* The genotype distribution was GG: 39.7% AG: 40.2% and AA: 20.1%. Despite of the fact that AA patients had higher levels of fibrinogen, this difference did not reach any statistical significance (AA: 477.5±123.1, AG: 449.0±119.3 and GG: 452.4±146.3 mg/dl, p=NS across genotypes). Similarly, there was no significant difference in CRP or CD40L among GG (2.6±2.5mg/L and 2.4±1.6 μg/mL), GA (3.2±5.5mg/L and 3.0±2.0_g/mL) and AA (3.1±2.7 and 2.7±1.3 mg/L) p=NS for all. *Conclusions*. Our study showed that the G58A polymorphism on fibrinogen a-chain gene does not affect sCD40L and C-reactive protein in patients with coronary artery disease. These findings indicate that other mechanisms rather than inflammatory process may be involved in the expression of this polymorphism.

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LEVELS OF VON WILLEBRAND FACTOR ON ADMISSION CAN DISCRIMINATE PATIENTS WITH ACUTE CORONARY SYNDROME FROM A GROUP OF PATIENTS WITH CHEST PAIN

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Background. Several studies have indicated that elevated plasma concentration of some proteins including von Willebrand Factor (vWF), fibrinogen and D-Dimers are associated, as independent risk factors, with coronary artery disease (CAD). This association becomes much stronger in patients with acute coronary syndrome (ACS), due to the role of vWF, fibrinogen and D-Dimers in the process of thrombus formation and degradation, leading to myocardial ischemia. vWF participates in this process by supporting platelet adhesion to the subendothelial matrix of injured vessel walls. Fibrinogen and D-Dimers levels are increased in this acute phase of coronary syndrome as acute-phase reactant proteins. *Aims*. The aim of this study was to evaluate the vWF, fibrinogen and D-Dimers measurement for the discrimination of patients with ACS from

patients with acute chest pain of non-cardiac etiology. *Design and Methods*. Forty-four (44) patients (30 males/14 females) who presented within six hours after the onset of chest pain were included in this study. Blood samples were obtained on admission and 24 hours after the onset of chest pain under standardized conditions. Fibrinogen, D-Dimers and vWF activity were measured on Siemens-BCS coagulation system. Using clinical evaluation (ECG) and laboratory results (troponin I-TnI) patients were divided into three groups. Group I included patients with non-cardiac etiology chest pain (ECG and TnI-negative), group II patients with unstable angina (ECG-positive and TnI-negative) and group III patients with myocardial infarction (MI) (ECG and TnI-positive). Sixteen healthy males served as controls. *Results.* The table summarizes our results. The following must be noted a)vWF values on admission are statistically significantly higher in patients with MI than in patients with unstable angina and values in patients with unstable angina are also significantly higher than in patients with chest pain of non-cardiac etiology as well as controls (p<0.001) b)fibrinogen and D-Dimers values on admission are not significantly higher in patients with ACS (group I and group II) than in patients with chest pain of non-cardiac etiology and controls (t-test, p=ns). *Conclusions*. There are several studies that have demonstrated that vWF increase is an independent predictor of short term adverse clinical outcome in patients with ACS. The results of this study show that vWF levels could be used for the discrimination of patients with myocardial infarction and unstable angina from a group of patients with chest pain.

Table 1.

	Group I		Group II		Group III		Control	P anova
N	10		17		17		16	
Male/ female	7/3		11/6		12/5		16/0	
Mean age (years)	54±5		56±4		55±5		51±6	
	Adm.	After 24h	Adm.	After 24h	Adm.	After 24h		
Fibrinogen (g/L)	4.45± 0.92	4.62± 1.03	6.06± 1.03	6.34± 1.19	6.22± 1.40	6.05± 1.35	4.20± 0.99	<0.05
D-dimers (mg/L)	0.55± 0.49	0.61± 0.53	0.83± 0.55	0.59± 0.45	1.68± 1.20	1.20± 1.59	0.30± 0.10	<0.05
vWF (%)	103.26 ±11.41	99.1 ±10.5	131.02 ±31.72	119.38 ±27.99	164.08 ±63.11	158.52 ±54.12	100.88 ±9.72	<0.001

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JUMPING TO MICROPARTICLES, A STRESS STUDY

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Background. Microparticles (MP) are small cell derived membrane vesicles. Circulating MP may originate from blood or endothelial cells and display specific cell surface proteins that indicate their cellular origin. Many mediators as shear stress and cytokines may induce cells to release MP as has been shown in e.g. arteriosclerosis and sepsis. MP are both cause and consequence of vascular changes, and play a role in thrombosis and hemostasis. *Aims*. The bungeejump study was designed to elucidate the effects of an acute stress response induced by a bungee jump on the immune system and coagulation, and the role of the β-receptor herein. In the present side-study we investigated the influence of the bungeejump on the levels of circulating MP and their origin, in relation to several coagulation parameters. *Design and Methods*. 20 healthy young men performed a bungeejump from a 70 metres high crane. Ten of these volunteers had been taking propranolol (three times 40 mg a day) for three days prior to the jump. Blood samples were drawn 2 hours before the jump, just before and right after, and 2 hours after the jump. We used previously described methods for MP analysis by flowcytometry. *Results*. Right after the jump there was a significant increase (p<0.001) in total MP in all volunteers. In the control group this increase was mainly due to a significant increase in MP expressing monocyte markers and a moderate increase in MP expressing platelet and leukocyte markers. In contrast, volunteers taking propranolol showed a moderate increase of MP expressing monocyte markers, but an impressive increase of MP expressing platelet or leukocyte markers. After two hours, the MP levels in all volunteers had returned to baseline. Higher levels of von Willebrand Factor (vWF) and factor VIII were observed directly after the jump by volunteers not taking propranolol. *Conclusions*. Bungeejumping by healthy volunteers temporarily increased numbers of circulating platelet, leukocyte en monocyte derived MP as well as levels of vWF and factor VIII, indicating a short transient hypercoagulable state due to the jump. Interestingly, whereas use of propranolol effectively blocked the increase in vWF and factor VIII as expected, it led to a massive unexpected increase in leukocyte and platelet derived MP. The β receptor may therefore be involved in suppressing MP formation by leukocyte and platelets.

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ARE THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) LEVELS, TAFI GENE POLYMORPHISMS AND CLOT LYSIS TIME RISK FACTORS FOR PREECLAMPSIA AND INTRAUTERINE FETAL GROWTH RESTRICTION?

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Background. The aim of the study was to evaluate TAFI levels, TAFI gene polymorphisms and clot lysis time (CLT) in complicated pregnancies with severe preeclampsia (PE) and/or severe intrauterine foetal growth restriction (IUFGR), and to compare them with healthy pregnancies. *Design and Methods*. 129 pregnant women who delivered at 26 or more weeks of gestation were prospectively recruited. The study group (Group 1) included 63 pregnant patients with severe PE and/or severe IUFGR. The control group (Group 2) included 66 healthy pregnant patients. TAFI antigen was determined by ELISA, and CLT as previously reported (Lisman T et al. Hepatology 2002;35:616-21). TAFI and CLT were determined in patients of Group 1 at the time of diagnosis of severe PE and/or IUFGR at the third trimester and 10 weeks after delivery (basal period) and, in Group 2 during each trimester of gestation and 10 weeks after delivery. Polymorphisms of TAFI gene, Ala 147Thr and +1542C/G, were determined by PCR. *Results*. A progressive increase in TAFI antigen levels and CLT was observed during normal pregnancy, which returned to basal levels after delivery. Significant higher TAFI levels and longer CLT were found in the basal period in Group 1 compared to Group 2 (TAFI antigen Group 1/ Group 2: 10.63 mug/mL /9.54 mug/mL (p<0.05); CLT Group 1/Group 2: 78.64 minutes /68.09 minutes (p<0.05)). There were no differences in TAFI gene allele distribution between the two groups. *Conclusions*. Increased basal TAFI and CLT may constitute biological predictors of the risk of severe PE and/or severe IUFGR during pregnancy.

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PREDISPOSING PREVENTABLE FACTORS IN PATIENTS WITH BLEEDING DUE TO WARFARIN USAGE: EVALUATION OF 114 PATIENTS

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Background. Warfarin, which is derivative of coumarin, is a commonly used anticoagulant drug for myocardial infarction, ischemic stroke, venous thrombosis, after heart valve replacement and atrial fibrillation both for prevention and treatment. However, warfarin has a narrow treatment range and the dose of the drug greatly differs among individuals. Insufficient dose might not prevent thrombosis while overdose may enhance hemorrhage risk. *Aims*. The aim of our study is to evaluate the predisposing preventable factors among patients with bleeding due to warfarin usage. *Design and Methods*. This study was performed on 114 cases who admitted to emergency service for complaint of bleeding due to warfarin usage. The sociodemographic characteristics, information on warfarin usage and their bleeding history are collected by a structured questionnaire form. The mean age of the patients was 66.65±13.57 years (mean±standard deviation, 27-89 years, 82 cases 60 years and over) and 61 (53.5%) of them were females. Results. At the time of admission 42 cases (36.8%) had melena, 31 (27.2%) hematuria, 29 (%25.4) ecchymosis, 25 (21.9%) epistaxis, 13 (11.4%) hematemesis, seven hemoptysis, five hematoma, five vaginal bleeding, three bleeding

from the current lesion, two hematochezia, two intracranial bleeding, one late bleeding after tooth extraction, one gingival bleeding and one case had subconjunctival bleeding. The mean of the time between the onset of the bleeding and admission to the hospital was 2.9±3.4 days. The mean dose of warfarin being used at the time of admission was 31.2±10.8 mg/week (8.7-70.0 mg; median 35.0 mg). The mean of the period from the onset of the warfarin usage to the time of bleeding was 31.6±49.0 months (1-228 months; median 10.5 months). 35 cases (30.7%) experienced bleeding during the first month, while 50 cases had bleeding (43.8%) within the six months. Only 37 cases were being controlled regularly. The mean number of the drugs being used other than warfarin was 4.8±2.5 drugs. Forty-eight cases were using aspirin, 28 non-steroidal anti-inflammatory drugs other than aspirin and 18 cases were using various antibiotics. Forty-eight of the patients knew that they had to use this drug under the regular follow-up of a physician and 43 knew that during monitoring a laboratory test had to be done while using the drug named as warfarin. Only 39 patients knew that this drug may cause bleeding. It was also surprising that only nine of the patients knew that there may be drug interaction and one knew that this drug may be affected from the dietary factors. *Summary and Conclusions*. As a result, our patients who admitted to the emergency service with bleeding have a big gap of knowledge which invited bleeding. Most of the patients were using drugs which are known to interact with warfarin metabolism while using warfarin. Developing a training program for the related specialists and general practitioners who follow-up such patients may have an effect on decreasing the morbidity and mortality caused by warfarin induced hemorrhages.

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PREVALENCE OF FACTOR V LEIDEN, PROTHROMBIN G20210A AND MTHFR C677T MUTATIONS IN DEEP VENOUS THROMBOSIS PATIENTS FROM WESTERN IRAN

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Background and Aims. Inherited disorders related to hemostatic system are known as risk factors for thromboembolic events (TE) such as myocardial infarction, stroke, pulmonary embolism, pregnancy complications, and especially recurrent deep vein thrombosis (DVT). The present study was aimed to investigate the prevalence of factor V Leiden G1691A, prothrombin G20210A and MTHFR C677T in DVT patients and their possible association with DVT in Western Iran. Patients and *Design and Methods*. Seventy two DVT patients with the mean age of 40.4±13.6 years including 37 females and 35 males and 100 age and sex matched healthy individuals from Kermanshah Province of Iran with ethnic background of Kurd were studied for factor V Leiden G1691A, prothrombin G20210A and MTHFR C677T by PCR-RFLP method using Mnl I, Hind III and Hinf I restriction enzymes, respectively. Results. Prevalence of factor V Leiden was 12.5% in patients and 2% in control group. A significant association was found between factor V Leiden mutation and DVT with odds ratios (OR) of 7.0 (95% confidence intervals [CI] 1.46-33.46, p=0.018). The prevalence of prothrombin G20210A variant in patients and control individuals were 4.16% and 1.0% respectively. . The prevalence of MTHFR C677T was found to be 38.9% and 44% in patients and control group, respectively. Around 18.9% of women were Oral contraceptive pill users. In 17.5% of patients there was a history of DVT in the family. Around 23% of patients had a history of prior venous thromboembolism. Venous thrombosis in legs was the most frequent clinical manifestation (n=67), corresponding to 93.1% of the TE, followed by pulmonary thromboembolism (6.9%) *Conclusions*. Our study for the first time have determined the prevalence of inherited thrombophilia in a homogenous ethnic group of DVT patients and suggests that factor V Leiden, and not the prothrombin gene mutation is a risk factor for DVT in Western Iran.

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PLASMA PROTEIN Z LEVELS IN NEONATES WITH RESPIRATORY DISTRESS SYNDROME

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Background. Respiratory distress syndrome (RDS) is one of the most common lung disorders in preterm neonate. Fibrin deposition has been demonstrated in the pulmonary microcirculation and small airways in RDS suggesting the activation of the coagulation system with a special role of protein C (PTC) and protein S. Recently, protein Z (PTZ) -a vitamin k dependent protein-, was added to these factors. *Aims*. Our aim is to primarily evaluate Protein Z and Protein C levels in healthy preterm and full term newborns and compare their serum levels with those newborns suffering from RDS. Second is to assess their serum levels after recovery. *Design and Methods*. 60 newborn infants, recruited from the neonatal unit Aïn Shams University, Cairo, Egypt were enrolled in the study and divided into 3 groups: Group (I) of those 20 preterm with RDS, Group (II) of 20 healthy full term control newborns and Group (III) of 20 healthy preterm control newborns. Protein Z and C were measured by ELISA. Results: Lower levels of protein Z was obtained in RDS group compared to preterm controls whose levels were by their role, significantly lower than in full-term controls. A significant increase in protein Z levels in RDS' group was noticed after their recovery from RDS, where it reached near normal levels compared to full-term controls. In RDS, no significant correlation existed between protein Z levels and routine laboratory investigations except for a significant negative correlation with platelets. Regarding protein C, no significant difference was seen in RDS group before and after recovery. *Conclusions*. Premature newborns suffering from RDS showed lower serum protein Z levels than either normal preterm or full-term newborns with further increase in its pattern to reach near normal after recovery. We recommend serial measures of PTZ in premature RDS for follow up of their condition.

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FIBRINOGEN AND POLYMORPHISM V34L OF FACTOR XIII GENE IN PATIENTS WITH ACUTE CORONARY SYNDROME (ACS)

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Background. The Factor XIII of the blood coagulation activated for the Thrombin, remains the Fibrin clot. The common polymorphism V34L of Factor XIII is localized in the subunit A, only to tree aminoacides of the zone of clivaje for Thrombin and certain would may to be implied in the stability of clot. The L allele frequency was significantly lower in patients group with Myocardial infarction compared to control group; this difference was extremely significant in patients younger than 50 years old. Those findings support the hypothesis that V34L polymorphism in FXIII gene has a protective effect against myocardial infarction (1). In a recent Meta-analysis with 5346 patients with ACS and 7053 controls (2), shows that the beneficial effect of the polymorphism might be smaller than the effect estimates obtained, because the analysis raised the possibility of publication bias. Data published in the literature suggest that gene-gene and gene-environmental interactions might significantly influence the protective effect of FXIII V34L polymorphism. Those authors (3) found that 34Leu homozygous patients form fibrin clots with lower permeability than 34Val homozygotes at low fibrinogen concentrations, but that the permeability was similar for both genotypes at intermediate fibrinogen levels and even higher for the 34 Leu homozygotes at high fibrinogen. *Design and Methods*. 1°. To determinate the prevalence of genotype of polymorphism V34L Factor XIII gene in patients with Acute Coronary Syndrome (ACS) 2°. The prevalence of genotype LL of polymorphism Factor XIII gene in the group of patients and the prevalence of control group will be compare for to determinate the protector factor (OR) of allele L. 3°. To value the meaning of Fibrinogen in relation to polymorphism V34L of Factor XIII gene in the patients with ACS. Patients: A group of 84 patients non older, surviving of an event of acute coronary syndrome; the diagnosis was based on the cardiology criteria's (descent of ST and /or Troponin >1). Controls: A group of 407 subjects voluntary healthy, blood donors of similar age and sex; before a physician informed them and they had authorized the recruitment in the study. All controls were free of any history of venous or arterial thrombosis. The detection of polymorphism Factor XIII V34L by Polymerase Chain Reaction (PCR), in liquid phase, in real time, adapted and automated in a LightCycler (Roche Diagnostic) thermal cycler will be made. To compare the percentages of allele L homozygote in every group of patients and controls, by Chi-square test will be made. The significative results in these proof of hypothesis with confidence interval 95% for odds ratio (OR) we will employ. *Results*. In the group of patients 80,2% are men and 19,8 women, medium age is 50 (24-74) years; in control group 66,6% are men and 33,4% women, medium age is 39 (22-73) years. A prevalence of allele L homozygote of polymorphism V34L of Factor XIII gene of 1,4% in the patients and 4,5% in the controls has been found. The difference is not significative but a tenden-

cy with protector effect is showed. In relation to levels of Fibrinogen in the patients of ACS, we had seen that in those with allele L the median is 350 mg (218-460) minor that the patients not carrier allele L 380 mg (189-740). *Conclusions*. A protective effect for ACS of the allele L of polymorphism V34L of Factor XIII gene has been showed in relation to fibrinogen levels. To study greater number of patients for a definitive result is necessary.

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COAGULATION PARAMETERS IN PEDIATRIC SOLID TUMORS

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Background. Association of prothrombotic markers with thrombosis in pediatric oncology patients is conflicting. There is little agreement about the most useful tool for defining coagulability on cancer. This study was done to measure parameters of hypercoagulation panel and to generate estimated prevalence of prothrombotic factors in pediatric patients with newly diagnosed malignancies excluding leukemia and to compare these results with those of healthy controls. *Design and Methods*. Patients with newly diagnosed solid tumors, brain tumors and lymphoma and age/sex matched healthy children undergoing elective surgery as controls were enrolled in this study. We measured fibrinogen, D-dimer, prothrombin time, activated partial thromboplastin time, antithrombin, protein C, protein S, homocysteine, Lipoprotein (a) and factor VIII were also performed in all patients and controls. The specimens from the enrolled patients were collected prior to the start of chemotherapy and in the setting of >2 weeks after surgical intervention for tumor resection. Results: In this descriptive cross-sectional study of 40 patients, 16 had solid tumors, 14 brain tumors and 10 lymphomas. [Mean age 8.2 years; 25 males]. Forty age/sex-matched controls were enrolled. Of these 40 cancer patients, abnormal hypercoagulation panel results were detected in (55%). Four patients had low functional protein S, 2 had high homocystine levels and 12 had high lipoprotein (a) levels. In contrast to healthy subjects, children with cancer had significantly higher D-dimer, factor VIII and fibrinogen [$p<0.05$]. At the time of this report, no patients experienced clinically documented thrombotic events. *Conclusions*. Children with newly diagnosed solid tumors have a high prevalence of prothrombotic markers, when compared to age/sex-matched controls. Positive D-dimer, high factor VIII, fibrinogen levels were the commonly found thrombosis risk factors in pediatric patients with solid tumors.

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INCREASED IL-1B, IL-6 AND TNF-A LEVELS IN PEDIATRIC MALIGNANCY ASSOCIATED WITH HYPERCOAGULABLE STATE IN THROMBOELASTOGRAPHY PROFILES

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Background. Multiple factors contribute to the activation of coagulation and thrombotic diathesis. Prothrombotic markers and their association with thrombosis in pediatric solid tumor patients are not well defined. The host response to malignancy including the acute phase reaction, paraprotein production, inflammation, necrosis and hemodynamic disorders contribute to thrombotic risk. Tumor cells have the ability to activate the coagulation system through the release of inflammatory cytokines, such as IL-1α, IL-6, IL-8, and TNF-α, and physical interaction between tumor cells and blood or vascular cells. Inflammation might represent important role for mediating thrombosis. Thromboelastography (TEG) allows the assessment of patient's global hemostatic function. A hyper- or hypocoagulable state can be easily visualized using TEG. *Aims*. We hypothesize that newly diagnosed pediatric patients with malignant tumor have abnormal TEG profiles and higher prevalence of prothrombotic markers such as elevated cytokine levels when compared to age and sex matched normal controls. *Design and Methods*. Specimens from the enrolled patients were collected prior to the start of chemotherapy. In the occurrence of surgical intervention, specimens were collected two weeks after tumor resection. Otherwise healthy children undergoing elective surgery were enrolled in the control group. In both groups, TEG parameters, reaction time (R), angle (α), maximum amplitude (MA), lysis 30 minutes (LY30), G value (G), and coagulation index (CI), and cytokine levels, IL-1α, IL-6, IL-8, and TNF-α, were measured in these newly diagnosed pediatric cancer patients and control groups. Results: In this descriptive cross-sectional study of 40 patients (25 males and 15 females), 16 (40%) with solid tumor, 14 (35%) with brain tumor, and 10 (25%) with lymphoma, with mean age of 8.4 years (2 to 17 years). Forty age and sex matched healthy children undergoing elective surgery such as hernia repair were enrolled as controls. Abnormal hypercoagulable TEG profiles were found in eight patients. Moreover, cancer patient's TEG MA, CI, and G values were significantly higher than the control group ($p<0.001$). IL-1α, IL-6, and TNF-α levels were also significantly higher in cancer patient group ($p<0.001$). There was no statistical difference in TEG parameters and these cytokine levels between patients with and without surgery prior to receiving chemotherapy. At the time of cohort, no patients experienced clinically documented thrombotic events. *Conclusions*. The significant differences of TEG profiles and cytokine levels between children with newly diagnosed malignant tumor and age and sex matched controls may indicate an increased risk of thrombosis in children with malignancies. This may indicate that the pathogenesis of cancer-associated thrombosis is substantially influenced by inflammatory cytokines. Further studies are needed to assess utility of using TEG and cytokine levels as a clinical tool to predict who is at risk of thrombosis.

Table.

	Patient n=21	Patient Post-surgery n=19	All Patients n=40	Control n=40	p-value*
Age (years, range)	9.6 (2-17)	7.0 (2-15)	8.4 (2-17)	8.1 (3-15)	
Reaction Time (R, min)	8.5 ± 2.0	9.7 ± 2.9	9.0 ± 2.5	10.6 ± 8.5	0.298
Angle (α, deg)	62.6 ± 7.8	58.3 ± 11.7	60.6 ± 10.0	59.3 ± 5.5	0.151
Maximum Amplitude (MA, mm)	66.3 ± 7.8	63.8 ± 7.1	65.1 ± 7.5	60.8 ± 3.8	0.002
Clot Firmness (G, K d/sc)	10.7 ± 4.0	9.3 ± 2.9	10.0 ± 3.5	7.9 ± 1.3	0.006
IL-1β (pg/mL)	3.9 ± 6.5	4.0 ± 6.6	3.9 ± 6.5	0.58 ± 0.41	0.011
IL-6 (pg/mL)	6.4 ± 11.6	14.9 ± 30.7	10.5 ± 22.8	0.21 ± 0.21	0.000
IL-8 (pg/mL)	6.1 ± 5.6	11.3 ± 16.1	8.6 ± 11.9	4.59 ± 2.45	0.205
TNF-α (pg/mL)	0.44 ± 0.90	0.63 ± 1.23	0.50 ± 1.01	0.08 ± 0.08	0.018

Data expressed as mean ± SD. *Kruskal-Wallis Test

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EVALUATION OF A NEW QUANTITATIVE HIGHLY SENSITIVE D-DIMER ASSAY IN HOSPITALIZED PATIENTS

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Background. D-dimer measurement is widely applied in the diagnostic work-up of venous thromboembolic disease, especially for the exclusion of deep vein thrombosis (DVT) and pulmonary embolism (PE). D-dimer Innovance is a new microparticle-enhanced immunoassay using monoclonal antibody and has been evaluated only in outpatients. Aims: To evaluate the levels of D-dimer Innovance in hospitalized patients. *Design and Methods*. We investigated the causes of elevated D-dimers in 425 patients with no symptoms or clinical signs of DVT or PE during their hospitalization. Pregnant women were excluded from the study. Patients samples were measured simultaneously on Dade Behring coagulation analyzer BCS XP (cutt-of: 0,5 mg/L). ; *Results*. From 425 samples assayed, elevated values were observed in 257 (60,47%). 70 (27,77%) of them were repetitions. Elevated levels were observed in the following situations, *Conclusions*. Innovance D-dimer is a highly sensitive assay and this increases the diagnostic value of its normal levels. However, this test has low specificity because elevated levels are observed in all diseases and conditions with activation of fibrinolytic system. Assessment of high levels requires attention, especially in patients with high D-dimer

values due to coexisting diseases. The use of D-dimer testing represents an efficient and safe screening tool for the exclusion of thromboembolic events in combination with a well-validated clinical pretest probability score. Further experience with this test when used in routine clinical practice in hospitalized patients should be obtained.

Table.

Disease	No of patients	Range of values (mg/l)
Bleeding	25	0,73-35,20
Infection	62	0,68-16,00
Iinflamation	17	1,98-20,73
Sepsis	7	1,04-35,00
Trauma	6	0,80-7,32
Malignancy	26	0,60-19,96
Surgery	55	0,60-35,20
Diabetes mellitus	16	0,66-3,44
AOC therapy	9	0,76-22,37
Drug poisoning	4	0,81-1,35
Heart failure	3	1,41-6,06
MI	3	1,01-4,90
Stroke	24	0,66-35,20

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CLINICAL EXPERIENCE IN COMBINED ANTI-PLATELET PLUS ANTICOAGULANT THERAPY IN PATIENTS WITH VALVULAR AND NON VALVULAR ATRIAL FIBRILLATION

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Background. Atrial fibrillation (AF) is the most prevalent arrhythmia in anticoagulated patients and it is also a strong independent risk factor for systemic embolism and stroke. From 1989 the role of anticoagulation for preventing stroke in this arrhythmia became established. AIMS: Following the 2004 NASPEAF trial: 'Comparative effects of antiplatelet, anticoagulant, or combined therapy in patients with valvular and nonvalvular atrial fibrillation", we have tried to assess the efficacy and safety of the combination of antiplatelet teraphy (trifusal) and moderate-intensity anticoagulation therapy (INR: 1'5-2'5) with acenocoumarol in patients with AF linked to recognized risk factors or mitral stenosis. *Design and Methods*. We selected 54 patients (33 men and 21 women) in acenocoumarol and triflusal combined therapy, dividing them in two groups: an intermediate-risk group including patients > 60 years age and a risk factor: hypertension, diabetes, heart failure, hyperlipidaemia (21 men and 14 women); and the high-risk group including patients with nonvalvular plus prior embolism, and patients with mitral stenosis with and without prior embolism (12 men and 7 women). The follow-up took place between 01/01/07 and 27/01/09. *Results*. Primary events: one case of deep venous thrombosis in a man from the intermediate-risk group who also had a minor rectal bleeding; one event of embolism stroke (TIA) in a high risk group female patient and another TIA of cardiac etiology in a man from the intermediate risk group who also suffered rectal bleeding and abdominal hematoma. All events occurred with INR in normal levels. Secondary events: 11 minor bleedings: one patient had subconjunctival bleeding and a limited hematoma. Another patient presented eye bleeding. Both patients were taking triflusal 300 mg/day. Another man from the intermediate risk group had spontaneous hematomas and one man from the high-risk group showed another subconjunctival hemorrhage. We also registered gingival bleeding in a man of the intermediate risk group and one epistaxis in a woman of the same group and in another patient who also had suffered a spontaneous hematoma event. Regarding the intermediate bleeding events, we registered two rectal bleeding cases in two men of the intermediate risk group and two patients presented hematuria, both from intermediate risk group, a man and a woman with INR: 2'57. We recorded a severe bleeding episode: an anemia due to great traumatic hematoma in the right thigh (the patient needed a blood transfusion due to hemoglobin: 7'1 and INR: 9'48). *Conclusions*. In our experience, combined antiplatelet and moderate-intensity anticoagulation therapy does not increase the risk of bleeding events. It seems an effective therapy, as the amount of thrombotic events is according to other previous studies using the same type of patients and treatment. In conclusion, the addition of antiplatelet therapy to acenocoumarol teraphy to reduce the anticoagulation intensity is effective in AF patients stratified by risk of stroke, and does it without increasing bleeding risk.

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PREVALENCE OF HYPERHOMOCYSTEINEMIA IN TUNISIAN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

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Background. Patients with inflammatory bowel disease (IBD) have an increased risk for thromboembolic events. Moreover, a hypercoagulable state has been hypothesized as a contributing factor in the pathogenesis of IBD. Recently, a growing amount of interest has focused on mild to moderate hyperhomocysteinemia as a risk factor for thromboembolic disease. Aims: We aimed to evaluate the levels of homocysteine and the prevalence of hyperhomocysteinemia in patients with IBD and to investigate the contribution of vitamin status in determining increased level of plasma total Homocysteinemie. *Design and Methods*. We studied 100 IBD Tunisian patients: 81 with Crohn's disease (CD) and 19 with ulcerative colitis (UC), of whom 4 patients had a history of venous thrombosis. Plasma homocystein concentration was determined by fluorescence polarization immunoassay with Axsym analyzer. The following parameters were analysed: age, sex clinical activity, length-extent and type of disease (CD or UC), plasma homocystein concentration, folates and vitamin B12. *Results*. The mean age of the population studied is 35.6 years, sex ratio female/male=1.12. Hyperhomocysteinemia was defined as homocystein level 14 µmol/l. Four cases with thromboembolic event were found (only in the patients with CD), but no one had hyperhomocysteinemia. The mean level of homocystein was 11.77 µmol/l. The prevalence of high homocystein concentration was 21% (11 male and 10 female) all of them had a CD. Only 2 patients had folate deficiency (there was no statistically significant correlation between folate and homocysteine plasma levels, p=0.19), but 7 patients had vitamin B12 deficiency (p=0.01). No correlation was found between homocysteine levels and either disease activity, smoking sex or involvement of the terminal ileum. *Conclusions*. Our study suggests that hyperhomocysteinemia is a common phenomenon in CD, and is associated with vitamin B12 deficiency, but no correlation is found between plasma level of homocysteinemia and thromboembolic complication in IBD Tunisian patients.

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INFLUENCE OF INFLAMMATION ON ATHEROSCLEROSIS DEVELOPMENT IN MAINTENANCE HEMODIALYSIS PATIENTS

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Background. Inflammation in maintenance hemodialysis (MHD) patients (pts) contributes to atherosclerosis development besides traditional atherothrombotic factors. *Aims*. Aim of the study is to show the influence of inflammation on atherosclerosis development. *Design and Methods*. The population of 42 MHD pts was divided in 3 groups according to tertiles of C reactive protein (CRP) values (cut points 1.87 and 5.32) as inflammation marker with first group consists of 16 pts with lowest CRP tertile, second group consist of 12 pts with median CRP tertile and third group consists of 14 pts with highest CRP tertile. We compared ultrasonographic parameters of early atherosclerosis: lumen diameter(LD), intima media thicknes (IMT) and cross-sectional calculated intima media area (cIMA) using formula 3,14x[(LD/2+IMT)2-(LD/2)2] for both right and left common carotid arthery (RCCA and LCCA) as well mean values for both carotide artheries using Kruskal-Wallis test. Afterwards we tested inter-group differences using Mann-Whithney test. *Results*. We found significant differences between three groups in RCCA LD (7.40+/-0.82 vs 7.80+/-1.58 vs 8.95+/-1.37; p=0.017), LCCA LD (7.38+/-0.83 vs 8.05+/-1.46 vs 9.11+/-1.45; p=0.009), LCCA IMT (0.77+/-0.16 vs 0.78+/-0.13 vs 0.91+/-0.31; p=0.045), mean LD (7.40+/-0.88 vs 8.01+/-1.51 vs 9.03+/-1.40; p=0.011) mean IMT (0.76+/-0.12 vs 0.78+/-0.18 vs 0.89+/-0.30; p=0.05) nad mean cIMA (19.82+/-5.20 vs 20.50+/-9.87 vs 29.58+/-12.37; p=0.049). There was significant differences between first and third group in RCCA LD (p=0.002), LCCA LD (p=0.001), LCCA IMT (p=0.031), mean LD (p=0.001), mean IMT

(p=0.025) and mean cIMA (p=0.013). *Conclusions*. Pts with highest CRP values have highest ultrasonographic parameters of early atherosclerosis. Inflammation is significant factor for atherosclerosis development besides traditional atherothrombotic factors.

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PROCOAGULANT ACTIVITY OF CIRCULATING MICROPARTICLES IN HEMODIALYSIS PATIENTS

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Background. End-stage renal disease is associated with high prevalence of endothelial dysfunction and subsequent cardiovascular disease. Elevated count of microparticles, recently discovered biomarker of endothelial dysfunction, was observed in patients under hemodialysis. However, data concerning count and activity of circulating microparticles in patients on hemodialysis are limited. *Aims.* To quantify procoagulant activity of microparticles in plasma of patients immediately before hemodialysis procedure. To compare it to healthy controls and plasma level of soluble P-selectin and fibrinogen levels, as markers of inflammation and atherosclerotic cardiovascular complications. *Design and Methods*. We analysed the procoagulant activity of microparticles in plasma of stable patients (n=57) with various nephropathies (n=23 with diabetic nephropathy) undergoing long-term hemodialysis treatment procedure and healthy volunteers (n=20) by thrombin-generating functional assay. Patients with end-stage renal disease had arteriovenous fistula and all samples were drawn immediately before the hemodialysis session. Plasma levels of soluble P-selectin were analysed by enzyme immunoassay. C-reactive protein levels were assessed, as a marker of inflammation and infection. The procoagulant activity of circulating microparticles expressed as concentration of phosphatidyl serine equivalents was modestly but significantly lower in hemodialysis patients (n=57) than in the healthy controls (n=20) (3.98±1.89 vs. 5.30±1.65 nmol/l; p<0.05). On the contrary, the plasma level of soluble P-selectin was significantly higher in patients than healthy controls (55.2±18.1 vs. 40.3±8.2 ng/ml; p<0.001). Patients with end-stage renal disease, as expected, had significantly higher concentrations of plasma fibrinogen (4.37±0.99 vs. 3.27±0.67 g/l). *Conclusions*. Our pilot results suggest that circulating procoagulant microparticles counts are not increased immediately before a hemodialysis session. Larger cohorts and the effect of hemodialysis procedure on the levels of microparticles will be assessed by this promising functional method in following study.

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THROMBOTIC EVENTS OF COBALAMINE DEFICIENCY

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Background. Diseases related to Cobalamine deficiency (CblD) or to congenital abnormalities of Cbl usually revealed by a macrocytic megaloblastic anemia. However CblD can be risk factor for vascular disease (VD). Hyperhomocysteinemia is sensitive and early indicator of CblD. There is relationship between it moderate elevation and the occurrence of both arterial and venous disease (*M. Catane. Hyperhomocysteinemia: A risk factor for arterial and venous thrombotic disease. Inter J Clin Res, 1997, 27: 139-144*). *Aims*. In this study we describe observations of patients with CblD revealed or associated with VD events, we aimed to establish incidence and prognosis of VD events in patients with a diagnosis of B12 deficiency. *Design and Methods*. We performed a retrospective study of all patients with Vit B12 deficiency between 1994 and December 2008. Diagnosis revealed by anemia and or macrocytosis megaloblastic, B12 dosage and therapeutic tests. Homocysteine levels performed just in 3 patients. Diagnosis of thrombosis was confirmed by ultrasoundgraphy, in others cases we need ECG, ophtalmoscopy, angiography and cerebral scanning. *Results*. 575 patients with Cbl deficiency were recruited. Among this cohort 9 (1.6%) had VD and thrombosis. The average age was 45years (18 - 67y), 6M/3F, 3 had deep vein thrombosis of extremities, 1acute ischemic stroke occurred without any context, 1 myocardial infraction, 1 had large vessel thrombosis with both arterial and venous thrombosis (mesenteric and portal vein thrombosis arterial cerebral thrombosis), 1 central vein retina thrombosis, 2 sibling patients with congenital Trancobalamin (TCII) deficiency had severe retinopathy. 3patients had history of an inadequate intake of folic acid or vitamin B12. Haemoglobin level was between 5 to 11,5 g/dL, MGV: 80 to 127 fl, Leucocytes:3,2 to 5,7/µL, platelets 115 to 210.00/µL, serum B12 level was : 87±26pg/ml. Average homocysteinemia performed in 3 patients was 55 µmol/l (N<15). Triglyceridemia, Cholesterol, HDL quotient were normal. Serum Folates and others prothrombotic factors (Protein C, S AT III) were normal. Birmer's disease was confirmed in 2 patients. Medium Interval between first symptoms and diagnosis was 18 months. Evolution: All patients received B12 therapy (1000 to 5.000 micgr /2days), and 4 patients were treated with fractioned heparin, switched to vitamin K antagonist (INR 2-3) during 6 to 12 months, 2 patients received clopidogrel sole. We observed 2 deaths after 3 and 7 days of diagnosis: 1after embolism pulmonary complicated DVT, 1 cardiogenic shock in patient with myocardial infraction. Neurological sequels observed in two patients (hemiplegia and leucoencephalopathy). Total Blindness noted in one patient after TVCR. Decrease of visual acuity in 2patients with TC deficiency related to proliferative retinopathy despite specific treatment. *Conclusions*. Our Study confirms the previously vascular disease reported the independent risk of thrombosis and associated mortality in patients with Cbl deficiency. A high index of suspicion should be maintained for early anticoagulant treatment. The prognostic value need to bee evaluated in properly designed studies.

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THE STIMULATING EFFECT OF ANTI-PLASMINOGEN MONOCLONAL ANTIBODY (A1D12) ON CLOT LYSIS AND ANGIOGENESIS.

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Background. Plasminogen is a single chain 92-KD glycoprotein consisting of 791 amino acid. This proenzyme that has a central role in fibrinolyrtic system can activate through various activators (Pas) to its active form plasmin and perfoem its vital function that is fibrin clot lysis. Furthermore the fibrinolyrtic system plays a major role in angiogenesis. The fibrinolyrtic system activation control cell migration and invasion (that is essential step in angiogenesis) by plasmin-mediated matrix proteolysis. In addition to this, plasmin regulates tumor growth and metastasis by the activation of matrix metalloproteases (MMPs) and growth factors. Monoclonal antibodies, as biological tools play a important role in basic researches. *Aim*. In this study we evaluated the activation of fibrinolyrtic system and angiogenesis process in the presence of antiplasminogen monoclonal antibodies.Methds: In the first step the effects of antibodie on the activation of fibrinolyrtic system with PAs were evaluated in several methods including macroscopic observation, quantitative measurement of DD/E fragments by D-dimer assay and activation of plasminogen by S-2251 synthetic substrate (ELIZA method). In subsequent we studied the effect of antibodie on angiogenesis process in an *in vitro* model. *Results*. Results showed that A1D12 which is against to N-terminal domain of Glu-plasminogen, in addition to activation of fibrinolyrtic system in presence of plasminogen activators can activate *in vitro* angiogenesis process. *Conclusions*. Plasmin directly participates in angiogenesis by direct fibrin and other matrix components degradation, and indirectly by activating matrix degrading metalloproteinase and angiogenic growth factors such as TGF-α, HGF, VEGF and bFGF. During the last decade, angiogenic research has focused on identification and detection of angiogenesis inhibitors and stimulators and their potential value in treating angiogenesis related diseases. Plasmin activating system is a potential target for this reason .According to the results of *in vitro* A1D12 accelerates this process in a dose dependent manner

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MAJOR CARDIOVASCULAR DISEASE IN A CHILD WITH ANTITHROMBIN DEFICIENCY - HEPARIN BINDING DEFECT

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Background. We present an unusually severe case of cardiovascular disease in a child presumed secondary to Kawasaki's disease. This child was noted to be heparin resistant in the setting of coronary artery by-pass surgery and was therefore presumed to have acquired antithrombin deficiency. Surprisingly he was subsequently found to have an inherited antithrombin deficiency with a heparin binding defect (HBS). *Design and methods and Results*. Genetic analysis of this childs SERPINC1 (GenBank X68793.1, MIM#107300) gene revealed a heterozygous C>T substitution at nucleotide c.218 within exon 2. This predicts the replacement of the native proline with a leucine at amino acid 41 of the mature protein, and was first described as Antithrombin Basel. This corresponds to codon 73

using HGVS nomenclature where +1 is the initiating Methionine. Pro41Leu is a well described mutation, with 18 reports on the current update of the Antithrombin mutation database. Pro 41Leu is characterised as a HBS defect, leading to Type II HBS Antithrombin deficiency. *Summary and Conclusions*. Childhood thrombosis is rare.The vast majority of events occur as a complication of therapy for serious underlying disease and over recent years the incidence has been noted to be increasing. Patients who are heterozygous for Antithrombin deficiency HBS variants do not exhibit an increased tendency to thrombosis although the incidence of venous thrombosis is approaching 100% in homozygotes. There have been no reports of Pro41Leu in a child of this age, and almost all reports have been in asymptomatic patients. Interestingly studies on almost 10,000 healthy blood donors in Scotland and a further 2500 in Canada failed to detect a single individual with this variant. The lack of correlation with the clinical presentation leads to difficulties in estimating the true prevalence of this mutation and whether anticoagulation is deemed necessary and for what duration. This childs anticoagulation was extended to six months therapy with a VKA (warfarin) in light of the genetic findings. The question remains as whether this is adequate therapy or not.

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ORAL ANTICOAGULANT THERAPY (OAT) IN PEDIATRIC PATIENTS

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Introduction. The use of antithrombotic agents in pediatric patients has significantly increased in recent years, due to developments in pediatric cardiology. *Design and Methods*. We performed a retrospective review of the pediatric patients treated with OAT (acenocumarol) at our hospital, from Feb-1995 to Jan-2009. There were 86 children (age 0-18 years, 49M/37F); 22 children (26%) initiated OAT before the age of 1 year. The indications for OAT were as follows: - Prosthetic heart valve: 6 cases. - Primary thromboprophylaxis: 13 cases; atrial flutter (1), Kawasaki disease (5), dilated cardiomyopathy (4), and associated with cardiac stent (3). - Treatment of Thrombotic events: 29 cases; venous thrombosis with risk factors (22 cases, including 10 cases catheter related thrombosis), spontaneous venous thrombosis (2), and arterial thrombosis (5). - Fontan surgery, 38 cases. In all cases, the initial dose of acenocumarol was 0.2 mg/kg, except in the Fontan cases that was 0.1 mg/kg. RESULTS: The mean follow-up was 10.5 months (1-157). We observed a high proportion of controls outside the target INR range in all groups, most under therapeutic INR. There were however few complications: only 4 cases of mild epistaxis and 2 cases of thromboembolic events, one in a child with dilated cardiomyopathy, and the other one in a child with congenital heart disease in thrombotic events group. Both recovered successfully. *Conclusions*. In our experience, OAT in pediatric patients is safe and effective; a high proportion of children have controls outside the target INR, most below it, what suggests acenocumarol underdosing due to an unjustified fear of bleeding complications.

Table.

	Prosthetic heart valves		Thrombotic disease prophylaxis		Thrombotic events		Fontan surgery
Months	<12	>12	<12	>12	<12	>12	>12
N	4	2	2	11	16	13	38
Mean age of onset	7,5 m	10 y	5,5 m	7 y	8 m	7 y	6 y
INR range	2,5-3,5		2-3		2-3		2-3
Mean follow-up (months)	44 (3-157)		9 (2-49)		6 (1-39)		8 (1-64)
INR above range	13,37% (0%-29,41%)		34,11% (0%-77,78%)		15,08% (0%-66,67%)		11,61% (0%-33,33%)
INR below range	45,2% (25%-57,14%)		35,27% (5,26%-50%)		44,37% (0-100%)		41,7% (0%-100%)
Thrombosis/ hemorrhages	2 mild epistaxis		1 cardiac embolism		1 stroke		2 mild epistaxis

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COULD WHOLE BLOOD THROMBOELASTOMETRY DISCOVER HYPERCOAGULABLE STATE IN PATIENTS AFTER AN EPISODE OF IDIOPATHIC VENOUS THROMBOEMBOLISM?

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Background. The recurrence rate after the first episode of idiopathic venous thromboembolism (VTE) is high. Hypercoagulability and reduced fibrinolytic capacity, which might predispose to recurrences of VTE are difficult to assess in assays employed in routine care. The whole blood rotation thromboelastometry (TEM) evaluates the clot formation initiation, development and lysis. This method seems suitable to discover hypercoagulable state. *Aims*. The aim of the study was to find out if patients at least 6 months after the first episode of VTE present hypercoagulable state in thromboelastometry profiles and to investigate a relation between thromboelastometric determinations and plasma concentration of D-dimer and fibrinogen. Elevated D-dimer is widely known as a predicting factor for venous thrombosis reccurence. *Design and Methods*. The study group consisted of 24 patients at least 6 months after the first episode of idiopathic VTE, aged 19-71 (median 31): 16 women (67%) and 8 men (34%). 8 healthy volunteers were enrolled to the control group. All patients from the study group had secondary antithrombotic prophylaxis with vitamin K antagonist (INR 2,0 - 3,0). One week before blood sampling vitamin K antagonist was replaced by enoxaparine s.c. (50% of therapeutic dose). Thromboelastometric evaluation, D-dimer and fibrinogen concentration measurements were performed in both groups. *Results*. A. Patients after VTE showed markedly increased values of ex-TEM Coagulation Time-CT (73.7s vs 59.3s; $p<0.0002$), ex-TEM Clot Formation Time-CFT (90.7s vs 71.1s ; $p=0.0347$) and ex-TEM a angle (72.04° vs 75.5°; $p=0.0464$) when compared to results of the control group. B. There was significantly higher concentration of fibrinogen in the study group in comparison with controls (5.07 vs 2.87 g µL; $p=0.0002$). C. There were no statistically significant correlations between thromboelastometry values and the plasma concentration of D-dimer. *Conclusions*. In patients after the first episode of idiopathic venous thromboembolism thromboelastometric determinations show features of hypercoagulability, which should be evaluated as risk factors for recurrent VTE, but do not correlate with D-dimer level. Elevated concentration of fibrinogen in patients might contribute to these modifications of thromboelastometric parameters.

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UPPER-EXTREMITY DEEP VENOUS THROMBOSIS: CLINICAL CHARACTERISTICS AND RISK FACTORS

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Background. Deep venous thrombosis of the upper extremity (UEDVT) is an unusual event ranging from 1% to 4% of all cases of deep vein thrombosis. Limited data concerning the contribution of hypercoagulable status in the pathogenesis of this disease are avaliable. *Aims*. To evaluate the characteristics of patients who developed UEDVT and to determine the prevalence of inherited and acquired thrombophilic risk factors and incidence of rethrombosis. *Design and methods*. We reviewed the clinical files evaluating demographic and clinical parameters, and inherited and acquired thrombophilic risk factors of all patients who developed UEDVT and required oral anticoagulation in our center from May 2002 to May 2008. Laboratory investigations were performed in absence of anticoagulant therapy for at least one month. The following parameters were evaluated: Antithrombin, protein C and S, homocysteine, anticardiolipin antibodies, lupus anticoagulant and thrombophilic mutations. Statistical analysis was performed with SPSS 15.0. Results: 42 patients showed UEDVT: 17 female (40.5%), 25 male (59.5%). Median age 51.04 yr (6-92). 4 patients (9.5%) had familiar history of thrombosis . 14 patients (33.3%) had idiopathic thrombosis and the remaining 28 patients (66.7%) showed acquired risks factors of thrombosis (cancer or central venous catheter). Local anatomic abnormalities were found

in 7 patients (16.9%) . 20 patients (47.6%) had thrombosis in one localization while 22 (52.4%) have more than one area afected. Localizations were: 15 axillar-subclavian, 10 yugular, 6 subclavian, 4 brachiocefalic, 2 axillary-subclavian-yugular, 1 axillary-subclavian-cefalic, 1 axillary-subclavian-brachiocefalic, 1 yugular-carotidean, 1 brachiocefalic-cava and 1 yugular bulb, transverse and sigmoid sinuses. Diagnosis was stablished by ultrasound in 17 patients (40.5%), contrast venography in 3 patients (7.1%), computerized tomography in 3 patients (7.1%), ultrasound and venography in 4 patients (9.5%), ultrasound and computed tomography in 13 patients (31%) and 2 patients (4.8%) by the three procedures. 14 patiens (33.3%), had not acquired risk factors, 8 patients (19%) had history of cancer , 9 patients (21.4%) had cancer and central venous catheter, 3 patients (7.1%) had central venous catheter, 2 patients (4,8%) had pacemarker and cancer, 2 patients (4.8%) were under oral contraceptive therapy and the last 2 patients had recidivant pathology of shoulder and middle ear infections. About hypercoagulable status: 6 patients (14,3%) showed low level of protein C, protein S and homocysteine levels were normal in all patients and antiphospholipid antibodies were positive in 8 patients (19%). Thromobophilic mutations were shown as follows: 10 patients (23.8%) Heterozygous MTHFR, 2 patients (4.8%) doble heterozygous prothrombin and MTHFR, 1 patient (2.4%) doble heterozygous FV Leiden and MTHFR, 1 patient (2.4%) homozygous FV Leiden and heterozygous MTHFR, 2 patients (4.8%) homozygous MTHFR and 1 patient (2.4%) triple heterozygous. Patients were divided in 2 groups, idiopathic or non idiopathic thrombosis to analize differerencies about gender, age, thrombosis localization, anatomic abnormalities, diagnosis by ultrasound, tomoghaphy or venography, and hypercoagulable status. No statistical differences were found between the 2 groups except localization of thombosis which seems to be more extensive in idiopathic thrombosis group (p=0.04). Rethrombosis was observed in 7 patients (26.7%) as pulmonary thromboembolism. *Conclusions*. 1) The presence of central venous catheter does not influence the risk for other thromboembolic events like pulmonary embolism, being in our serie similar to previously reported in the literature. 2) Neoplastic status is teh most prevalent event as risk factor in our cases. 3) The antecedents of idiopathic thrombosis are correlated in our experience with more extensive disease. 4) In spite of the relatively high incidence of genetic hypercoagulable status, no statistical differences were observed between idiopathic or non idiopathic thrombosis.

1761

SPLANCHNIC VEIN THROMBOSIS AS THE FIRST CLINICAL FEATURE OF MYELOPROLIFERATIVE DISEASE

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Myeloproliferative diseases (MPDs) represent the commonest cause of splanchnic vein thrombosis. The resulting hypersplenism and haemodilution decrease the accuracy of blood counts and splenomegaly for MPD diagnosis. We present 7 cases (5 women, 2 men, aged 22-47 years) admitted for thrombophilia testing because of idiopathic portal or hepatic vein thrombosis. Inherited thrombophilia including: antithrombin-, protein C-, protein S deficiency, factor V Leiden or protrombin gene G20210A mutation was excluded in 6 cases. In one patient a hereditary heterozygotic deficiency of protein C was discovered. 4 cases presented with antiphospholipid antibodies and primary diagnosis of antiphospholipid syndrome was established. Splenomegaly was of no value in differential diagnosis, as it could result from hepatic or portal vein thrombosis. Histopathology of bone marrow revealed megakariocyte hyperplasia and fibrosis (+1) in 6 patients. Molecular testing was positive for JAK 2V617F mutation in 6 cases, suggesting myeloproliferative disease as the primary cause of thrombosis. Additionally paroxysmal nocturnal haemoglobinuria (NNH) has been diagnosed in one case. Conclusion: In patients with splanchnic vein thrombosis diagnosis of underlying clinically "latent" MPD could be established with JAK2 analysis. This test could replace bone marrow investigations as initial diagnostic test for MPD. Aniphospholipid antibodies were the most frequent associated prothrombotic factor in our group of patients.

1762

PROTEIN C, PROTEIN S AND ANTITHROMBIN III PLASMA LEVELS IN ACUTE LEUKEMIA IN CHILDREN

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Background. Acute leukemia is the most common childhood malignancy. The occurrence of various coagulation abnormalities in acute leukemia is well established. Hemorrhage is the most common haemostatic disorder in patients with acute leukemia. Thrombosis is a rarer complication.However, the epidemiology and the exact pathogenesis of this entity are complex and have not yet been clearly defined. Aims: We aim to evaluate the plasma levels of the natural anticoagulant panel {protein C (PC), protein S (PS) and antithrombin β (ATβ)} and their role as predisposing factors for hemorrhagic and thrombotic complications of acute leukemia in children. *Design and Methods*. The study was carried out on 40 patients with acute leukemia (24 with ALL and 16 with ANLL) at Pediatric Hematology and Oncology Unit in Zagazig University Hospital. All patients were subjected to full medical history and examination, routine work-up for acute leukemia and estimation of Protein C, Protein S and Antithrombin β plasma levels at diagnosis and during treatment. *Results*. It was found that the level of ATβ was significantly lower at diagnosis in both ALL and ANLL (p<0.01) with a significant increase in its level during remission in ANLL patients (p<0.05). As regards PC, there was a significant decrease in PC level in both ALL and ANLL patients compared to controls (p<0.01 & p<0.05) respectively, while no statistically significant differences were detected regarding PS in all patient groups. *Conclusions*. Our results indicate that apart from thrombocytopenia, low levels of ATβ and PC and alteration in fibrinolysis and coagulation may be responsible for the hemorrhagic and thrombotic complications observed with acute leukemia in children.

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INVESTIGATIONS OF THROMBIN GENERATION TESTS IN WARFARIN-TAKING PATIENTS

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Background. Thrombin generation tests measure the total thrombin formed and are potentially superior when compared with the currently used coagulation tests in monitoring the hypercoagulation or hypocoagulation state of patients. We used chromogenic method on the fully automated Behring Coagulation System (BCS) to measure the thrombin generation in patients under warfarin therapy with different international normalized ratios (INRs) to evaluate their coagulation state. AIM: The investigation of the relationship of endogenous thrombin potential (ETP) parameters between controls and patients under stable warfarin therapy. *Design and Methods*. We studied 30 control blood samples and 39 blood samples from patients who had been taking warfarin for more than 3 months since underwent valve replacement or suffered from atrial fibrillation. INR and thrombin generation parameters were performed in all samples.The INR values varied from 1.5 to 3.8. We used the chromogenic method on the fully automated Behring Coagulation System (BCS) for the measurement of thrombin generation parameters. RESULTS: Table Comparison of the two groups showed statistical significant differences p< 0.0001 for all parameters. The ETP and the Cmax showed a strong inverse correlation with the INR (n=20; r= -0.632 and -0.709 respectively). *Summary and Conclusions*. The range of ETP values is rather width at the investigated INR range. Patients at therapeutic INR range may have low ETP values and high risk of bleeding or high ETP values and thrombotic risk.Although the INR is a good surrogate, ETP measurement might be more accurate in estimating anticoagulation potential. Therefore, ETP might offer better differentiation between those who bleed more easily and those who develop thrombosis despite "therapeutic" anticoagulation. For clinical validation of these findings a prospective clinical trial is warranted.

Table.

	CONTROLS		PATIENTS	
	median	*min-max*	*median*	*min-max*
tlagsec *SD*	**19,4** 2.6	12.5-26.37	**46.3** 60	12.57-248.24
tmax sec *SD*	**54,8** 4.4	45.52-62.09	**99.4** 84.5	17.74-462.04
CmaxmA/min *SD*	**124,7** 6	112.68-134.57	**56.9** 32.7	5.72-182.53
Cmaxcal% *SD*	**111,5** 5.4	100.72-120.29	**34.86** 24.9	5.11-102.7
AUCmA *SD*	**390,9** 29.5	322.74-451.07	**186.8** 71.5	7.3-348.41
AUCcal% *SD*	**114,8** 8.7	94.84-132.56	**50,4** 20	2.15-102.39

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IRINOTECAN AND INCREASED TRIGLYCERIDES LEVEL IN PARANEOPLASTIC DEEP VENOUS THROMBOSIS(DVT): A STRANGE LINK IN AN OLD PROBLEM

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Background. DVT is present in about 3-15% of cancer patient. Paraneoplastic thrombosis pathogenesis is multifactorial. Chemotherapy is frequently involved in DVT genesis as proinflammatory/prothrombothic factor. Ther are few data about incidence of DVT in patients treated with Irinotecan. *Aims*. Aim of our study is to define if thrombotic risk in patient treated with Irinotecan is increased and eventually through wich mechanism probably Irinotecan acts in DVT genesis. With this purpose we considered in our patient complement fraction C3 and C4 and immune circulating complex (ICC) because they activate macrophage and platelets and increase tissue factor level. Moreover we recognize also total cholesterol and triglycerides level, because they are linked with factor VII activation. *Design and Methods*. We considered C3, C4, ICC, cholesterol and triglycerides level, dose density and chemotherapeutic agents used in 116 patients with solid neoplasm (62colon, 26lung, 18gastric,) and without anticoagulant prophilaxis. Median age was 68.5 years (R 57-83). M/F ratio was 66/40. The threshold value of third quartile was chosen as risk cut-off (C3: 130mg/dl; C4: 32mg/dl; ICC: 2.9 mcg/ml; total cholesterol: 205mg/dl; triglycerides 120mg/dl). The statistical analysis was conducted with Yates corrected chi square test, Fisher's exat test, Odds Ratio (OR), realtive risk (RR). Results 24 patients (20%) showed DVT. Patients treated with Irinotecan were 42. DVT occurred in 14 of these patients (32%) and in 10 of 74 patients treated without Irinotecan (14%) with a Yates corrected chi square test of 4.6 (p 0.03), a Fisher's exact test with p0.03, an OR of 2.98 (95%CI 1.2-7.3) and a RR of 2.35 (95%CI 1.1-4.8). CRP and ESR at Pearson's test were not related with C3, C4, ICC, total cholesterol and triglycerides levels. All patients treated with Irinotecan had advanced stage colon cancer. High levels of triglycerides (>120mg/dl) were present in 17 out of 42 patients treated with Irinotecan (40%) and in 17 out of 74 patients treated without Irinotecan (23%) with a Yates corrected chi square test of 3.1 (p 0.07), a Fisher's exact test with p0.05, an OR of 2.2 (95%CI 1-5) and a RR of 1.7 (95%CI 1-3). *Conclusions.* Althoug patients receiveing Irinotecan and developing DVT were those with advanced stage colon cancer, influence of chemotherapy in DVT genesis in our study cannot be excluded. Probably Irinotecan contributes to DVT increasing triglycerides levels. In fact increased levels of triglycerides are linked *in vivo* with factor VII activation. This relief suggest also that other risk factors and other drugs (i.e. hypolypemizant drugs) might be used in paraneoplastic DVT prevention. Nevertheless these data need confirmation on a larger cohort of patients.

1765

SERUM THROMBOMODULIN LEVEL IN CHILDREN WITH β THALASSAEMIA MAJOR

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Background. In recent years, it is suspected that vascular endothelial cells may have some role in vascular compications noted in thalassemic patients. Activated or injured endothelial cells release their protein constituents mainly thrombomodulin into the circulation. Thrombomodulin is considered the most important indicator of vascular endothelial injury. *Aims*. to study serum thrombomodulin level in children with β-thalassemia major and its relation to the duration of disease and extent of iron overload. *Subjects*: The present study was carried out on 35 patients with β-thalassemia major. They were divided according to the age into two groups. Group I: included 15 young children aged less than 10 years. Their mean age was 6.4 years, and their mean disease duration was 5.5 years. Group II: included 20 older children and adolescents aged from 10 to 20 years. Their mean age was 16.5 years and their mean disease duration was 15.6 years. Ten apparently healthy children of matching age and sex served as control group. *Design and Methods*. Estimation of serum levels of Thrombomodulin (TM) by ELISA. Echocardiography, two-dimensional, M-mode, and Doppler studies. *Results*. Cardiomegaly was found in 7 (35%) patients of group II. Pulmonary hypertension was encountered only in 4 (20%) patients of group II. Thromboembolic manifestations (femoral deep vein thrombosis) were found in only 1 (5%) thalassaemic patient in group II. The serum TM level was significantly higher in both groups, in comparison with controls (F=10.36, $p<0.001$). No significant difference was found between both thalassaemic groups (t=0.421, $p=0.673$). Moreover, no significant difference was found between TM levels in splenectomized and non-splenectomized cases (t=0.62, $p=0.541$). TM levels of both thalassemic groups showed no significant correlation with serum ferritin level (r=-0.02, $p=0.914$). A significantly higher mean value of right ventricular wall thickness was encountered in group II thalassaemic patient as compared to group I (t=2.57, $p=0.019$). *Conclusions*. increased serum TM level in our polytransfused thalassaemic patients reflects a state of endothelial cell activation and/or injury in these patients. The statistically significant negative correlation between serum TM level and pulmonary acceleration time (r=-0.45, $p=0.047$), may point to the possible role of pulmonary vascular endothelium injury as a contributory factor in the pathogenesis of pulmonary hypertension in our thalassaemic patients.

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PROGNOSTIC VALUE OF VON WILLEBRAND FACTOR AND FIBRINOGEN LEVELS IN ADVANCED GASTRIC CANCER PATIENTS

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Background. Activation of clotting and fibrinolysis systems are thought to be involved in tumor angiogenesis, tumor-platelet adhesion and tumor-endothelial cell adhesion. Von Willebrand factor (vWf), an adhessive ligand with platelets, is elevated in advanced disseminated malignancies and it is involved in the metastatic process. *Aims*. To correlate coagulation markers levels in plasma of advanced gastric cancer (GC) patients (pts) undergoing palliative chemotherapy (CMT) with response to treatment and time to progression (TTP). *Design and Methods*. 41 pts with locally advanced or metastatic GC, diagnosed between january and december 2008, and 20 healthy controls were enrolled in the study. Blood samples were taken before (basal time) and after platinum-fluoropirimidine-based CMT. We measured plasma levels of vWf, vWf activity-ristocetin cofactor test (vWf: Rcof) (BCS coagulometer), vWf Antigen (vWf:Ag), factor VIII activity test, D-Dimer (DD), Plasminogen, Thrombin Time (TT), Fibrinogen and Reptilase time (ACL-TOP coagulometer) and plasma GC tumor markers (CEA and CA19.9). Results.- Median age of pts was 64 (range 38-89). All pts but one received at least one cycle of CMT, with a median of 3 cycles (range 0-10). Median basal ECOG was 1 (range 0-3). After a median follow-up of 9 months the median TTP was 4 months and the median overall survival (OS) 8 months. At basal time were found elevated levels of vWf:Ag (median 210%; range 121,1-492,2%), DD (median 530 mgr/L; range 49-17489) and Fibrinogen (median 5,4 gr/L; range 2,78-7,67) when compared with healthy controls. Basal time plasma levels of vWf:Ag were significantly correlated with basal CA19.9 levels ($p< 0.05$). Higher basal Fibrinogen levels predicted worse response to treatment ($p= 0.02$) and shorter TTP ($p= 0.012$); however basal time levels of vWf:Ag and DD were not correlated with CMT response, TTP or OS. Higher vWf:Rcof levels after 3 cycles of CMT were correlated with worse treatment response ($p= 0.019$) and shorter TTP ($p< 0.05$). *Conclusions*. vWF:Rcof levels during CMT and basal levels of Fibrinogen might both predict response to treatment and TTP in advanced GC pts.

1767

SUCCESSFUL PREVENTION OF ATTACKS WITH STANOZOLOL IN A CHILD WITH SEVERE CRYOFIBRINOGENEMIA

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Background. Cryofibrinogenemia is a rarely symptomatic disorder that is underestimated due to the rarity of the symptomatic form of the disease; however it may sometimes cause multiple thromboembolism in the skin, lung and myocardium and cause deleterious effects leading to significant morbidity and mortality. The pathological manifestations associated with cryofibrinogenaemia have been treated with plasmapheresis and fibrinolytic drugs such as streptokinase, streptodornase and/or urokinase. *Aims*. Stanozolol, an androgenic steroid with fibri-

nolytic properties. is a safe and effective drug for the long-term management of hereditary angioedema and there is data on its use in childhood cases in this indication. There is also literature data on good results with stanozolol in adult patients with symptomatic cryofibrinogenemia, however data on its use in childhood cases with this indication is limited. *Design and Methods*. Herein, we report an eleven year-old male presented with complaints of red painful swelling of the tips of his right hand's third digit and his left hand's three digits. He had been having similar attacks on acral areas like finger tips, auricles, nose tip since he was 18 months-old. This attacks mostly occur after exposure to cold like playing with snowball. He was admitted to many other clinics before and was treated with many drugs like calcium channel blockers, asetil salisilic acid, intravenous pentoxyfilin, dextran infusion and methylprednisolone, but none of these measures could stop tissue loss and his left hand's fourth and fifth digits were amputated (Figure 1). Laboratory tests revealed slightly elevated fibrinogen and high α1 antitrypsin levels. On protein electrophoresis serum α 2 band was found as increased. Cryoglobulin was negative but cryofibrinogen test was positive in qualitative assay. The tests that are made to exclude secondary cryofibrinogenemia causes including collagen vascular diseases revealed ANA positivity (1/160 nucleolar pattern) and mildly increased anti dsDNA levels. Antiphosholipid and cardiolipin antibodies were negative. Hypercoagulability tests were normal. The skin biopsy taken from knee was consistent with thrombogenic vasculopathy showing PAS positive fibrin thrombi inside vascular lumen and on the wall which was positive with fibrinogen and C3 on immunohistochemistry stains. He was diagnosed to have cryofibrinogenemia and stanozolol (2 mg/day, po) prophylaxis was initiated with the concent of the family. The patient had no attacks in the winter under stanozolol, besides some of the new forming lesions at the onset of stanozolol healed without sequela. The patient developed no side effects related to stanozolol and being closely monitored. *Summary and Conclusions.* Cryofibrinogenemia is a difficult disease for patients and physicians in its symptomatic form. Stanozolol seems to be promising and safe with this indication in pediatric cases. The symptomatic form is rare in childhood and early prophylaxis with stanozolol may prevent morbidities like amputations of digits.

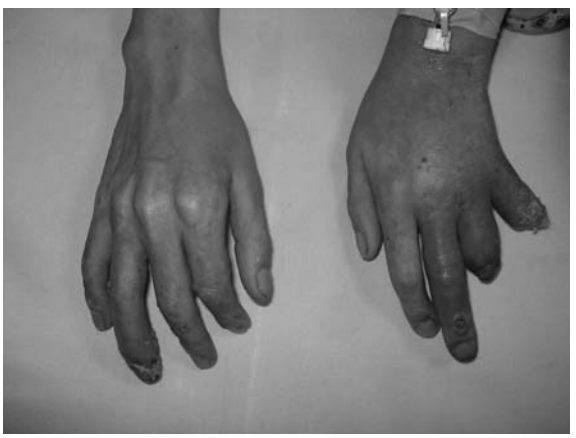

Figure.

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THROMBOPROPHYLAXIS DURING PREGNANCY-A RETROSPECTIVE STUDY IN A DISTRICT GENERAL HOSPITAL

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Background. Venous thromboembolic (VTE) disease is the most common cause of maternal death in the United Kingdom. The Royal College of Obstetricians and gynaecologists (RCOG) have produced guidelines (2004) on thromboprophylaxis during pregnancy. Aims To see if these how these guidelines were being followed at the Royal Cornwall hospital and to see if they were effective at preventing VTE. *Design and Methods*. Twenty one patients who had received thromboprophylaxis during pregnancy were selected at random from the haematology database. Information was obtained from hospital and laboratory records. This included, age, pre-pregnancy weight, timing of prophylaxis, risk factors, details of previous VTE, drug and dose used and thrombophillia status. *Results*. The mean age was 31 years (21-40) and all had previous VTE. The mean pre-pregnancy weight was 83kg (47-120kg); weight was not documented in 10 patients. Eighteen were started on prophylactic dose antenatal low molecular weight heparin (LMWH) dalteparin in the second trimester. One patient had severe skin reaction to several preparations and was changed to danaparoid post partum. Three patients had only post partum LMWH prophylaxis. Two patients had thrombotic events despite prophylaxis, one lady had Klippel-Trenaunay syndrome, immobility and hyperemesis, the other had a prolonged labour and caesarean section. All ladies had 6 weeks post natal thromboprophylaxis. Thrombophillia testing was performed in 14. Three results were positive (heterozygote for factorV Leiden). *Conclusions.* All women had a history appropriate to recommend use of thromboprophylaxis. Although guidelines suggest starting prophylaxis as early in pregnancy as practical, individual women expressed personal views on the timing of antenatal prophylaxis and in the absence of a strong evidence base this should be taken into consideration. The guidelines recommend thrombophillia screening; thrombophillia results did not influence the management of any of our patients and we question its value in the management of this patient group. It was difficult to extract information from the hospital notes; particularly the pre-pregnancy weight was unavailable in many notes. With increasing obesity more women will be over 100kg at booking, not only is this risk factor for VTE but anti-Xa levels should be measured to monitor effectiveness of LMWH in this patient group-clearer documentation is essential. The 2 patients who had VTE despite prophylaxis were complex but a more consistent multi disciplinary approach may have ensured their VTE risk was reassessed as the pregnancy progressed and their LMWH dose reconsidered

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ENDOGENOUS THROMBIN POTENTIAL (ETP) IN PATIENTS WITH AN ACUTE MYOCARDIAL INFRACTION

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Background. The ETP test, recently, was added to the conventional coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), for the investigation of coagulation function. ETP has been shown useful to detect an hypercoagulable state in individuals at risk of venous thrombosis. *Aims*. To investigate ETP parameters in acute myocardial infraction on admission and 4days later. *Design and Method*s. 12 patients with AMI were included. Blood was sampled on admission and after 4 days when all patients received heparin/LMWH, acetylsalicylic acid and/or clopidogrel. ETP parameters was measured by the chromogenic method on the fully automated Behring Coagulation System (BCS). *Results and Conclusions*. ETP and Cmax were decreased and tlag and tmax were prolonged when patients were on anticoagulant therapy. These results are not statistically significants. That might be explained by the key role of antiplatelet drugs in AMI which do not affect ETP. ETP is sensitive to antithrombotic medication but the number of patients was very small thus for clinical validation of these findings a prospective clinical trial is warranted.

Table.

		SD	P
ETP admission	365.2	73.2	**0.118**
ETP 4days	319.3	113.2	
Cmax admission	132.8	37	**0.201**
Cmax 4days	114.5	40.3	
Tlag admission	32.7	12.4	**0.277**
Tlag 4days	55.6	68.8	
Tmax admission	66.3	18.3	**0.276**
Tmax 4days	100.2	103.7	

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ESTIMATION OF THROMBIN PRODUCTION IN DIFFERENT SUBJECTS

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Background. During the decade of 90s new methods, of global impact, for the evaluation of the activation of blood coagulation appeared and mainly after the presentation of Hemker and Beguin method. We thought of a newer and simpler method using the already existing aggregometer (Chrono-log, P.I.C.A). Our method is based in thrombin production using platelet rich plasma (PRP) in which CaCl2 is added. *Aims*. The aim of our study is the presentation of possible new test and checks it in normal subjects, and patients in different pathologic situations. Material: We applied our method in 114 subjects (30 normal subjects (NP) with mean age 37,9±9,4 to estimate the mean values and SD, 16 haemophiliacs, 36 patients under LMWH, 56 patients under antiplatelet drugs -48 under Aspirin and 8 under Plavix-, and 6 patients under anti-vitamin K treatment). *Principe of method*. The addition of CaCl2 in PRP activates it and produces coagulum. *Design and Methods*. In 450 μL of PRP we add 250 μL CaCl2 (0.025 mm) in a plastic tube of aggregometer (Chrono-log P.I.C.A). We also tested the reproducibility of the method by repeating the test 12 times in the same normal subjects CV for the area under curve 5,9%, and for the lag time was 13%. The results presented in table I. For normal subjects for example the mean lag time of GTT was 161,3±63,4sec while in haemophiliacs was 573.2±218.7sec (with a t-test statistically very significant, $p<0.001$). The advantages of our method besides the fact that it is without cost is: The mean values of lag time in these categories differ accordingly; witch is statistically significant and proves their diagnostic value.

Table. Mean value and t-test.

categories	X ± SD	p
NP vs hemophiliacs	161,3±63,4 vs 573,2±218,7	<0,000168
NP vs thrombophiliacs	161,3±63,4 vs 298,2±122,7	<0,0235
NP vs antiplatelets	161,3±63,4 vs 320,9 ±55,8	<0,020
NP vs LMHW	161,3±63,4 vs 349,6±144,3	<0,01

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USE OF TISSUE PLASMINOGEN ACTIVATOR(R-TPA) IN 6 CHILDREN, CASES SERIES

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The information about the thromboembolic events, the optimal treatment choice, the dose and duration of antithrombotic therapy in children are limited. More clinical data are required. Recombinant tissue plasminogen activator (r-tPA) is increasingly used in pediatric thrombosis. We retrospectively analyzed the clinical course of 6 children (mean age: 6.1±3.4) with venous thrombosis (n:1) and intracardiac thrombosis (n:5). The children were treated with r-tPA. The dose ranged between 0.2-0.4mg/kg/h infused for 3 to 4 hours. This dose was repeated between 2 to 7 times till the thrombolysis was achieved. Treatment side effects were closely monitored. Plasma D-dimer, fibrinogen levels, prothrombin time and activated partial thromboplastin time were measured prior to each r-tPA administration. If they were prolonged, fresh frozen plasma was given in order to maintain normal hemostasis. Complete clote lysis was achieved in all cases. None of them had severe bleeding except mild recurrent epistaxis occurring in 2 cases. In conclusion, r-tPA is an effective and safe therapy under close haemostatic control in children.

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CLINICAL MANIFESTATIONS AND HEMOSTASIS LABORATORY ABNORMALITIES IN ADULT β THALASSEMIAS WITH HIGH LEVEL OF ANTIPHOSPHOLIPID ANTIBODIES

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Background. A total number of 41 adult Adult β Thalassemias (87.81% were Thalassemia β/HbE, age ranged between 19 to 61 years old) with a high level of antiphospholipid antibodies (ACA IgG, ACA IgM, and anti β2 GP1 IgG), had been enrolled into a study on the clinical manifestations related to the blood circulation disturbances and the hemostasis laboratory abnormalities. No data has been reported about these findings. *Aims*. The questions were: (1) What clinical manifestations and how frequent the blood circulation disturbances developed in those 41 study subjects. (2) What kind hemostasis abormalities developed in those 41 study subjects. *Design and Methods*. Descriptive data of clinical manifestations of blood circulation disturbances as well as hemostasis laboratory abnormalities have been collected in those 41 study subjects of Indonesian populations. Laboratory examinations were conducted in Jakarta and compared to 41 people of control normal group. *Results.* Of 41 study subjects, 31 patients (75.61%) had head complains (cephalgia, migrain, vertigo), 24 patients (58.54%) experienced lower extremities disturbances (pain, edema and paresthesia), 22 patients (53,66%) demostrated chest complains (shortness of breath, chest pain, etc), 14 patiens (34,15%) developed tinnitus or visus problems, 9 patients (21,95%) developed bleeding (epistaxis, purpura, bleeding of the gum)), and 5 patients (12.20%) showed fetal loss syndrome (miscarriage, perinatal death). Of 41 study subjects, mean of VCAM-1 was 2101.339±1120.403 ng/ML (higher than control normal group, $p<0.001$), TAT complex 4.779±15.019 ng/mL (higher than control, $p<0.001$)) F1+2 prothrombin 1.168±1,657 nmol/L) (higher than control, $p=0,001$), D-dimer 539.415±1051.799 ng/mL (higher than control, $p<0.001$), AT III 75.448±19.360% (lower than control, $p<0.05$), Protein C 69.075±98.616% (lower than control, $p<0.001$), Protein S 72,225±18,883% (lower than control, $p<0.001$), PAI-1 3.395±5.328 iu/mL (compared to control $p > 0.05$), fibrinogen 207.666±95.707 (lower than control, $p<0,01$). Platelet aggregation revealed 31 of 41 patient (75.61%) had hyporeactive aggregation, normoreactive in 6 patients (14.63%), hyperreactive in 3 patients (7.31%), sticky platelet in 1 patient. *Conclusions*. Compared to thalassemia patients or patient with anti-phospholipid syndrome (APS) in general Adult β Thalassemias with a higher level of ACA IgG, ACA IgM, anti β GP1 IgG showed similiraties, except in the latest group showed hyporeactive platelet aggregation, low F1+2 prothrombine, and no differerences of PAI-1 compared to control normal group.

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PLASMA COAGULATION TESTING AND GENETIC THROMBOPHILIA MUTATIONS CAN PREVENT THE IDIOPATIC RECURRENT ABORTIONS IN HEALTHY FERTILE WOMEN

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Background. A hypercoagulation state is frequently reported in apparently health women with adverse pregnancy outcomes. In this scenario to many tests and too much conflicting data are until now described.In this regard several facors have been considered:increased venous stasis,high circulating plasma levels of FI,II,VII,VIII,IX,X,XI,XIII,misinterpreting temporary decrease in Protein C (PC), Protein S (PS) and antithrombin III (ATIII) as these rare inherited deficiencies,enhanced platelet activation,raised fibrin generation/deposition,latent or manifest vascular endothelium suffering/damage,immunological competence changes,increased complement activation,abnormal cytokines' release and up-expression or under-regulation of several cellular membrane receptors. Recently the genetic thrombohilia mutations have been invoked as causative agents for idiopathic recurrent abortions (ARI) as well as for repeated fetal losses (RFL),intrauterine growth retardation (IUGR), recurrent placenta abruption, early onset preeclampsia and oligohydramnios(so called maternal thrombophilias). Aims.To define the prerequisites for the genetic thrombophilias as adjunctive factors in women with ARI,RFL and/or maternal thrombophilias.To establish a suitable tests' ordering pattern. *Design and Methods*. 94 consecutive

apparently healthy fertile women,age ranging 16-52 years,with body mass index in normal average were considered.ARI from 2 to 9 and/or RFL at 2nd-3rd of pregnancy were referred in each subject.No infections,nor endocrinopathies,nor anatomic uterine malformations were documented.15/94 have had one birth in the list of their ARI and/or RFL.18/94 had venous tromboembolism episodes(>2)in juvenile age.Plama coagulation routine tests,LAC,PAI-1 and D-dimer were assayed.Anticardiolipin(ACA IgG-IgM),anti-phospholipid(APA IgG-IgM),anti-b2-glycoprotein-I(A-b2GP-I),anti-gliadine(AGA)antibodies and plasma homocysteine amount were determined.Genetic thrombophilia mutations(n=13) were performed by Reverse Dot Blo-Real Time PCR. Results.In 83/94 females a hypercoagulation pronesess and/or several thrombophilia mutations were seen: ATIII deficit(n=2),PC reduction(n=3),PS deficiency(n=57),LAC positivity(n=21),APA(n=21),A-b2GP-I(n=18),AGA(n=14),increased PAI-1(n=19),elevated(> 1200 mg/Lt) D-dimer(n= 46).Prothrombin mutation(n=29 etherozygousity and n=5 homozygous state),FV Leiden(Q506 n=19 etherozygous and n=4 homozygous condition),FV R2 aplotype(n=11 etherozygous state and n=2 homozygousity), combined FII and FV Leiden (n=9),MTHFR(C677T n=49 etherozygous and 21 homozygous state,A1298C n=21 etherozygous and n=13 homozygous, combined aplotypes n=18).Hyperhomocisteine (21-155 nmol/Lt) was found in 27/94 women. *Conclusions*.The pathogenetic mechanisms of ARI,RFL,IUGR and of so called maternal thrombophilias in apparently healthy fertile women remain of biggest concern. Predisposing/primary-secondary causes have to be considered. In this area, a wide hypercoagulation and heterogenous inherited thrombophilias are documented because of the patients' etherogeneity as well as laboratory variability.Furthermore,disparate perspectives of obstetrician,hematologist,reumatologist,internist persist until now in this scenario.Individual methodological laboratory approaches and an accurate clinical evaluation must be considered. The reasoned screening for hypercoagulability,the genetic thrombophilia profile and the immunological competence changes in conjuction with the sonography scanning pictures can define the etiopathogenesis of ARI,RFL,IUGR and the maternal thrombophilias to establish the therapeutical strategy.

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ENDOGENOUS THROMBIN POTENTIAL (ETP) AND COAGULATION MARKERS IN PATIENTS WITH AN ACUTE MYOCARDIAL INFRACTION ON ADMISSION

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Background. The ETP test, recently, was added to the conventional coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), for the investigation of coagulation function. ETP has been shown useful to detect an hypercoagulable state in individuals at risk of venous thrombosis. AIM: To investigate ETP parameters in acute myocardial infraction (AMI) and other coagulation markers in patients and controls. *Design and Methods*. 31 patients with AMI and 30 controls were included. Blood was sampled on admission. ETP parameters was measured by the chromogenic method on the fully automated Behring Coagulation System (BCS). Other markers were D-dimer and fibrinogen. RESULTS: 87.1% was male. Patients and controls had normal PT and aPTT. Table 1.

Table 1.

	CONTROLS		PATIENTS		
	median	sd	median	sd	p
tlagsec	19.3	2.6	41.4	28.4	**0.0001**
tmax sec	54.3	4.4	86.2	61.9	**0.007**
CmaxmA/min	123.5	6	124.2	35.1	0.916
ETPmA	394.7	29.5	352.6	70.5	**0.002**
FIBmg/dl	403.9	110.3	511.6	174.6	**0.008**
DDmg/l	0.27	0.15	1.22	2.2	**0.022**

Conclusions. Although AMI is an hypercoagulable state, ETP in AMI is comparable to the values of ETP in controls. ETP appears to take a rather width range of values in AMI. ETP reflects hypercoagulability in AMI similar to fibrinogen and d-dimers. For clinical validation of these findings a prospective clinical trial is warranted.

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SNEDDON SYNDROME IN A CHILD

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Background. Sneddon Syndrome is a rare disorder characterized by generalized livedo reticularis, multiple cerebrovascular events and ischemic stroke particularly in the territory of middle cerebral artery. This syndrome mainly affects women in early adult life and is very rare in childhood. *Aims*. We present a 4-year-old girl with Sneddon Syndrome. Case: A 4-year-old girl was admitted to our hospital with sudden onset of left sided weakness, right central facial paralysis. No fever , infection or arthralgia was described. She had livedo reticularis on physical examination and had a history of right sided optic atrophy. Her family history revealed two relatives diagnosed as Behçet's disease. There was no family history of trombosis, stroke or myocardial infarction. Her laboratory studies including clotting and thrombophilia screen were all normal. Her echocardiogram was normal. Magnetic resonance imaging revealed infarction in right anterior cerebral artery territory, which was on subacute phase, as well as right carotid artery signal void loss which is consistent with occlusion. Her skin biopsy was normal. The diagnosis is made by clinical and radiological findings. *Conclusions*. Sneddon Syndrome was first described by Champion in 1960 and Sneddon reported 6 cases with livedo reticularis, multiple cerebrovascular incidents of limited and benign nature. Sneddon syndrome is a rare syndrome with an incidence of 4 per million. The pathogenesis of this syndrome is unclear and different mechanisms such as thrombophilic, autoimmune or inflammatory vascular processes are considere. Sneddon Syndrome is rarely seen in children. Her skin biopsy was normal; there are reported cases with normal skin biopsies. The diagnosis of Sneddon Syndrome should be considered in presence of livedo reticularis and multiple cerebrovascular incidents.

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PATIENT INR RESPOND ON TREATMENT WITH OAT

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Background. To present the response to OAT of the patients in the Department of transfusiology Prilep. *Design and Methods*. 496 patients were treated with OAT (Oral anti coagulant therapy) during a period of 1 year. (Prothrombic Time) PT INR was measured once to twice a monthly. Mainly were treated with OAT patients with DVT(Deep vein Thrombosis), 245, (49%), DVT and PE(pulmonary Embolism) 47(9,4%), PAD 42(8,4%), cardiac and vascular implants 24+14 (7,6%), arrhythmias 29 (5,8%), prosthetic heart valve 18 (3,6%), by pass 17 (3,4%), CVI Cerebra vascular insult) 18 (3,5%), St post IMA(infarcts myocardial acute) 12, 8 thrombophilia patients (ATIII, FV Leiden, Protein C, MTHFR mutations) and other diagnosis 22 patients. *Results.* During period of 1 year, from 1-01-2008 till 31-12-2008, 6070 analysis were performed and PT INR measured were fro interval INR 0,9-1,4 = 2066 (34%); PT INR 1,5-1,7 1486 (24%); PT INR 1,8-2,0= 1078 (18%); PT INR 2,1-2,5 =882 (14,5%); PT INR 2,6 - 3,5 =420 (6,9%); PT INR 3,6-5 =97 (1,6%); PT INR > 5 =29 (1%). In the interval of 1,8 to 3,5 =2380 (39,4%). This shows that a high% have no satisfactory answer on treatment. We recognized that many patients do not complied to the treatment appropriately, mainly because the drug were not available on the pharmacy, or they do not have a money to by it. Some of them by only one pack of the drug, and take it one or two week, and when the control is performed they have not taka a tablets for more than a week. Some doesn't believe that they need to take tablets, so they just come to check their "thrombus" or perform a "trombotest", to check their thrombosis. But some of them take a high dose of drug, but they do not respond to the therapy. *Discusion*. There is a need to improve a patient education and patient compliance to the treatment. Better communication between Department and family (matical) doctors and other specialist involved in treatment (multidisciplinary approach) is needed. Screening patients for thrombophilia is also a need.

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MARKERS OF ENDOTHELIAL ACTIVATION IN THE PREDICTION OF SUBSEQUENT DEVELOPMENT OF PREECLAMPSIA

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Background. The hypertension and preeclampsia in pregnancy are multisystemic diseases characterized by hypertension, proteinuria and generalized systemic vasoconstriction.The ischaemia of the fetoplacental unit cause the release of specific factors into maternal vessels and subsequent activation of the endothelium and vasoconstriction . There is a rush development of the laboratory tests and a new markers of the endothelial activation have been found. Eg. t-PA, PAI-1, vWF, EPCR, thrombomodulin and endothelial microparticules with procoagulant activity. The aim of the study: To detect of above mentioned markers of endothelial activation in healthy pregnant women compared to those with pregnancy complicated by hypertension, diabetes mellitus and preeclampsia. The work hypothesis: We suppose that plasma specimens of the women with preeclampsia and diabetes mellitus will contatin a higher levels of endothelial activation markers compared to healthy pregnant. *Design and Methods*. All included patient have to assign an informed constent. The blood samlping will be taken by the routine way at the time of the first blood pregnancy sampling the end of the first trimester. The second specimen will be taken between 24.- 28. weeks of gestation. The following tests will be performed: t-PA - ELISA, PAI-1 - ELISA, vWF.Ag - EIA (immunologic detection by imunoturbidimetry), ePCR - ELISA , MMP-2,9 - ELISA (fluorogenic detection), endothelial microparticules - Flow cytometry. *Results*. The levels of vWf Ag and vW activity are significantly different (p=0,02 resp. 0,01) in group patietns with diabetes in comparison with control group. The levels of TRM are significantly different (p=0,03) in group patiens with hypertension as well as in group patietns with diabetes (p=0,02) in comparison with control group. The levels of ePCR are significantly different (p=0,04) in group patiens with hypertension as well as in group patietns with diabetes (p=0,01) in comparison with control group. The levels of tPA and PAI are not significantly different in group patiens with hypertension as well as in group patietns with diabetes in comparison with control group. *Conclusions*. As expected, there were stat. significant differences in the diabetes group in the case of vWf Ag, vWact., thrombomodulin and ePCR. Significant difference was found in the hypertension group compared to controls in ePCR. Whereas EMP, MMP-9 were not different in all groups.

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STUDY ON THE MOLECULAR PATHOGENESIS OF HEMOPHILIA B

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Background. The hemophilia B (HB), which is caused by the mutations in the factor β gene, is known as an X-linked recessive disease and occurs in about 1:30000 male live births. Clinical characteristics of this disease were the family history, spontaneous or injured bleeding and hemarthrosis. At present, due to lacking of the eradicative therapy, the gene diagnosis and detection of the carriers, which is an effective method to prevent the infant patients to be born, block the transmission of harmful gene and improve the population quality, should be actively carrying out. Aims The aim of this study is to diagnosis the propositi and carriers in the gene level and explore the molecule pathogenesy of HB. *Design and Methods*. The 3 unrelated HB families gave informed consent to be included in the study. The genome DNA were collected from each propositus and the family member. The polymorphisms of the 6 STR loci were detected by polymerase chain reaction with multiple reaction system and the fluorescently-labeled primers. For the propositi and doubtful carriers, all regions of the Fγ gene, including all exons and the flanking sequences, were amplified by PCR using the primer sequences which were devised with Primer 5. The products of PCR were sequenced by the dideoxy chain termination using ABI 3700 sequencer, the trial effect was compared with normal sequences using Chromas for finding the mutations. Results The 9 doubtful carriers were found through the allele analysis. But confirmed by the direct sequencing, the doubtful carriers of the first and third family did not have the same gene defects as the propositi, we considered that the gene defects were spontaneous mutations. To the second family's carrier, the heterozygous mutation was found in the corresponding locus of the propositus, it illustrated that the gene defect of the propositus was transmitted from his mother. 3 missense mutations were identified in the Fγ gene of the propositi when compared with normal sequence. G22119A was identified in exon 6 of the first family's propositus, it existed in the shearing situs of the flanking sequences, affected the normal physiologic function of F_. G7932C (Glu8Asp) was identified in exon 2 of the second family's propositus, it impacted Fγ binding with phospholipid. T32685C (Cys336Arg) was identified in exon 8 of the third family's propositus, it affected possibly the synthesis and secretion of Fγ intracellular. *Conclusions*. 1. The defect of the Fγ gene is the molecular pathogenesy of HB. 2. Combination analysis of multiple STR loci could be an effective and simple method for indirect diagnosis of the carriers in the HB family. However, there is possible to misdiagnose with the genetic linkage analysis for the families without the family history of HB. 3. Gene sequencing is one of the straightest and rigorousest method for the diagnosis of HB.

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A NEW CASE OF DYSFIBRINOGENEMIA Aα 13 GLY/GLU

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Background. Fibrinogen is a 340 kDa glycoprotein which is composed of six polypeptide chains. It plays a crucial role in blood coagulation. Serine protease thrombin cleaves fibrinogen to fibrin monomer and releases N-terminal parts of Aα and Bβ chains, termed fibrinopeptide A and fibrinopeptide B, respectively. Hereditary dysfibrinogenemia is a disease wherein an inherited abnormality in the fibrinogen molecule results in defective fibrin clot formation. *Aims.* The aim of our study was to characterize a case of a gene defect causing dysfibrinogenemia in a patient with abnormal coagulation test results. *Design and Methods*. Fibrin polymerization and fibrinolysis were measured by turbidimetrical method. Kinetics of fibrinopeptide release was measured by HPLC method according to Suttnar *et al.*[1] Gene sequencing was performed by dideoxysequencing method. Scanning electron microscopy (SEM) was performed on VEGA Plus TS 5135 electron microscope as described earlier.[2,3] *Results*. The patient - 36 year old woman had prolonged thrombin time (39.3 s) and low Clauss fibrinogen level (0.85 g/l). Fibrin polymerization was impaired and measurement of fibrinopeptide release showed higher rate of released fibrinopeptide B. The patient bears a heterozygous point mutation in exon 2 of FGA causing substitution of Aα 13 Gly to Glu. SEM revealed thicker fibres than control. *Conclusions*. The patient was found to bear a heterozygous point mutation in the fibrinogen Aα chain. Gly 13 is necessary for right fibrinopeptide A release by thrombin. Substitution Aα 13Glu was found to inhibit thrombin catalyzed fibrinopeptide A release. The mutation leads to impaired fibrinopeptide release and polymerization, abnormal fibrin clot and pathologic coagulation.

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LEVELS AND EFFECT OF ADRENOMEDULLIN ON ENDOTOXIN-INDUCED DISSEMINATED INTRAVASCULAR COAGULATION IN RABBITS

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Background. Disseminated intravascular coagulation (DIC) known as consumptive coagulopathy is an acquired hemostasis dysfunction, char-

acterized by systemic intravascular activation of coagulation, leading to widespread fibrin deposition in the circulation and impaired fibrinolysis. Adrenomedullin (ADM) is a peptide consisting of 52 amino acids and is secreted from vascular endothelial cells and adrenal medulla. There are only a few studies about the effects of ADM on coagulation and platelets which is secreted from endothelial cells. *Aims*. To develop an experimental animal model for the DIC, to assay ADM levels in rabbits with DIC and to investigate the probable effects of ADM in management of DIC. *Design and Methods*. Four groups (Control, DIC, Heparin, ADM) each consisting of 8 New Zeland rabbits were formed randomly. A standard DIC model was developed in DIC group by infusion of endotoxin from Escherichia coli. Than the effect of standard dose heparin infusion and 0.05 µg/kg/minute ADM infusion were evaluated in this model and therapy results were compared with statistical methods. *Results*. Mean platelet count was 101.130±66.038/mm^3, prothrombin time (PT) was 19.8±2.1 sec, activated partial thromboplastin time (aPTT) was 236.9±40.3 sec, fibrinogen level was 158.0±83.9 mg/dl, activity of antithrombin was 90.0±8.3%, and activity of protein C was 28.8±17.9% on sixth hour in DIC group. These results were in accordance with DIC findings and were significantly different from the findings of control group (p<0.05). In DIC group beginning, second and sixth hour mean ADM levels were 7.09±1.63, 8.10±1.55, 9.35±0.76 ng/dl, respectively. ADM levels on second and sixth were significantly higher than control group (p<0.05). Heparin and ADM groups were not significantly different for DIC group with therapy. *Conclusions*. A DIC model that can be use by alternative therapies was developed successfully. ADM levels were found elevated during the progress of DIC. Heparin and ADM were not effective in DIC therapy. New substances are thought to use in this therapy model in the future.

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SIGNIFICANT VARIATION IN ENDOGENOUS THROMBIN POTENTIAL (ETP) IN WARFARIN-TAKING PATIENTS

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Background. The ETP test, recently, was added to the conventional coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), for the investigation of coagulation function. The International Normalised Ratio (INR) derived from the PT is sensitive only to factor VII,X,V,II and I. In contrast, the ETP is sensitive to all the coagulation factors. We speculate that the same INR may not accurately reflect ETP in all patients and that ETP might be useful in monitoring warfarin anticoagulation. *Aims*. The investigation of correlation of ETP and INR in patients under stable warfarin therapy. *Design and Methods*. We studied 20 chronic atrial fibrillation patients who were stable on warfarin. Their INR was between 2.0-3.0. We performed the chromogenic method on the fully automated Behring Coagulation System (BCS) and determined the ETP (mA) and the peak amount of thrombin generation (Cmax,mA/min). We also determined the D-dimers and the fibrinogen. RESULTS: The ETP and the Cmax showed a strong inverse correlation with the INR (n=20; r= -0.611 and -0.515 respectively). There was a wide range of ETPs at this INR range (47.33-222.27 mA, median 149.0755mA). There was no correlation between the fibrinogen and ETP/Cmax. However, d-dimer show a significant correlation with ETP r=0.702. The correlation with the INR is weaker with d-dimers r= -0.393. *Conclusions.* Although the INR is a good surrogate, ETP measurement might be more accurate in estimating anticoagulation potential. Patients at therapeutic INR range may have low ETP values and high risk of bleeding or high ETP values and thrombotic risk.. Therefore, ETP might offer better differentiation between those who bleed more easily and those who develop thrombosis despite "therapeutic" anticoagulation. The correlation with d-dimer merits further investigation especially in respect to developing a predictive model for thrombosis prevention.

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IMMUNOTOLERANCE INDUCTION WITH VON WILLEBRAND FACTOR/FACTOR VIII CONCENTRATES IN PATIENTS AT HIGH RISK OF FAILURE

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Background. Inhibitor eradication with immune tolerance induction (ITI) is the best long-term therapeutic approach in haemophilia A patients with inhibitors. Several parameters are known that are correlated with success of ITI, such as the young age, a historical peak titer below 200 BU, inhibitor titer at ITI start below 10 BU. One that has more recently been proposed is the type of FVIII concentrate. Recent clinical findings indicate that plasma-derived factor VIII (FVIII) products containing von Willebrand factor (VWF/FVIII) could have an impact in ITI success rate, even in poor prognosis patients. *Design and Methods*. Two key prospective clinical studies have been designed to confirm the previous results supporting the use of VWF/FVIII in ITI: the RESIST experienced (RESISTexp) and the RESIST naïve (RESISTnaïve). RESISTexp is a prospective, nonrandomized study designed to assess rescue treatment with VWF/FVIII at high dosage (200 IU/kg daily) in patients who failed a previous ITI attempt with any dose of a VWF-free FVIII concentrate (plasma-derived or recombinant). RESISTnaïve is a prospective, controlled, randomized, open-label study comparing two types of FVIII concentrates (non-VWF-containing and VWF/FVIII) in their ability to induce tolerance in high responding haemophilia A patients, with no previous ITI attempt and with poor prognosis for success. Both types of concentrates will be administered at high dosage (200 IU/kg daily). Enrolment criteria are: severe haemophilia A (FVIII < 1%), any age, high responding inhibitors (peak inhibitor levels > 5 BU), any inhibitor level at study enrolment, and at least one of the following risk factors for ITI failure: (a) peak inhibitor titer > 200 BU, (b) titer at ITI start > 10 BU, (c) age > 7 years, (d) time between inhibitor occurrence and ITI > 2 years. Patients undergoing concomitant immunosuppressive treatment are not eligible for either study. Primary end point is the success in achieving ITI defined as: complete or partial, according to the complete disappearance of inhibitors with normal FVIII recovery and half-life or the presence of low titer inhibitors below 5 BU with low recovery and/or half-life. Secondary endpoints are: ITI maintenance, time to success, safety/compliance to treatment and cost of care. *Conclusions*. The results of RESIST studies will be crucial in understanding the role of VWF/FVIII in ITI outcome and will contribute to providing effective treatment for the devastating complication of FVIII antibody development in haemophilia A patients.

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A SYSTEMATIC REVIEW OF DISSEMINATED INTRAVASCULAR COAGULOPATHY ASSOCIATED WITH PROSTATE CANCER

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There are over 2 million men living in the United States with the diagnosis of prostate cancer (PC). Disseminated intravascular coagulation (DIC) is a catastrophic deregulation of coagulation that results in an overwhelming significant and sometimes fatal clinical syndrome. There are case reports of DIC in association with PC in the medical literature, but no rigorous study has been done. *Aims*. In this study we review the medical literature on DIC associated with PC to analyze the incidence, patient characteristics, clinical presentation, laboratory evaluation, diagnosis, treatment and outcomes of this syndrome. Methods: Published articles were screened through a MEDLINE search using the keywords "prostate cancer" and "disseminated intravascular coagulation" or "coagulopathy", and references from journal articles and books. Any English language article reporting any number of patients with DIC associated with PC was included for data extraction. Since most of the literature comprised of case reports and small series of patients, the heterogeneity of studies was too broad to allow any formal statistical calculation. The data was synthesized using descriptive methodology. Results: Several decades' worth of case reports and small series suggest that DIC associated with PC may not be as rare as previously thought. There is a noticeable distinction between more recent case reports and those prior to the mid-1990s; this might be related to aggressive screening for PC and early diagnosis. Older cases often have reported DIC as the first

manifestation of very advanced prostate cancer that either resulted in death or responded to castration. More recent reports have been of patients with known metastatic PC, who experienced the complication of DIC as their cancer progressed into castration-resistant PC that sometimes responded to chemotherapy. Treatment of DIC in the PC patient is challenging. The importance of treating the underlying PC has been highlighted in almost every article encountered during this review. Aminocaproic acid, an anti-fibrinolytic agent that works via inhibition of plasminogen activator, has been used successfully in some acute cases of DIC associated with PC. DIC in PC patients have been reported after surgical procedures, but improved surgical techniques have almost eliminated this complication. *Conclusions*. This study provides important information about a very serious complication of PC. Increased awareness of the potential development of DIC in patients with PC would allow aggressive medical intervention to prevent its devastating consequences.

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RAPID REVERSAL OF ORAL ANTICOAGULATION WITH WARFARIN BY A PROTHROMBIN COMPLEX CONCENTRATE AT DOSES OF 15IU/KG AND 30 IU/KG: EFFICACY AND SAFETY IN 36 PATIENTS

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Background. Rapid anticoagulant reversal for patients on oral anticoagulants is required for spontaneous life or organ threatening haemorrhage and for emergency surgery. Prothrombin complex concentrates (PCC) are now accepted as the quickest and most effective way to do this. The BCSH guidelines on warfarin recommend a dose of 50iu/kg but the optimal dose is uncertain due to lack of clinical and laboratory data 2. In this study we evaluated the efficacy in 36 patients requiring immediate reversal of their oral anticoagulant with 15iu/kg if INR <4 and 30 iu/kg if >4, using the PCC Octaplex. *Design and Methods*. Patients considered for immediate reversal of warfarin were discussed with the on-call consultant haematologist. A dose of PCC was recommended and a repeat INR and factor assay sample was requested for within an hour of treatment. All patients received 5mg IV Vit K. All 36 patients had repeat INR and 10 of these patients also had factors II, VII, IX, X analysed. The haemostasis laboratory uses a Sysmex analyser with Dode Behring reagents. The lab participates satisfactorily in the UK National External Quality Assessment Scheme (UK NEQAS). A retrospective inspection of the notes for arterial or venous thromboembolic disease during their hospital stay, clinical outcome and original need for warfarinisation was made. *Results*. Factor IIThe median (range) pre-treatment Factor II was 0.23 iu/mL (0.02-0.43) and post-treatment levels were 0.65 iu/mL (0.53-0.99). Factor VII The median (range) pre-treatment Factor VII was 0.18 iu/mL (0.01-0.49) and post-treatment levels were 0.39 (0.26-0.85). Factor XI The median (range) pre-treatment Factor IX was 0.25 (0.05-0.64) and post-treatment levels were 0.49 (0.31-0.92). Factor XThe median (range) pre-treatment Factor X was 0.065 (0.01-0.3) and post-treatment was 0.465 (0.23-0.76). INR The median (range) pre INR was 3.9 (2 - >10) and median post INR was 1.5 (1 - 2.8). INR ≤1.5 was achieved in 23 patients. Of the remaining patients only 3 had INR's >2. *Summary.* We present 36 patients who have had their INR reversed rapidly by dose controlled octaplex. No thromboembolic events were described. Ongoing bleeding in one patient may have contributed to one mortality. Of the 10 patients who had pre INR's of >10, only two had post INR of >1.5, and none had haemorrhagic deaths. The lack of thromboembolism and paucity of ongoing haemorrhage with overall reasonable reversal of anticoagulant effect could be supporting evidence that this is sufficient PCC to restore thrombo generation and reduce the risk of a hypercoagulable state with the higher doses (50iu/kg) normally recommended.

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THE AUTOMATED ENDOGENOUS THROMBIN POTENTIAL (ETP) TEST TO REFLECT COAGULATION CHANGES IN PATIENTS WITH CIRRHOSIS OF THE LIVER

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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. *Design and Methods*. 18 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, 6 HCV, 2 PBC and 4 cirrhosis of unknown origin. The patients with alcoholic cirrhosis had prolonged times and decreased Cmax and ETP compared to HCV cirrhosis. (tlag 22.65 vs 18.8, tmax 54.6 vs 55.5, Cmax 89.2 vs 99.6, ETP 268.6 vs 299.1, these observations were not statistical significants). *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 1.

Parameters	Patients	Controls	P Value
Antithrombin III	64.2 (23-98.9)	100 (79-122)	0.0001
protein C	62.2 (17-139)	105 (78-135)	0.0001
protein S	71.3 (24.6-89.2)	110 (66-157)	0.0001
lupus anticoagulant	1.05 (1-2)	1.1 (1-1.2)	0.2
plasminogen	77.6 (38-118.6)	107(79-132)	0.0001
a2-antiplasmin	79.16 (46-110)	105 (82-115)	0.0001
APCR	0.77 (0.64-0.91)	0.9 (0.65-1.02)	0.0001
homocystein	8.7 (1.3-14.7)	10 (7-13)	0.043
tlag	21.9 (12.7-51.1)	19.3 (12.5-26.37)	0.146
tmax	63.7 (40.3-116.2)	54.3 (45.5-62.1)	0.017
Cmax	93.7 (60.5-146.9)	123.5 (112.7-134.6)	0.0001
ETP	301.4 (202.3-472,7)	394.7 (322.7-451.1)	0.0001

1786

TRANSCATHETER ARTERIAL EMBOLIZATION OF CONCURRENT SPONTANEOUS HEMATOMAS OF THE RECTUS AND OBLIQUE LEFT ABDOMINAL MUSCLES IN A MODERATE HAEMOPHILIA B PATIENT

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Background. Cumulated evidence had proven that the substitution

therapy in haemophilia population according the prophylaxis regimens as well as the prompt infusion of concentrates had markedly reduced the occurrence of spontaneous muscle hematomas or their inevitable sequelae in terms of disability and/or signs of abdominal compartment syndrome or pseudotumor' late development. *Aims.* However, the incidence of life-threatening spontaneous or post-traumatic muscle hematomas of the anterior and/or posterior abdominal walls remain to be feared even in moderate haemophilia which commonly are less infused. Generally, the treatment is conservative and intensive by replacement therapy because of the difficulty of revealing or controlling the bleeding arterial vessel(s) surgically. Furthermore, in these cases the haemorrhage is often multifocal and involves complex collateral pathways. *Design and Methods*. We here report our recent observation of vaste concurrent spontaneous hematomas of the rectus and oblique left muscles of the anterior abdominal wall in a moderate hemophilia B patient 52 years old. By computed tomography(TC) an active and massive bleeding was documented from the epigastric artery with extravasation along the left retroperitoneum as far as the pelvic region. Despite the several infusions of FIX concentrates (AimaFIX® Kedrion) 19,000 IU/3 days, the epigastric artery haemorrhage lasted worrying. So, we considered to perform endovascular selective embolization by transcatheter arterial embolization (TAE)via transfemoral access to treat these muscle hematomas, as well as it has recently been done in non-hemophilia subjects i.e. in patients undergoing anticoagulation (*Basile A. et al. Cardiovascular and Interventional Radiology. 2004; 27:659-662*). In our case we used microcoils agreeing with the theory of Sharafuddin *et al.* (*J Vasc Interv Radiol 2001; 12:1231-1234*). Thus, because a part of the standard advantages (radiopacity, accuracy, and safer development), coils and microcoils are able to pack the entire length of the major supplying vessel(s), preventing retrograde filling from collaterals with selective arterial embolization. *Results*. TAE was technically successful. No active bleeding was detected by angiography after the procedure. *Conclusion/Summary*. No reports we found in the literature regarding TAE to treat muscle abdominis hematomas in haemophilia B patients. In our case the persistent and massive bleeding signs at CT as well as the unstable hemodynamic conditions of the patient indicated the use of angiography and the subsequent successful embolization to stop the severe haemorrhage and to prevent their inevitable sequelae. From this rare observation the embolization is effective and safe to control bleeding in these haemophiliac otherwise problematic cases where the conservative treatment seems to be insufficient and surgery can fail.

1787

THE PREVALENCE OF VON WILLEBRAND DISEASE IN WOMEN WITH MENORRHAGIA

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Background. Menorrhagia is one of the important clinical manifestations of von Willebrand disease (vWD), however, it is easily neglected by the general practitioners that vWD could be a cause of menorrhagia. Aims: We conducted a study in patients with unknown cause of menorrhagia, in order to find the prevalence of vWD in this group of patients. *Design and Methods*. After first check-up by gynecologists to rule out the gynecologic causes of menorrhagis, 94 women with unknown cause of menorrhagia were enrolled into the present study. Bleeding time (BT), aspirin BT, prothrombin time (PT), activated partial thromboplastin time (APTT), complete blood count (CBC), liver and renal functions, von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity (RCoA), factor VIII assay (FVIII:C) and ristocetin-induced platelet agglutination (RIPA) were detected in these women, their results are compared and analyzed. Results: 4 patients (4.3%) with low vWF:Ag (<50%) were detected, low RCoA (<50%) noted in 2 of them. RCoA/vWF:Ag <0.5 was found in 2 patients. Aspirin BT was not found to be better than BT. The best test for detecting vWD in this study is vWF:Ag. *Conclusions*. About 8.6% of the menorrhagic patients would have vWD, although the percentage is not high, the clinicians should be alert of this disease in patients with unknown cause of menorrhagia.

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CYP2C9 , VKORC1 AND CALU GENOTYPE FREQUENCIES IN THE NATIVE OMANI POPULATION

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Background. The utility of pre-prescription CYP2C9 and VKORC1 genotyping and the proposed pharmacogenomic algorithms have not yet been established in prospective randomized clinical trials. However, hereditary pharmacokinetic(CYP2C9) and pharmacodynamic(VKORC1) factors account for more that 50-60% inter individual variability of warfarin response.

Table.

Deviations from Hardy-Weinberg proportions with over representation of minor alleles

	Observed Genotype fequency	Calculated Genotype frequency	Chi Square P value
CYP2C9*1/*1	88.8	87.6	0.78[NS]
CYP2C9*2/*2	1.6	0.4	0.014[SIG]
CYP2C9*1/*2	9.6	11.98	0.0001[SIG]
CYP2C9*1/*3	10.4	10.28	0.81[NS]
CYP2C9*3/*3	0.8	0.36	0.001[SIG]
VKORC1*1/*1	43.41	41.9	0.17[NS]
VKORC1*1/*mut	42.64	45.66	0.007[SIG]
VKORC1*mu/*mu	13.95	12.44	0.006[SIG]
CALUArg4/Arg4	76.27	76.93	0.68[NS]
CALUArg4/Glu4	22.88	20.06	0.0001[SIG]
CALUGlu4/Glu4	0.85	1.3	0.0028[SIG]
CALUa29809a	41.53	41.48	0.96[NS]
CALUa29809g	45.76	45.85	0.95[NS]
CALUg29809g	12.71	12.66	0.94[NS]

Aims. To establish the CYP2C9, VKORC1 and CALU genotype frequencies in the native Omani population which consists of an admixture of African and Asian ethnicities. *Design and Methods*. In 240 Omani subjects(volunteer blood donors) we assessed CYP2C9 genotypes(*1,*2 &*3), VKORC1 haplotypes (*1[A] v/s NonA], Calumenin (CALU) Arg4Gln(rs2290228) and a29809g polymorphisms. Genomic DNA was isolated using the semi-automated 6100 nucleic acid extractor. CYP2C9, VKORC1 and CALU genotypes were defined by direct sequencing using primers designed by Primer Express software (ABI 3100 Genetic analyzer). *Results*. Observed allele frequencies for CYP2C9*1(wild type), CYP2C9*2& CYP2C9*3 were 88.8, 0.16 and 0.08 respectively in this population. Allele frequencies for VKORC1*1 (wild type) was 43.4. Allele frequencies for CALU Arg4Arg(wild type) 76.27 whereas for the a29809a(wild type) it was 41.53. Genotype data was tested for deviation from Hardy-Weinberg proportions using weighted least square estimates of allele frequencies and chi-square goodness-of-fit tests and showed significant over representation of the minor alleles. *Conclusions*. This study establishes the population allele frequencies of main genetic variables associated with pharmacokinetic(CYP2C9) and pharmacodynamic(VKORC1) variable responsible for inter individual variations in warfarin dosing. The increased practice and prevalence of consanguinity in the local culture and customs are probably responsible for the significant deviations from the Hardy-Weinberg proportions indicating nonrandom mating.

1789

PRIMARY OR SECOND ATTEMPT OF IMMUNE TOLERANCE INDUCTION IN PATIENTS WITH HEMOPHILIA A WITH INHIBITORS WITH A FACTOR VIII/ VON WILLEBRAND FACTOR HUMAN COMPLEX (FANHDI®). RETROSPECTIVE DATA COLLECTION STUDY FROM 14 SPANISH CENTERS

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Background. The prevalence of inhibitor development in Hemophilia A (HA) patients is as high as 33%. Currently, inhibitor eradication with immune tolerance induction (ITI) is the best long-term therapeutic alternative. Due to the number of parameters potentially related to the ITI

success, as well as to the inherent complexity of the ITI procedure, therapeutic optimization is a priority. Several parameters are known to be potential ITI outcome predictors such as the type of administered FVIII concentrate. Recent experimental and clinical findings indicate that plasma-derived FVIII products (pd-FVIII) containing von Willebrand factor (VWF/FVIII) could have an impact in ITI success rate, even in poor prognosis patients. *Aims*. Evaluate the rates of success, partial success, and failure among severe hemophilia A patients with inhibitors, who received a plasma-derived FVIII product (Fanhdi®) as a part of either a primary or rescue ITI protocol. *Design and Methods*. This is a multi-center, observational, retrospective chart review study of patients with severe Hemophilia A and factor VIII inhibitors who have been treated with Fanhdi® as part of their ITI treatment. The study will evaluate the ITI outcomes of around 14 centres and approximately 30 Fanhdi® patients with a known outcome of complete succcess, partial success or treatment failure. Patient population are diagnosed with congenital HA (FVIII < 2%) submitted to immunotolerance protocol with high purify FVIII/VWF concentrates (Fanhdi®) and who have completed primary ITI or are still ongoing (naïve patients) or who have had previously undergone at least one ITI procedure and failed, partially or totally (according to the criteria of the physician carrying out the ITI process). *Conclusions.* The results of this retrospective study will add valuable information in clarifying the role of VWF/FVIII in the success of primary or salvage ITI.

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HAEMOSTATIC ABNORMALITIES IN TREATMENT-NAIVE TYPE 1 GAUCHER PATIENTS

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Background. Gaucher disease (GD) is the most common lysosomal storage disorder, caused by glucocerebrosidase deficiency. Gaucher patients may suffer from bleeding, which is usually attributed to thrombocytopenia; however, excessive bleeding has also been observed in patients with normal platelet count. It is postulated that coagulation factor deficiencies and platelet dysfunction might also contribute to bleeding tendency in GD patients. *Aim.* The aim of this study was to investigate platelet function and coagulation factors in Serbian, type 1 Gaucher patients who had not been treated with enzyme replacement therapy. *Design and Methods*. The study was carried out on 31 Serbian treatment-naive type 1 Gaucher patients (M/F 17/14; median age 49; splenectomized 9/31). Complete blood counts, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were assessed according to standard methods. Coagulation factor activity was measured using commercial kits. Platelet aggregation was performed by a standard technique, using citrated platelet rich plasma, on a whole-blood aggregometer (Chrono-Log Corporation, Havertown, model 560, PA, USE). Results: Bleeding episodes were registered in 10/31 patients. Mean platelet count was $150x10^9$/L (range: 46-428); 22/31 patients had platelet count < $150x10^9$/L and 3/22 < $50x10^9$/L. Platelet count inversely correlated with spleen volume ($p<0.05$). PT and PTT were prolonged in 16/31 and in 13/31 of patients respectively. The most frequent clotting factor deficiencies (either isolated or combined) were: FV (9/31), vWF (5/31), FVIII (3/31), FX (3/31), FXI (4/31), and FXII (5/31). Platelet aggregation abnormalities were registered in 19/31 patients. Abnormal aggregation in response to 1 and ≥2 agents was registered in 13 and 6 patients, respectively. Patients with bleeding episodes had significantly lower platelet counts, higher plasma chitotriosidase levels (a specific marker of storage glucocerebroside) and greater spleen volumes compared to the non-bleeding patients ($p<0.01$). There was no significant difference in the clotting factor concentrations and platelet function between the bleeding and non-bleeding phenotype. None of the splenectomized patients had bleeding episodes. In addition, the non-splenectomized patients had significantly lower platelet count ($94x10^9$/L vs. $286x10^9$/L) and reduced response to collagen (0.55 vs. 0.79) compared to the splenectomized ones. Spleen volume inversely correlated with a reduced aggregation response to collagen, adenosine diphosphate in lower concentrations (5µg/ml), and arachidonic acid. *Conclusions*. GD type 1 is associated with decreased levels of coagulation factors and abnormal platelet function, which is a contributory factor in increased risk of bleeding.

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OFF LABEL USE OF RECOMBINANT FACTOR VIIA IN THE HAEMATOLOGY WARD

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Recombinant factor VIIa (rFVIIa) has proven effective in treating bleeding in patients with haemophilia and inhibitor. Use of rFVIIa in other clinical conditions, like in patients with intractable bleeding and other serious haematological disorders, has not been studied in larger cohort of patients. The aim of this retrospective study is to investigate conditions and efficacy of rFVIIa in haematological patients with severe bleeding. The study included all off-label patients since 2005, which were treated in the Clinic of haematology Novi Sad. In the assessment of efficacy, our scoring system which combines clinical and laboratory, radiological or endoscopic data, was used. There were 18 patients (9 with acute leukemia, 3 with nonHodgkin lymphoma, 1 with chronic lymhocytic leukemia, 2 with aplastic anaemia, 1 with paroxysmal nocturnal haemoglobinuria, 1 with vonWillebrand disease and 1 with adult-onset Still disease). The most of them, 9 patients (50%) had severe gastrointestinal bleeding, 4 (22%) had soft-tissue haemathomas (neck, diffuse skin or perianal), 3 (17%) had intracranial bleeding and 2 (11%) respiratory tract bleeding. When the usual dose of rFVIIa (80-100 µg/kgBM) had been used, good efficacy was registered in more then 80% of patients. Low dose regimen (40 µg/kgBM or less) was associated with treatment failure. Although almost all patients received platelet transfusions, rFVIIa has been effective, even in severely thrombocytopenic patients. Conclusion- In our small group of haematological patients with severe haemorrhagic diathesis, rFVIIa proved effective, if given in usual dose and with platelet transfusions.

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FACTOR V DEFICIENCY AND CROHN'S DISEASE - A CASE REPORT.

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Background. Factor V deficiency is a rare inherited coagulopathy. Clinical presentation include ecchymoses, mucosal bleeding and blood loss. Factor V deficiency is caused by a large number of genetic abnormalities. The deficiency is a rare bleeding disorder whose genetic bases have been characterized in only a limited number of cases. The inheritance of factor V deficiency is autosomal recessive, with varying expressivity in the heterozygote; however, other modes of inheritance have been described. Heterozygotes have lower levels of factor V but probably never bleed abnormally. Consanguinity has been observed in families with factor V deficiency. Heterozygous deficiency states are generally unrecognized because of a lack of significant clotting time prolongation or bleeding risk. Presentation: A 23 year old gentleman with inflammatory bowel disease presented with pancytopenia. Initial full blood count showed haemoglobin 4.3g/dl, MCV 111.7, white cell count of 0.5, absolute neutrophil count of 0.3 and platelets of 125. He was diagnosed with Crohn's disease at the age of 14. His treatment included mesalazine 800mg TID and azathioprine 75mg od. He didn't have a blood count done for 18 months prior presentation with pancytopenia. Bone marrow biopsy showed a markedly hypoplastic marrow with reduced haematopoiesis and no evidence of infiltrate or malignancy. He did not have any symptoms or signs of coagulopathy at the time of presentation or in the past. He required treatment for neutropenic sepsis including intensive care facilities. He recovered well on intravenous tazobactam / gentamycine and G-CSF. He also required multiple red cell transfusion. He was found to have a prolonged prothrombin time, INR and APTT despite treatment with Vitamin K. A low factor V was noted (7%) and this was present on repeat studies following the recovery of his marrow and quiescence of his disease. All other factors investigated were normal (II VIII X IX XI XII). However, despite a low factor V level, the patient remained asymptomatic and did not required any intervention. Patient received routine vaccinations in line with current guidelines. Currently he is doing very well, his full blood count has recovered and patient does not require any haematological interventions. *Discussion*: Factor V deficiency is a rare coagulation abnormality and is associated with hemorrhagic complications of varying severity. Only a limited number of cases are reported in the medical literature. There are no report of an association with inflammatory bowel disease and factor V deficiency so far. Interestingly our patient never had any symptoms related to factor V deficiency and never required any treatment for it.

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SUCCESSFUL USE OF VINCRISTINE IN THE MANAGEMENT OF KASABACH-MERRITT PHENOMENON

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Kasabach-Merritt Phenomenon (KMP) is a life threatening clinical picture characterized by thrombocytopenia, consumption coagulopathy with a low fibrinogen level and microangiopathic hemolytic anemia.in the presence of a rapidly enlarging vascular lesion. Affected infants may manifest high output cardiac failure as a result of increased blood flow through the vascular lesions. It can be lethal; the estimated overall mortality rate ranges from 10-37%. Kaposiform hemangioendothelioma is the responsible lesion most of the time, Retroperitoneal involvement is significant determinant of the mortality. Early treatment is important to prevent fatal bleeding.MRI and ultrasonography are both effective tools for determining the extension of the lesion hence the prognosis and and for assessing their response to therapy.We would like to present a 2 month old boy with KMS, successfully treated with vincristine therapy. *Case report.* A 2-month old boy with Kaposiform Hemangioendothelioma of the lumbosacral region invading the retroperitoneum was admitted with Kasabach-Merritt Phenomenon After corticosteroid and interferon treatments failed with progression of the disease to a life threatening condition, vincristine monotreatment granted a rapid rise in platelet counts, fibrinogen levels and a resolution in the enlargement of the lesion. Once a week induction treatment for a month was followed by once a month maintenance for 2 months. The remission was maintained and no adverse effects were observed during the treatment. The patient did not relapse 2 months after vincristine was stopped. *Conclusion*. This case and review of the literature point to vincristine as a first line treatment and suggest that multidrug regimens with additional drugs should be hold for bleeding emergencies.

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FERRITIN SERUM LEVELS IN BLOOD DONORS POPULATION

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Aims. The aim of this study is to determine the serum ferritin levels in a population of blood donors especially of those who donate blood two or three times per year. The evaluation of iron stores and the application of protocols concerning the frequency of ferritin serum measurement. *Design and Methods*.Were examined 280 blood donors ,156 men (aged 20-65 median age 40) and 124 women (aged 20-65 median age 37,4) . They were subdivided into two groups : Group A (n:185) consisted of regular blood donors with a frequency of blood donation of 2-3 times per year and Group B consisted of occasional blood donors who donate blood for a friend or relative.

Table.

Ferritine ng/ml	Regular Blood donors (n=185) 122 M+63W		Occasional Blood donors(n=95) 34M+61W	
>70	17	9.1%	40	42.1%
40-70	19	10.2%	4	4.2 %
30-40	33	17.8%	6	6.3%
20-30	35	18.9%	14	14,7%
<20	81	43.7%	31	32.6%

All of them filled out the blood donor form and were examined for serum ferritine levels. The determination of ferritin was done with reagents Beckman Coulter Access Ferritin in the analyzer Access . The expected normal values of ferritin : 25-320 ng/mL. *Results.* In Group A only 9.1% were found with ferritin levels > 70 ng/mL , for 46.9% the ferritin was 20-70 ng/mL and for 43.7% the ferritin level was found extremely low < 20 ng/mL meaning empty iron stores. In Group B , 42.1% were found with ferritin levels > 70 ng/ml, for 25.2% the ferritin was 20-70 ng/mL and for 32.6% the ferritin level was found < 20 ng/ml. *Conclusions*. It is obvious that the percentage of regular blood donors with ferritin levels <20 ng/mL is very high(43.7) and for this we have to take greater for them because their offer is significant. The regular blood donors have lower ferritin levels than the occasional blood donors regarding the times of blood donation per year. The determination of ferritin levels in all regular blood donors has been programmed for once every six months . If the ferritin is < 20 ng/mL, it is proposed : limitating the donation, treatment with ferrum and re-examination before the next donation.

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THE EFFECT OF PENTOXIFYLINE ON THROMBOCYTE FUNCTIONS, DURING THE CARDIOPULMONER BYPASS AND CORONER ARTER SURGERY

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Background and Aims. Cardiopulmoner bypass (CPB) surgery caused endotelial injury and tissue edema because of the pathophysiologic reactions on thrombocyte functions. The aim of this study was to investigate the effect of using intraoperative pentoxifylline on the clinic findings and thrombocyte function. *Design and Methods*. Twenty patients were randomly selected among patients undergoing coronary bypass surgery. The effect of pentoxifylline on the thrombocyte number, platelet factor 4(PF4), mean platelet volume (MPV)and blood film were determined. The results were examined during the four periods of CPB surgery (1st period was anesthesia induction period-T1, 2nd period was after 30 minutes of surgery-T2, 3rd and 4th periods were after six and twentyfour hours of surgery-T3,T4) 100 mg pentoxifylline in% 0.9 isotonic was given in 180 minutes during the anesthesia induction with the warm cardioplegy .% 0.9 isotonic was given as placebo to 10 other patients. PLT and MPV were determined in automatic blood counters(CoulterLH 750, UK), PF4 levels were studied by ELISA (Stago, France).Blood film examination was done by the haematology doctor. *Results*. Platelet number was showed no statistically difference between two groups in T4 period, but in the group which pentoxifylline used, the platelet number was lower than other groups. In both group PF4 levels were found increased, but in T3 period the PF4 levels in patients were statistically higher than controls. MPV showed no difference between the groups. *Conclusions*. We concluded that, pentoxifylline could inhibite the thrombocyte activation particularly which was started during CPB surgery. But this effect wasn't enough to decrease complications such as hemorrhage and the ned of transfusion .

1796

SUCCESSFUL BURKITT'S LYMPHOMA TREATMENT DESPITE HPA-1A ALLOIMMUNIZATION

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Background. Some hematologic malignancies, such as Burkitt's lymphoma, must be treated with very intensive chemotherapy, thus requiring exhaustive transfusional support. Problems in finding compatible blood or platelets might hinder therapy administration. HPA1a antigen is part of most people's platelets; anti HPA-1a antibodies can arise in negative individuals after pregnancy or transfusion, causing post transfusional purpura or neonatal alloinmune thrombocytopenia in newborns. Once antibodies appear, blood or platelets must be transfused from HPA-1a negative donors although some alternative strategies must be used. Case report: We present a case of a 28 year old female patient with HPA-1a antibodies that caused prior neonatal alloinmune thrombocytopenia. She was diagnosed of Burkitt's lymphoma at stage IV A. Initial staging showed retroperitoneal masses extending to pancreas, stomach and left ovary. Additionally a right breast mass was confirmed to be involved by lymphoma. Bone marrow biopsy and cerebrospinal fluid were free of disease. Intensive chemotherapy was initiated according to Burkimab-08 Pethema protocol. This included high dose methotrexate, cyclophosphamide and cytarabine, so profound cytopenias were expected, therefore requiring intensive blood and platelet support. In order to minimize transfusion requirements erythropoietin was administered at maximum dose (30000 IU of Epoetin β twice weekly)

as well as parenteral iron therapy and antifibrinolytic drugs to avoid bleeding associated with thrombocytopenia. All chemotherapy was administered timely and complete remission was obtained quickly. Blood components transfused during therapy were as follows: -Two blood units from HPA1a positive donors, previously washed -Four blood units from HPA1a negative donors -Two platelet pheresis units from one HPA1a negative donor (same as one blood unit) Haematologic recovery after transfusion were as expected and no adverse reactions occurred during or following transfusion. *Conclusions*. Despite being a drawback, HPA-1a antibody has not precluded intensive chemotherapy administration. Washed blood components are an alternative to HPA-1a negative donor transfusion. Other support therapies such as erythropoietin and antifibrinolytics are of valuable help.

1797

BLOOD DONATION AND BLOOD TRANSFUSION IN SPAIN (1997 - 2007): POSSIBLE EFFECTS OF UNIVERSAL LEUKOREDUCTION?

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Background. As epidemiological information is useful in planning the production and assessing the efficiency of product use, we reviewed the Spanish data on population, blood donation and blood component transfusion from 1997 to 2007, and the possible influence universal leukoreduction (ULR) on them.

Table.

Per 1000 population per year	Before ULR mean ± SD	After ULR mean ± SD	Difference Mean (95% CI)	P
RBCT (units)				
•Overall	30.1 ± 0.6	32.5 ± 0.5	2.5 (1.63 – 3.28)	0.001
•< 65 years	14.4 ± 0.3	15.7 ± 0.2	1.3 (0.81 – 1.68)	0.001
•≥ 65 years*	108.5 ± 2.4	115.0 ± 3.1	6.5 (2.41– 10.61)	0.006
Platelets (doses)**	2.7 ± 0.1	2.9 ± 0.2	0.2 (0.03 – 0.36)	0.024
FFP (units)***	5.3 ± 0.1	5.6 ± 0.3	0.3 (0.01 – 0.60)	0.045

Design and Methods. Data of the Spanish population were obtained from the National Institute of Statistics, and data blood donation and blood component transfusion from the Spanish Ministry of Health and Consume. Results. Along the study period, the Spanish population increased by 5.6 millions (14.4%), and blood donation by 28.1%, although red blood cell obtained increased only by 21.5% whereas red blood cell transfusion (RBCT) increased by 28.3%. However, RBCT rate was significantly higher after the implementation of ULR (2002 - 2006) when compared to the pre-ULR period (1997 - 2001) (Difference = 2.54 units/1000 population/year; 95%CI 1.81 - 3.27; $p<0.001$) (Table 1). No clinically relevant differences were observed for platelet or plasma transfusion. *Conclusions*. The increase observed in RBCT index after implementation of ULR may have been due to a reduction of the haemoglobin content in the RBC units. Therefore, our data on blood use seems to add to the case against ULR, which has led to an incremental cost at unknown and likely low benefits for the patient.

1798

LOW PREVALENCE OF ANTIBODIES TO HUMAN T-LYMPHOTROPIC VIRUS TYPE I AND II IN VOLUNTEER BLOOD DONORS IN A DEFINED AREA OF NORTHERN GREECE

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Background. TLV I/II infection is aetiologically associated with adult T-cell leukemia/lymphoma and HTLV-associated myelopathy/tropical spastic paraparesis. Blood donors are systematically screened for antibodies to human T-cell lymphotropic viruses type I and II (HTLV-I and HTLV-II) in Greece since 1995. AIMS:The aim of our study is to evaluate retrospectively the prevalence of HTLV I/II infection among the healthy blood donors of the area of Giannitsa of Northern Greece. *Design and Methods*. During the last eleven years (from September 1997 to September 2008) sera from 28254 consecutive blood donors were initially screened for anti-HTLV I/II antibodies using a comercially available immunoassay (sandwich ELISA- MUREX HTLV I+II®, Abbot, Germany). The reactive samples were further examined by a Western blot (WB) assay (HTLV BLOT 2.4, MP Diagnostics,) which detects specific IgG antibodies against HTLV-I and HTLV-II viruses, as a confirmatory test. RESULTS: Totally, only 6 serum samples were found to be reactive for anti-HTLV I/II antibodies by the ELISA, whereas only one was confirmed as positive by the WB assay. Thus, the prevalence of HTLV I/II infection among blood donors of our area is very low (0.00003%). *Conclusions* The practically zero prevalence of HTLV I/II infection among healthy blood donors of our area, during an eleven-year period of time, clearly demonstrates that this province of Northern Greece is not an endemic area for HTLV I/II. This result is consistent with the findings of other surveys conducted in different areas of Greece and poses the question of the necessity of routine screening for anti- HTLV I/II antibodies of the blood donors in Greece.

1799

DECLINING TENDENCY OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I CARRIER RATES AMONG BLOOD DONORS IN MASHHAD, IRAN

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Background. Human T-cell leukemia virus type I (HTLV-I) is a blood-transmissble retrovirus unevenly distributed around the world. Recently North East Iran, particularly the region of Mashhad, has been recognized as a new endemic region with 2-3% of the population and 0.7% of the blood donors infected with HTLV-I .Since 1996, all donated blood samples in the blood transfusion center of Mashhad, Iran, are routinely screened for HTLV-I. Aims: We studied the results of screening blood donors of Mashhad for antibodies to HTLV-I to understand the prevalence of this infecion during 3 years. *Design and Methods*. We evaluated and reported the results of screening 232,648 blood donors in Mashhad for antibodies to HTLV-I, from January 2004 to December 2006 which were taken from data sheets. *Results*. The donors were 90% male (n=211,265) and 10% female (n=21,383), with a mean age of 30 years (range: 18-65). The number of blood donations was 73489, 77502 and 81657 in the years of 2004, 2005 and 2006, respectively. Anti-HTLV-I antibody was performed by using ELISA. The positive samples were confirmed by Western blot (WB) test. Among the 232,648 blood donors analyzed, 1054 (0.45%) were repeatedly reactive by ELISA and confirmed by WB testing. The numbers of HTLV-I seropositive blood samples were 365, 343 and 346 in 2004, 2005 and 2006, respectively with a prevalence of 0.5%, 0.44% and 0.42% in these years. One hundred sixty three out of 1054 seropositive donors were females (15%) and 891 were males (85%). The prevalence of HTLV-I infection in males and females was 0.42% and 0.76% respectively. *Conclusions*. This decline in HTLV-I prevalence in Mashhad, from 0.77% in 1999 to 0.42% in 2006, could be attributed to better strategies for donor screening, sufficient education to the population about the routes of transmission and modes of protection, and other unknown reasons which need more studies to be determined.

1800

THE ROLE OF THERAPEUTIC APHERESIS IN THE TREATMENT OF IMMUNE-MEDIATED DISORDERS

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Background. Therapeutic plasma exchange (TPE) is an apheresis procedure which guarantees high-level blood purification when removed plasma is replaced with normal plasma or saline and/or albumin. The basic goal of TPE is to reduce the patient`s load with pathogen(s), i.e. autoantibodies, cytokines, and other immune mediators and modulators to levels that will allow improvement and/or to supply an essential substance that is absent from patient`s plasma. *Aims*. To investigate of clinical efficacy of TPE, in combination with drug therapy, on the course and overall aucome of different immune-mediated disorders. *Design and Methods*. As the part of the management program, TPE (combined with anti-inflammatory and immunosuppressive agents) was applied in the treatment of patients with different immune-mediated disorders: myasthenia gravis, acute polyradiculoneuropathy, polymiositis, systemic lupus erythematousus, syndrome Goodpasture, progressive glomerulonephritis, hypersensitive vasculitis, lupus like syndrome, C1q-deficiency, cold agglutinin disease, autoimmune neutropenia, autoimmune trombocythopenia and autoimmune hemolytic anemia. TPE-procedures were performed by Cobe Spectra every other day, occasionally daily. The volume of exchanged plasma was: 1.0-1.5 patient's plasma volume (2.95 L in average) during one TPE-procedure and 3-5 patient's circulating plasma volume (21.4 L in average) during the whole treatment. The replacement fluids used were 5% and 20% albumin and physiological saline solution. *Results.* The use of TPE resulted with significant positive effect on clinical findings and some "para-clinical" events in patients with different immune-mediated disorders. In this group of patients disappearance or reversal of some symptoms and findings was obtained. To be precise, immune-complex removal, modulation of immune-mediators, correction of cellular immunity, improved circulation in targeted organs, renal function restitution, disappearance of arthralgia, viscosity reduction, disappearance of purpura and skin ulcer healing or complete clinical remission were observed. *Summary and conclusions*. TPE-procedures were effective, but not associated with long-term remission of disorders. The efficacy of TPE-treatment depends on the nature and stage of the basic disease, of adequate selection of patients and of timely applied apheresis procedures.

1801

ANALYSIS OF TURBID PLASMA DONATIONS

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Plasma units are used for transfusion and for manufacturing of plasma derived products. Turbid plasma units like "milky white" are refused and destroyed. We investigated some donors characteristics. Material and *Design and Methods*. In our routine work, plasma unit is defined as turbid by visual inspection. The total incidence due to this problem last year was 4.1% from whole blood donation (113.724 WB donation) and 1.65% from single donation (7.996 SD donation). Between April and August 2008, plasma samples from WB (n=20) and from SD (n=20) with evident "milky" appearance were frozen. Samples of plasma and serum were analyzed in both donations but in case of apheresis platelet supernatant was analyzed too. Chemistries for triglyceride (TG) and cholesterol (Ch) levels were determined by enzymatic method, LPLasa-POD and CHOD-PAP respectively, supplied by Dade Behrring SA. The reference values for adults are triglyceride <150 mg/dL (<1.7 mmol/L) and cholesterol <200 mg/dL (<5.2 mmol/L). *Results*. The values are shown in media, range and median (see table below) We observed high triglyceride level with a normal cholesterol level in samples from serum and plasma. The TG level in supernatant platelet is irrelevant because the platelet concentrate is stored in additive solution, therefore the residual plasma was approximately 32%. Donations provided by men were more often turbid than those provided by women, in the report 37 men and 3 women. In our experience the total incidence had not relation to donation month but was related to dietetic habits. In this study, 52% of donors gave a turbid plasma more than three times of their previous donations. Therefore it is important that the donor of a "milky unit" is advised not to eat fatty food before their next donation. *Conclusions*. The incidence of turbid plasma donation depends on donor characteristics such as gender, nutritional factors and individual metabolic factors. We need to establish some procedures in order to decrease the amount of wasted plasma units, this could include donor questionnaire concerning dislipemic history, dietetic recommendation, and perhaps to promote and to facilitate plasmapheresis donation in young female donors to produce plasma derived products.

Table.

Age	Weight Kg	TG serum	TG plasma	Ch serum	Ch plasma
40.6 (27-63) 40.5	87 (70-107) 86.5	774 (252-2760) 658	660.5 (258-2160) 538	168 (88-246) 165	134 (81-179) 133.5

1802

WAA APHERESIS REGISTER - RESULTS IN CZECH REPUBLIC

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Background. Therapeutic aphereses are important contribution of transfusion medicine to the therapy of some serious diseases. They are performed in 7 centres in Czech Republic. Two of them have been registered their procedures in WAA register. One centre (I) is located in Moravia (Ostrava/Frydek) and the other (II) in Bohemia (Hradec Králové). We give a report on this activities. *Design and Methods*. WAA is a webb-based registry. So far 205 patients (672 procedures) have been included. A median of 3,3 treatments have been performed (1-26). Main indications: haematological and lipid apheresis, stem cell collection (autologous, less from donors) and neurological. Both centres are including their procedures regularly. Results: The reason of apheresis was therapeutic in 83% and retrieval of blood components in 17%. Blood access: mainly peripheral vessels (90%), central catheter through subclavia route or femoralis vein (9%) and AV fistula (in 1% only). Anticoagulant was ACD-A mainly. Replacement fluid was mainly by albumin. Advers events were registered in centre I in 3, 1%, in centre II in 5, 6% of procedures. They were clinically not relevant and no death occurred due to treatment. The connection with the leading centre in Sweden was without problems. The administrator was able to collect data to the end of each year or for the separate centres upon request. The actual version of WAA register is "user friendly", data submission are accompanied by a clear help. To enter data of one performed procedure usually lasts about 2-3 minutes. We can summarize that there were no significant differences between the centre I and II. *Conclusions*. Analyses of data in WAA register enables improvement of quality of apheresis. To learn more about various indications for apheresis as well as learn about the risks and to reduce adverse events, the use of WAA register is helpful as a quality assurance measure.

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1803

PREVALENCE OF HEPATITIS C VIRUS (HCV) IN PATIENTS POLYTRANSFUSED

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Background. The incidence of HCV transmission through transfusion is estimated 1/700 000. The aim of this work is to assess the prevalence in patients polytransfused followed in the department of hematology and oncology pediatric from CHU Ibn Rochd for various hematological affections and after the introduction of the regional blood transfusion of Casablanca (CRTS) a new screening test to the combined search of the Ag-Ac HVC. *Design and Methods*. Patients candidates for polytransfusion are taken before any transfusion. They must be seronegative. The levies are renewed every 2 months to detect any seroconversion. Results: From May 2007 to Mars 2008, 100 patients were collected. All patients are HCV-negative with a follow up of 10 months for 36 of them. Discussion: Before the introduction of screening for hepatitis C among blood donors, the prevalence of HCV in patients polytransfused was 26.6%. This percentage was significantly reduced to 7% after the screening started in July 1994 to reach 1.6% with reagents of the 3rd generation introduced in 2003. *Conclusions*. The risk of transmission of HCV in the blood transfusion has become very small due to the constant improvement of the recruitment and selection of donors, and the progress being made in screening techniques which became with high performance.

1804

B12 AND FOLATE ACID SERUM LEVELS IN BLOOD DONORS POPULATION

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Background. The aim of this study is to determine the serum B12 and Folate levels in a population of blood donors. For the evaluation of these levels, the nutritional abilities and the alcool consumption of the blood donors were used. Method : 280 blood donors were tested ,156 men (aged 20-65 median age 40) and 124 women (aged 20-65 median age 37,4). They were subdivided into two groups : Group A (n:156) consisted of male blood donors and Group B (n: 124) consisted of female blood donors. All of them filled out the blood donor form and a questionnaire concerning their nutritional abilities (how many times per week they eat meat,fruit,vegetables) and how often and how much alcool they drink every week. The determination of B12 and Folate was done with reagents Beckman Coulter Access Ferritin in the analyzer Access. The expected normal values of B12: 145-914 pg/mL and Folate 2-17ng/ml. Results: In Group A , B12 was found from 100 pg/mL to 715 pg/mL with mean value 298,8 and Folate from 1,4 ng/mL to 10,8 ng/mL with mean value 4,01 In Group B , B12 was found from 119 pg/mL to 565 pg/mL with mean value 269,68 and Folate was found from 1,7 ng/mL to 17,5 ng/mL with mean value 4,33 . The study of the questionnaire results yielded the following conclusions How often do you eat Vegetables/fruits? -rarely : 80 men, 57 women -occasionally : 50 men ,26 women -often : 26 men,41 women -How often do you eat meat? -90% of participants (both men and women) eat meat 4-5 times weekly. -How often do you consume alcool ? -Never : 47 women, 12 men -Often :21 women, 68 men -Rarely : 56 women,76 men. *Conclusions*. The serum levels of B12 and Folate in both Groups are in normal values. Although we believe that Folate levels are quite low. For this reason we advise all blood donors to increase their consumption of folate rich vegetables/fruits.

1805

THE RECORD OF FERROPENIA AMIDST BLOOD DONATING PEOPLES

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Background and Aims. To study the frequency of ferropenia to regular and occasional blood doners, to reveal those with poor ferum storage and treat them with supplement therapy. *Design and Methods*. We studied 480 blood doners (314 men and 166 women). From those 305 were regular ones (at least 2 times a year), 156 were first timers, while 19 were occasional blood doners (had donated blood in the past, but less than 5 times in total). Ferritin values were calculated with the Elisa technique (MEIA ABBOTT). Ferropenia values were considered to be: ferritin <20 ng/mL for men and ferritin <10 ng/mL for women. *Results*. As you can see at the table. *Conclusions*. (i) Ferropenia among women is much more frequent than men. (ii) Regular blood doners present clearly greater ferropenia than occasional blood doners. (iii) It is therefore needed besides heamatocrit and hemoglobin measurment to have also the ferritin value periodicaly checked, especially in women.

Table.

BLOOD DONERS: 480	MEN	ferritin< 20 ng/ml	WOMEN	ferritin< 10 ng/ml
REGULAR: 305	211	38 (18%)	94	34 (36,1%)
FIRST TIMERS: 156	89	3 (3,4%)	67	12 (17,9%)
OCCASIONAL: 19	14	1 (7,1%)	5	1 (20%)

Mean Ferritin Value			
	MEN	REGULAR	56,9 ng/ml
		FIRST TIMERS	85,3 ng/ml
	WOMEN	REGULAR	17,1 ng/ml
		FIRST TIMERS	30,2 ng/ml

1806

STUDING THE EFFECT OF PRESTORAGE WASHING (FOR LEUKOREDUCTION) ON THE FUNCTION OF PLATELETS IN PLT BAGS WHICH HAVE KEPT FOR 7 DAYS

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Background. The presence of WBCs in platelet component may induce some problems such as alloimmunization against HLA, CMV transmission, febrile non-hemolytic transfusion reactions (FNHTRs) in the patients (recipients). During platelets storage in laboratory, their function may be affected by such factors as cytokine and metabolite release from WBCs and, probably, decrease in pH. Because of these factors PLT shelflife has been limited to at most 5days (depending upon the bag type) in room temperature. *Aims*. The main purpose of caring out this research is washing PLT component for leukoreduction which may help to abate the above concerns. *Design and Methods*. This study was on an experimental basis and carried out on the PLT components of random blood donors which were organized into two groups: test (washed products, 20) and control (unwashed products, 10). At the end of day 7 of storage all the PLT units were primarily assessed for any bacterial or fungal growth through culture, then WBC count was carried out on the components in two groups. Finally, in order to evaluate platelet function, samples were analyzed in an aggregometer with four agonists, arachidonic acid, ADP, collagen and ristocetin. The obtained data were assessed by t-test. *Results*. Statistical analysis indicated that PLT washing had no significant effect on the number of platelets but had caused WBC reduction in washed components ($p<0.01$). This analysis also showed that there was a significant difference in PLT average reaction with arachidonic acid ($p<0.01$) and collagen ($p<0.05$) in two groups, but there appeared no significant difference between PLT average reaction with ADP and ristocetin agonists in two groups ($P>0.05$). *Conclusions*. PLT washing causes WBC reduction in platelet component and fortunately doesn't have any undesirable on this product, so this protocol may be recommended for increasing PLT shelflife, from 5days to 7days.

1807

QUANTITATIVE DETERMINATION OF NEUTROPHIL VOLUME DISTRIBUTION WIDTH: A NEW AUTOMATED HEMATOLOGIC PARAMETER FOR 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, USA) 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. *Design and Methods*. Absolute neutrophils count, and SDVI data from 328 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 was considered significant. *Results*. A significant increase in the SDVI was observed in the bacteremic patients compared with the controls (27,78 vs 20, 63, $p<0.001$). Such increase was observed even in patients with absolute neutrophil counts less than 6600/ ^{t}L) (25,46 vs 20,63, $p<0.001$). The more dramatic increases were seen in patients with neutrophilia (28.65 vs 20,63, $p<0.001$).

Table.

	Control	Patients	<6600	>6600	P
Number	54	328	89	239	
SDVI mean	20.63	27.78	25.46	28.65	<0,001

Conclusions. 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.

1808

EFFECTIVENESS OF POSACONAZOLE IN ANTIFUNGAL PROFILAXIS IN THE ANLL AND ALLO BMT TREATMENT

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Background. The risk of IFI in onco-haematological patients with ANLL and patients subjected to allo-BMT is >30% with respect to intensive chemiotherapy regimen, to >10 days neutropenia, to mucositis onset and to GVHD. The antifungal prophylaxis is important to control such infections. Studies by Cornely (ASH 2005) and Ulmann (ICAAC Washington 2005) documented the effectiveness of prophylaxis with posaconazole. *Aims*. to evaluate the positive effect of prophylaxis with posaconazolo in ANLL and allo-BMT treated patients. *Design and Methods*. 24 cases were subjected to prophylaxis with posaconazole, 14 of them were treated with induction protocol for ANLL (Fab subtype : 2 Mo-5 M1- 3 M2- 1 M5a- 3M5b) and 10 were subjected to HSC allotransplant (2 ANLL-3 NHL- 2 MM- 2 HL- 1 CLL). Average age of patients : 44 yrs (range 19-78). Among additional risk factors, 4 patients showed fungal colonization at the admission, 12 received previous chemiotherapy treatment, 3 was treated with ASCT and 6 received immunosuppressive therapy. The prophylaxis with posaconazole was carried out in 24 cases (200 mg three times daily on a full stomach) since the first day of chemiotherapy up to > 500 ANC. At the admission, all patients underwent to haemoculture on peripheral blood and CVC, culture test on faeces and urine and buffers in the various sites. At the fever onset, that is, on alternate days, haemoculture on peripheral blood and CVC as well as evaluations of PCR, Procalcitonin, Galactomannan (GM) and DNA for Gram + and Gram -, bacteria and fungi. On the 1st day of fever, chest X-Rays were carried out, followed by high-definition chest CT after 72 hours of persistent hyperpyrexia. *Results*. The prophylaxis lasted 18 days on average (range 5-30 days). The treatment was discontinued due to >500 ANC in 12 cases, to intolerance in 8 cases and to fungal infections shift therapy in 4 cases. 20 patients showed hyperpyrexia. 3 cases of fungal infections have been documented (12.5%) : 1 with Krusei Candida on peripheral blood and Candida Glabrata on coproculture, 1 with *Candida albicans* on DNA peripheral blood, 1 case with *Candida albicans* on expectoration and probable invasive *Aspergillosis* documented by GM index 0.6 and HR CT (high-risk patient subjected to allo-BMT showing 4th-degree hepatic GVHD). *Conclusions*. In our experience, prophilaxis with posaconazole has revealed positive effects in the onset of IFI (12.5%) with acceptable tolerability.

Table. Prophylaxis with posaconazole.

PROPHILAXIS WITH POSACONAZOLE	
DIAGNOSIS	ANLL 14 (ANLL FOLLOWING MDS 3) ALLO ESC 10 (ANLL 2- NHL3- MM2- HL 2- CLL1)
RISK FACTORS	COLONIZATION 4 (C. Albicans 3 C. Glabrata 1) Previous CHT 15 ASCT 3 HD STEROIDS 1 I.S.T. 6
PROPHILAXIS PERIOD	AVERAGE LENGHT 18 DAYS (RANGE 5-30)
CAUSES FOR SUSPENSION	INTOLERANCE 7 (VOMITING-DIZZINESS- HEADACHE) ANC >500 12 SHIFT THERAPY 5
FUNGAL INFECTION	- C. Krusei p.b. DNA and C. Glabrata on coprocoltu 3 cases - C. Albicans p.b. DNA - GM 0.6 and C. Albicans on espectoration
COLONIZATION	- C. Glabrata on buffer oral mucosa
NEGATIVIZATION OF PREVIOUS COLONIZATION	3 on 4 cases

1809

RENAL AFFECTION IN CHILDHOOD MALIGNANCY: IMPACT OF ANTICANCER DRUG MANAGEMENT

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Background. Malignant disease can damage the kidneys by different mechanisms, e.g. infiltration of the kidneys, obstruction of urinary pathways or the syndrome of acute tumor lysis after progressive cytotoxic treatment. Moreover, there may be even direct renal damage caused by cytotoxic treatment. Renal impairment can results in altered excretion and metabolism of chemotherapeutic agents. Despite the recent advance in understanding the mechanism of anticancer drug nephrotoxicity, prevention still relies on drug dosage decrease and active screening for renal abnormalities as part of the usual biological work up in patient treated with anticancer drugs. *Aims*. We aimed to assess the effects of antineoplastic chemotherapy on kidney function when these drugs are used either alone or in combination with each other. *Design and Methods*. The study was carried out on 40 newly diagnosed malignant patients at Pediatric Hematology and Oncology Unit in Zagazig University Hospital from December 2007 till October 2008, their age ranged from 6 months to 14 years (24 males and 16 females). All patients were subjected to complete history taking and examination, laboratory investigations; especially BUN, serum creatinine, electrolytes (Na, K, Mg and Ca), blood glucose, arterial blood gases and creatinine clearance, and radiological evaluation of the kidneys at diagnosis and after three and six months of chemotherapy. *Results*. In our study the most commonly used anticancer drug was methotrexate (67.5%), followed by vincristin and cyclophosphamide (62.5%). Our results revealed that there was significant increase in BUN (p= 0.013), and significant decrease in creatinine clearance (p=0.002) after 6 months of chemotherapy especially solid tumors. The most common renal complication in our patients was hematuria (42.5%) followed by hypertension (17.5%). CRI was reported in 17.5% in our patients while ARF was reported in 15%. Serum phosphorus and uric acid were increased in a highly significant statistical fashion in all stages, (p=0.001), while there was no significant statistical difference regarding serum Na, Mg and K before and after chemotherapy. Among our patients ARF was the most common cause of death (57.1%). *Conclusions*. Renal affection should be focus of interest during treatment and follow up of childhood malignancies. Protocols of treatment of these patients especially those with solid tumors should be evaluated for their potential nephrotoxic effects. Good preparation of these patients, anticipation of nephrotoxic side effects and modification and substitution of these nephrotoxic agents if possible with less or none nephrotoxic ones are crucial factors.

1810

MEAN NEUTROPHIL VOLUME AS A NEW A NEW AUTOMATED HEMATOLOGIC PARAMETER FOR 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, USA) 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. *Design and Methods*. Total white blood cell count, percentage of neutrophils, and positional parameters data from 328 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 was considered significant.,*Summary and conclusions.* 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=0.085). 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	328		108	186	
MVI mean	146,17	163.19	**<0.001**	161.31	164.16	0.085
MCI "	145,85	141.08	**<0.001**	141.43	140.52	0.199
MSI "	146,06	137,21	**<0.001**	136.93	137.49	0.593

1811

EPIDEMIOLOGY OF BLOOD ISOLATES FROM DEPARTMENT OF HEMATOLOGY AT THE CHU IBN ROCHD CASABLANCA MOROCCO BETWEEN 2003 AND 2007

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Background. Bacterial ecology of the department of hematology at the CHU Ibn Rochd is important to know, to better guide the choice of antibiotic in 1st intention in the management of febrile patients. *Design and Methods*. We chose to exploit the results of blood cultures performed in this department from the database of the Microbiology Laboratory CHU Ibn Rochd Casablanca over five years (2003 to 2007). *Results.* During this period, we analyzed 4787 blood cultures performed in 2216 patients, an average of 957.4 blood / year and 2.2 balloons blood / patient. 751 blood cultures were positive (34%). Among the seeds frequently isolated (duplicates excluded), we find the *Staphylococcus* coagulase negative with 48%, followed by *Pseudomonas aeruginosa* (7%), Staphylococcus aureus (6%), *Escherichia coli* (6%), Klebsiella pneumoniae (4%) and *Acinetobacter spp* (2%). Of all the strains isolated, 39% of E. coli and 29% of *K.pneumoniae* are producing BLSE, 15% of *S.aureus* were resistant to Meticilline while *P.aeruginosa* and *Acinetobacter* are resistant to Imipenem with respectively 22% and 5%. *Conclusions*. These results which will be discussed in the light of the literature show the importance and the need to rationalize the use of antibiotics to control the spread of multiresistant strains.

1812

INFECTTIOUS COMPLICATIONS IN 81 CONSECUTIVE ADULT PATIENTS UNDERGOING INDUCTION CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA

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Patients with Acute Myeloid Leukemia (AML) undergoing chemotherapy are at high risk for infections particularly during the induction phase. The records of 81 consecutive adult patients (pts) (mean age 49 years) with AML treated homogeneously with an induction chemotherapy with Idarubicin, Etoposide and Cytarabine (ICE) were analysed. All patients were treated in an air-conditioned facility without HEPA protection. Twelve pts (14.8%) had no infections. Neutropenic

fever was observed in 69 pts (85.2%). Twenty-seven ptss (33.4%) had fever without neither isolates nor clinical or instrumental signs of infection. They were therefore definded as Fever of Unknown Origin (FUO). Ten pts (12.4%) had fever with clinical or instrumental signs of infection and were defined as Clinically Documented Infections (CDI). In 23 pts (28.4%) isolates of infectious agents were obtained and therefore they were defined as Microbiologically Documented Infections (MDI). "Possible" invasive fungal infection (IFI) were observed in 2 pts (2.4%), "probable" IFI in 3 pts (3.7%) and "proven" IFI was documented in 4 pts (4.9%). In febrile episodes, a pneumonia was documented in 19 pts (27.5%), neutropenic enterocolitis in 8 pts (11.6%), perianal abscesses in 2 pts (2.8%), vulvovaginitis in 2 pts (2.8%) and cholecystitis in 1 pt (1.4%). In one patient a Central Venous Catheter (CVC) infection was documented. A Severe Inflammatory Response Syndrome (SIRS) was observed in 5 febrile pts (7.2%). Blood cultures were performed on occasion of the first febrile spike: in 44 pts (66.6%) the blood cultures were negative, in 22 pts (33.4%) a pathogen was found in at least two blood culture samples (17 Gram+, 5 Gram-, 3 candida). More isolates were found on infected sites swabs or tissue specimen cultures (1 fusarium, 3 p. aeruginosa, 2 s. epidermidis, 1 MRSA, 1 bacteroides ovatus,). S. maltophilia was isolated in 3 pts from sputum throat. L. pneumophila antigen was detected in the urine in two cases. Empiric antibiotic therapy was started with anti-pseudomonal penicillins in 42 pts (60.8%), with carbapenem in 16 pts (23.2%), with 3rd generation cephalosporins plus aminoglycoside in 11 pts (16.0%). Glycopeptides were added in 31 cases and antifungals (liposomal amphotericin, echinocandins or voriconazole) in 33 pts. Fever resolved with treatment in 34 pts (49.3%); 5 pts (7.2%) died because of infectious complications. Fever resolved with granulocyte recovery in 30 pts (43.5%). The predominance of Gram+ bacterial infections was confirmed in the present series. The incidence of probable and proven IFI (8.6%) was clinically relevant. A new HEPA filtered facility is now available for induction therapy of AML patients and therefore a reduction in fungal infections may be expected.

1813

WEST NILE VIRUS INFECTION IN A PATIENT WITH ACUTE GRAFT-VERSUS-HOST DISEASE

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Background. West Nile virus (WNV) is generally transmitted by an infected mosquito. Some of the cases are infected by transfused blood products. No symptom is seen in approximately 80% of infected cases. In 20%, this microorganism causes mainly fever, neurological, gastrointestinal, respiratory symptoms. These symptoms are generally more severe and progressive in elderly or immunocompromised patients. Viremia persists for less than 12 days in an immunocompetent host, but may last much longer in immunosuppressed patients. *Design and Methods*. We describe a 40 year-old patient who underwent nonmyeloablative bone marrow transplantation for acute myelogenous leukemia. On posttransplant day 51 he was admitted with complaints of high fever (39oC), diarrhea, and diffuse skin rash. After 3 days, his liver enzymes increased mildly. Skin biopsy, but not liver and colon biopsies, was consistent with acute graft-versus-host disease (GVHD). His complaints due to acute GVHD relieved with immunosuppressive agents. On day +80, a second attack of fever together with headache and sudden onset of severe weakness of limbs happened. His CSF, blood, urinalysis and imaging studies were not remarkable for bacteria, CMV, EBV, HSV, toxoplasma, fungal infections, leukemic relapse and other neoplastic cells. He became pancytopenic. Three units erythrocyte and two units platelet concentrates were transfused in the posttransplant period. Diagnosis was made by a positive result of a reverse-transcriptase polymerase chain reaction for WNV on blood sample. Symptoms of the patient relieved gradually with supportive measures by day +110. There seems no complication so far. *Summary and Conclusions*. WNV is a severe infection with nonspecific findings. Therefore, differential diagnosis with another infections or GVHD may be difficult. Since it may be fatal in transplanted patients, this infectious agent should be kept in mind and all blood products which will be transfused to transplanted patients should be screened for WNV.

1814

AMINOGLYCOSIDE-FREE INTERVENTIONAL ANTIBIOSIS IN PATIENTS UNDERGOING HAEMOPOIETIC STEM CELL TRANSPLANTATION

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Background and aims. The position of aminoglycosides within interventional antibiosis in the early phase after stem cell has not been clarified so far despite their use can induce serious renal impairment. Data from conventional haematological patients suggests that they can either be omitted or replaced by other drugs. *Design and Methods*. To investigate this question early-infection data from 152 patients undergoing 195 allogeneic and autologous stem cell transplantations were investigated. Prophylaxis and treatment of infections followed international standard, however, aminoglycosides were omitted when ever possible. Results: The overall-incidence of infections was 77,9% (152/195) and 67 patients suffered from more than one episode. Fever of unknown origin and bacteriaemia/septicaemia dominated the spectrum of infections. Spectrum of isolated pathogens showed no increase of Gram-negative rods. The overall-response to interventional regimen consisting of β-lactam or carbapenem plus glycopeptides was 47.7%. Aminoglycosides were given in three patients in the late course of disease. Overall mortality was 15/195 (7.7%) and clearly related to infection in nine cases mostly due to mould infection. A comparison with previous publications gave no hint for inferiority of 'aminoglycoside-free' antibiosis. *Conclusions*. In conclusion, the present analysis supports the policy to omit aminoglycosides in the therapy of early infections in patients undergoing stem cell transplantation to avoid additional nephrotoxicity.

1815

ALLOPORINOL, CHAMOMILE AND NORMAL SALINE MOUTHWASHES FOR PREVENTION OF CHEMOTHERAPY-INDUCED STOMATITIS

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Background. Stomatitis is a common side effect in patients receiving chemotherapy. It is alters survival because of the risk of infection and has a significant impact on quality of life causing treatment delays, nutritional deficiencies and increasing cost of care. *Aims*. The aim of this study was to determine and compare efficacy of allopurinol, chamomile and normal saline mouthwashes in prevention of chemotherapy-induced stomatitis. *Design and Methods*. A randomized, double-blind clinical trial was conducted on 83 patients receiving chemotherapy . The cases were selected from Shahid Ghazi Oncology Service patients undergoing chemotherapy for various malignancies. They were randomly divided into three groups and received allopurinol (group 1), chamomile (group2) or normal saline (group 3) . ANOVA and 2test have been used for data analyzing. *Results*. Significant differences were obtained between allopurinol, chamomile and normal saline groups in scores of severity of stomatitis (p=0.017), stomatitis pain (p=0.027) and persistence of stomatitis. No significant differences noted among the mean stomatitis (p=0.59) and stomatitis pain (0.071) severity scores between group 1 and 2. *Conclusions*. These findings indicate equal efficacy of allopurinol and chamomile in prevention of chemotherapy-induced stomatitis compared to Normal Saline control group. Considering the cost and easy accessibility of chamomile and potential therapeutic applicability in reduction of the severity of chemotherapy-induced, it's implied.

1816

EPIDEMIOLOGY OF BLOODSTREAM INFECTIONS IN HEMATOLOGY-ONCOLOGY PATIENTS

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Background. Bloodstream infections (BSIs) are directly responsible for a prolonged hospital stay and, in critically ill patients, are associated with high healthcare costs and significant mortality. *Aims*. To describe the clinical and microbiological characteristics and to determine the incidence density and predictors of BSIs in patients with hematologic malignancies *Design and Methods*. Standardized Surveillance system based on the Cen-

ters for Disease Control and Prevention's National Nosocomial Infections Surveillance system. *Results.* The incidence rate of BSIs was 16,7%. The incidence density of bloodstream infections was 7,4 per 1,000 patient-days (21,9 per 1,000 patient-days at risk). Most of patients with BSI had acute myeloid leukemia (75%). The origin of the BSI was unknown in 70,6% of the episodes and was associated with known sites in 11,8%. The most common isolates were E Coli (17,6%) and K Pneumoniae (17,6%). Most of BSI was monomicrobial (88,2%). Multivariate analysis showed length of stay (p=0.017), duration of neutropenia (p=0.017) and acute myeloid leukemia (p=0.017) as independent predictors of BSIs. *Conclusions.* The incidence density of BSI was high. Risk factors of BSIs are associated with severity of patients and could not modify by all staff members.

1817

HEMATOLOGICAL MODIFICATIONS IN PATIENTS WITH IMPORTED MALARIA FROM WESTERN ROMANIA

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Background. Imported cases of malaria are occasionally diagnosed in Romania and their hematological modifications are difficultly managed due to complicate mechanisms and low therapeutic response. Anemia in malaria is multifactorial due to accelerated destruction of red cells, bone marrow dysfunction, shortened red cell survival and increased splenic clearance. It is used as an indicator of the impact of malaria control in intervention trials, monitoring and evaluation. Thrombocytopenia is commonly seen in severe *Plasmodium falciparum* malaria due to increased consumption of the platelets in the periphery, especially in the spleen. The hallmark of P. falciparum malaria is the sequestration of infected erythrocytes within the capillaries and postcapillary venules from different organs. *Aims*. To highlight the malaria clinical cases with their life threatening hematological modifications and to emphasize the importance of their adequate and fast correction in patients' survival. *Design and Methods*. Overview of the medical charts of patients diagnosed with malaria who required advanced medical attention due to the hematological modifications detected. Patients were hospitalized at Victor Babes Hospital of Infectious Diseases from Timisoara, Romania during the period 2001-2007. Clinical and laboratory features of the cases were analyzed. Results: Hematological modifications were observed in six patients diagnosed with P. falciparum (malignant tertian) malaria. The clinical pattern of these patients included: fever and shivers (all six patients), hepatomegaly (five patients), icterus (three patients) and splenomegaly (two patients). Regarding the laboratory tests, anemia was found in five patients, thrombocytopenia in two patients, leucocytopenia in one patient and leucocytosis in one patient. *Plasmodium falciparum* was found in thin and thick blood smears in all cases. The values of the routine hematological laboratory tests are shown in Table 1.

Table 1. Routine hematological laboratory tests.

Patient	Gender	Follow-up	Erythrocytes (/mm³)	Hemoglobin (g/dl)	Leucocytes (/mm³)	Thrombocytes (/mm³)
1	♂	fatality	3 250 000	4.8	normal	18 000
2	♂	survival	2 980 000	9.8	normal	normal
3	♂	survival	normal	normal	normal	89 000
4	♂	survival	2 630 000	9.4	2 800	normal
5	♀	survival	3 360 000	9.7	normal	normal
6	♂	survival	3 800 000	9.8	12 000	normal

Conclusions. Generally, hemoglobin levels of less than 7 g/dl must be a warning for impending crisis and a decrease of less than 5 g/dl is an indicator for transfusion of packed cells. In case of a single patient from our study a hemoglobin value of 4.8 g/dl was detected, leading to his death. Giemsa-stained thin and thick smears taken from this patient indicated a massive parasitemia (affecting over 60% of the erythrocytes). Anemia and fever tended to increase the cardiac output and this combination worsened the status of five patients from our study. In cases with severe parasitemia and profound hemolysis repeated transfusions were administered.

1818

MUCOR CIRCINELLOIDES INFECTION IN AN AUTOLOGOUS BONE MARROW TRANSPLANTATION RECIPIENT

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Background. Invasive fungal infection (IFI) is a severe complication in bone marrow transplant recipients. In last decades, Candida was the main etiology of IFI in bone marrow transplant (BMT) recipients. Nowadays Aspergillus has became the main fungus in haematologist patients although other less frequent moulds can be also found. These infections are likely to become increasingly recognized even though the occurrence after BMT has only been described sporadically. Aim: In this work, we report a 59-year-old patient who developed a post-transplant zygomicosis due to Mucorales circinelloides. *Design and Methods*. The patient was diagnosed with Mantel cells lymphoma EIVB IPI 3 treated with 5 Mega-CHOP and 2 R-IFE and achieved an apparent CR. Autologous transplantation was performed after conditioning with total body irradiation and cytarabine treatment. After hospital delivery, he was treated with fluconazole and levofloxacin because of oropharyngeal candidiasis and flu, thorax radiology was normal. On day +91 he came to the hospital because of right intercostal metameric zoster, without fever but without resolution of oropharingeal candidiasis and flu. Thorax radiology showed an alveolar consolidation at right upper pulmonary lobe and started ceftazidima, aciclovir and itraconazole. On day +97 we found bilateral homogeneous ground glass opacity on the right upper pulmonary lobe with minimum bilateral pleural effusion in a CT. He had many fever episodes that make us to add linezolid and soltrin and change acyclovir and itraconazole for foscarnet, caspofungina and voriconazol. A bronchoscopy was carried out with macroscopic inespecific results. A new CT was worse. On day +105 the patient was moved to intensive care department because of respiratory instability. All the microbiological tests required, including fungal and viral antigenemias and cultures, were negatives. On day +106 we asked for parasites cultures and added doxiciclina and albendazol to the treatment. The patient presented acute renal failure and needed continuous hemofiltration, but finally the patient died. An autopsy was carried out with the diagnosis of invasive fungal infection (respiratory and gastrointestinal tracts). Tissue samples were sent to a microbiology laboratory in order to make a PCR. Results: The diagnosis was not achieved by means of different cultures, imaging or histopathology methods carried out before the death of the patient. The histopathology is an important aid although because of the fact that the morphology of hyphae is microscopically similar in a lot of moulds, a differential diagnosis became a difficult task. In this case Mucor Circinelloides Zygomicetos was only isolated using the mould DNA sequentiation by PCR. *Conclusions*. DNA sequentiation by PCR is potentially helpful for detection and identification of fungal infections in BMT recipients. It increases detection sensitivity and decreases time for results, a difference that can has a significant positive impact on patient care.

1819

UNUSUAL CAUSE OF DIARRHOEA AFTER UNRELATED HAEMATOPOIETIC STEM CELL TRANSPLANTATION FOR RELAPSED FOLLICULAR LYMPHOMA

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We submit the case of a 46 years-old woman diagnosed of a stage IVA grade 2, FLIPI 1, follicular lymphoma. She received four lines, including autologous haematopoietic stem cell transplant (auto-HSCT). She underwent a reduced intensity conditioning, peripheral blood haematopoietic stem cell transplantation, from an ABO identical unrelated donor with one HLA mismatched, five years after initial diagnosis. At day + 100 she had a DLBCL transformation in an inguinal lymph node EBV negative, reaching complete remission after reducing inmunossupression and anti-CD20. On day + 190 after comitial crisis, cerebral abcess were detected on CT scan and RM, suggestive of toxoplasmosis which was resolved after antibiotics. From day 180 postHSCT she has two episodes of diarrhoea being diagnosed after gastric biopsy of late onset acute graft-ver-

sus-host-disease (aGvHD) that reached complete response with topical steroids. Microbiologic screening was negative. On month 16, watery diarrhoea reappeared with increasing stool volume, body weight loss, nausea and abdominal pain. Gastric endoscopy with biopsy take showed again GVHD, so second and subsequent line treatment for GvHD was initiated without response. A third gastric endoscopy was taken, suggesting GvHD versus infection. Colestasis appeared accompanying the former clinical symptoms. Abdominal ultrasonography, CT and MRI scans showed a dilated choledocus, an enlarged gall-bladder wall, and an obstructed common bile duct with a highlighted billiary system. Sphyncterothomy and Kherr tube placing relieved pain, and billiary fluid samples were taken. Spherical structures in crypts compatible with Cryptosporidium were revealed showing apoptotic bodies, crypt destruction, inflammatory infiltrate, and growing of coccidian organisms in crypt lumen in the Giemsa stain. All three gastric biopsy were reviewed and showed Cryptosporidium growth with varying degrees of accompanying GvHD immunofluorescence (IF) was positive in all gastric, duodenal and billes samples for Cryptosporidium as well as PCR testing (Cryptosporidium parvum) Therapeutical approach: Paromomycin and reduction of immunosupressive drugs. Clinical course: The patient got a better general performance, but minimal to mild diarrhoea persisted in an oscillating course. Five months after diagnosis, diarrhoea became acute again and fever reappeared. Cryptosporidium specific PCR was positive in new samples and the patient began Nitazoxanide therapy. After this treatment diarrhoea disappeared and patient gained 10 kilograms. Comments: Persistent diarrhoea unresponsive to immunosuppressive treatment after HSCT should lead to screening for uncommon microorganisms if no treatment response is achieved with conventional antibacterial, antifungal or antiviral agents . Cryptosporidiosis is typical of severely immunodepressed patients. The main clinical onset is with watery diarrhoea, which might be confounded with lower-tract intestinal GvHD. Very few post HSCT crytosporidiasis cases with this infection have been reported. Immunosupression withdrawal when possible should be the mainstay of treatment, at the risk of worsening pre-existing GvHD

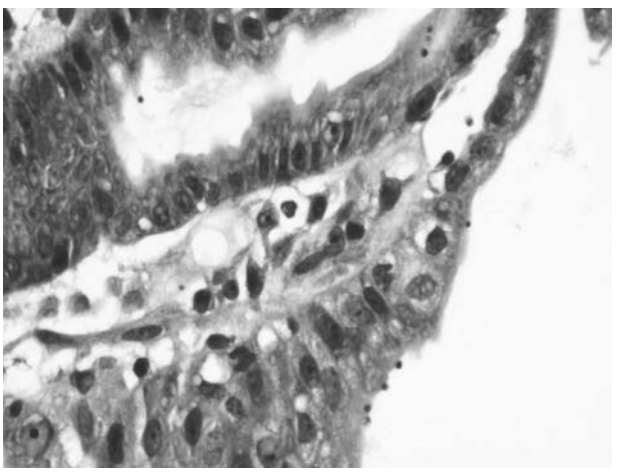

Figure.

1820

PERSISTENT ABSOLUTE MONOCYTOSIS AND SPLENOMEGALY: THE IMPORTANCE OF EXCLUDING A NON-HEMATOLOGICAL ORIGIN

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Background. Persistent absolute monocytosis is sometimes the first and unique hematological abnormality in the peripheral blood (PB) in a variety of chronic infectious, inflammatatory diseases and neoplastic disorders. In patients with splenomegaly, malignant conditions must be excluded (e.g. lymphoma or myeloproliferative/ myelodysplastic disorders). A guided medical history and complementary studies are necessary to reach a correct diagnosis. *Aims.* To describe our experience in the follow-up of a patient with chronic monocytosis and mild splenomegaly under the suspiction of a chronic myelomonocytic leukemia (CMML) in which a positive serological evidence of brucella sp gave us the chance for a specific therapy. Case Report A 57 years-old healthy male, was studied in the haematological deparment because of persistent monocytosis (> 1000/mm^3), thrombocytopenia (122x10^9/L) but normal hemoglobin value > 140 gr/L. A mild palpable splenomegaly was observed by physical examination and abdominal studies (ultrasounds and CT Scan). The patient was totally asymptomatic without history of fever, arthralgias or weight loss. He had always lived in town, denied contact with animals but admitted occasional ingestion of dairy products without sanitary guarantee. The bone marrow (BM) aspiration and trephine biopsy only disclosed unspecific displastic changes. BM cariotype was normal (46 XY). Complete autoimmunity studies, serologies for common virusses and tumoral markers were all normal. A diagnostic of possible myelodysplasia of CMML type was established and the patient was follow-up, without observing any clinical or biological changes. Under suspiction of a non-hematological origin of both monocytosis and splenomegaly, a Rose Bengal slide agglutination test was performed which resulted positive. The standard tube agglutination titres and coombs test were both positive (1/ 320 and 1/640 respectively) and confirmed in another later serum sample. A spine X-Ray ruled out infectious spondylodiscitis; echocardiogram was normal and repeated blood cultures were all negative. It was indicated treatment with intramuscular streptomycin during three weeks and oral doxycycline and rifampicine for three months. At the time of these writing, the patient is still asymptomatic with no modification of PB abnormalities and with persistent asymptomatic splenomegaly. *Conclusions*. It's concluded for this study that screening tests for diagnosis of Brucellosis should be performed early in patients who live in endemic areas and presenting with chronic monocytosis. These laboratory findings could avoid an unnecessary bone marrow biopsy.

1821

INFORMATION AND PREOCCUPATION IN OUTPATIENTS WITH NON-ONCOHEMATOLOGICAL PATHOLOGIES AT ARRIVAL AT THE HEMATOLOGY EXTERNAL CONSULTATION

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Background. Haematology is frequently considered by the patients as a medical speciality that deals with the diagnostic and treatment of very serious diseases, this circumstance causes in them a variable degree of distress. *Aims*.- We have studied a group of outpatients with non-oncohematological pathologies sent to our external consultation to evaluate their knowledge about the reason for consultation, the existence or not of preoccupation and its degree at arrival. *Design and Methods*. We have conducted a prospective study including those consecutive patients who were sent to our external consultation between June 2008 and January 2009. First we designed an inquiry and with it all patients were interviewed. This inquiry included the following data: age, sex, origin (hospital, urban or rural health centres), reason for consultation, knowledge of the reason of consultation (yes, partially, no), existence or not of preoccupation and when present its degree (low, moderate, high). The interview was performed to the parents in those patients aged below 14 years. *Results*. One hundred and twenty eight consecutive patients were interviewed, 81 female (63.3%) and 47 males (36.7%), mean age 48,3 years (1-87). Eighty four were referred from hospital (65.6%), 28 (21,9%) from urban health centres and 16 (12,5%) from rural health centres. Hospital medical specialities that sent these patients to our consultation were: Internal Medicine 50 (59,5%), Gastroenterology 7 (8.3%), Anaesthesiology 6 (7.1%), rest ≤5 patients (≤6%). The causes for consultation were: anaemia 69 (53,9%) cases, thrombocytopenia 16 (12.5%), alterations in white blood cells distribution 9 (7%), alterations in coagulation tests 7 (5.5%), others ≤5 patients (≤3.9%). Only 57 (44.5%) patients knew the subject of consultation while 23 (18%) only knew it partially and 48 (37.5%) did not know it at all. Seventy (54,7%) patients referred to come worried at our consultation. The degree of preoccupation in these patients was low in 18 (25,7%), medium in 34 (48.6%), and high in another 18 (25.7%). When analyzing the knowledge of the subject of consultation and the existence of preoccupation we found almost significant statistical differences (0.10) (more patients referred preoccupation when they had no or partial information). Comparing the results of our interviews to parents of paediatric patients (n=16) with those of the rest of adults we have found no statistical differences in the knowledge of the subject of consultation nor in the existence or not of preoccupation but there were almost significant differences (p=0.06) with respect to the degree of preoccupation (all parents who came worried presented a medium or high degree). *Conclusions*. In our area, most of the patients with non-oncological problems sent to the external consultation of

Haematology refer at arrival a low or no information about the subject of consultation, most of them refer to come worried as well being the degree of this preoccupation medium-high. This circumstance is specially noted in parents of paediatric patients. We believe is essential to improve information of patients to reduce their stress.

1822

PSYCHOMETRIC PROPERTIES OF THE MULTIDIMENSIONAL FATIGUE INVENTORY IN BRAZILIAN HODGKIN LYMPHOMA SURVIVORS

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Background. Cancer-related fatigue is the most common symptom among Hodgkin lymphoma survivors. *Aims*. To describe the validation steps of the Brazilian Portuguese version of the Multidimensional Fatigue Inventory (MFI). *Design and Methods*. The validation was done in two steps. The questionnaire was first translated from English to Brazilian Portuguese with the "forward-backward" procedure. The internal consistency, construct validity and convergent validity were then evaluated. The MFI was administered along with a general fatigue question and the informed consent form. Data from five different institutions were collected on 122 Hodgkin lymphoma survivors, with a median follow-up of 6.8 years from diagnosis. *Results*. The overall Cronbach alfa for the 20 itens was 0.85, and the Cronbach alfa of each of the five dimensions ranged from 0.83 to 0.75. Interitem correlations of each dimension ranged from 0.24 to 0.74, with almost all values higher than 0.4. There was a significant correlation between the MFI and the "General Fatigue" question, with a value of 0.75 for the "General Fatigue" dimension and 0.70 for the "Physical Fatigue" dimension. The factor analysis yielded a 5 factor solution that explained 75% of the variance. The first factor corresponded to the original "General Fatigue" and "Physical Fatigue" dimensions. The fifth factor identified, however, consisted of three isolated questions from different dimensions that need to be reappraised. *Summary*. The Brazilian MFI showed a satisfactory psychometric performance, and is a valid research tool for measuring cancer related fatigue, allowing different dimensions of fatigue to be assessed. General and physical fatigue were grouped together, as has been previously reported. The three questions grouped as a fifth factor require reformulation and revalidation.

1823

WITHDRAWN

1824

THE CURRENT OPINION OF THE MEDICAL STAFF AND OF PATIENTS FROM SOUTHERN TRANSYLVANIA REGARDING THE BONE MARROW DONATION

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Background. The bringing into existence of new bone marrow or peripheral stem cells banks and of the registers for the donors are essential for finding donors which are compatibles, without whom there could not be realized important stages of therapeutic consolidation at many patients with malignant haemopathies. *Aims*. We established this study in order to see the attitude of the medical staff and of the patients concerning the bone marrow donation. *Design and Methods*. We have realized a transversal study an a group of 252 subjects, among whom 168 were hospitalized patients (group A) and 84 were medical staff of the same hospital (group B), who have agreed to answer at a questionnaire with 17 questions regarding the bone marrow donation. 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 43.92±17.49 years. The gender repartition was 66.93% women and 33.07% men. While 74% from the questioned medical staff consider that bone marrow transplant takes sometimes to the curing of the patients with leukemia, 64% of the patients consider that after the transplant the healing is sure. 93% from the medical staff knows that, after the transplant there are possible complications related to the transplant and 71% of the patients are aware of this thing. If they were be proposed to become donors, only 60%, respectively 61% of the groups A, respectively B, will agree, while, if they personally will need a bone marrow transplantation, 100%, respectively 87% will adopt this opportunity. If they will be diagnosed with a malignant disease, and they will be in the situation of choosing between the classical treatment and the bone marrow transplantation, only 53% from group A and 79% from group B will choose the transplantation. Almost all from the 2 groups agree to donate bone marrow or peripheral stem cells if a family member would have acute leukemia, and they will be compatibles. Despite this thing, if the patient would be from another country of the European Union, only 54%, respectively 67% will accept to donate, and if the patient will be from an Asiatic country, the percentages would decrease at 47%, respectively 58%. The majority will agree that donation should be anonymous (78%, respectively 70%), and a charitable gesture (84%, respectively 88%). Almost all agree that there should be made a bone marrow donor's register, and that there should be more specialists and biologists involved in this programs. The majority also consider that the state and the European Union should involve more, financially, for this purpose. Only 68% of the patients and 85% of the medical staff are aware of the advantages of the peripheral stem cells donation, in contrast with the bone marrow. Almost 90% of all would agree to explain to their acquaintanceship that becoming a donor is a noble gesture. The differences between the 2 groups were not statistically significant. *Conclusions*. Many of the questioned subjects are insufficiently informed about the risks and benefits of the bone marrow transplantation, this being well reflected in their attitude regarding the donation and the acceptance of the graft. The majority agrees that there should be made a donor's register in Transylvania and they are willing to plead for the bone marrow donation.

1825

SKELETAL MANIFESTATIONS IN CHILDREN WITH DIFFERENT HEMATOLOGICAL DISEASES AND HEMATOLOGICAL MALIGNANCIES AT ZAGAZIG UNIVERSITY HOSPITAL [RETROSPECTIVE STUDY FROM 1998 TO 2008]

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Background. Pediatric patients with hematoncological diseases are at risk for the development of numerous skeletal complications. In β thalassemia, skeletal manifestations occur frequently due to anatomic proximity of bones and joints to the active centers of hematopoiesis.These include fractures, premature epiphyseal fusions and thalassemic osteoarthropathy. Leukemia is the most common cancer in children and may affect virtually all organ systems. Skeletal abnormalities have been described in association with ALL, including juxtametaphyseal lucent bands or 'leukemic lines' in long bones, osteoporosis, periostal reaction, reactive sclerosis, lytic defects and vertebral compression fractures. In sickle cell anemia, the manifestations include intramedullary and extramedullary effects of increased hematopoiesis; sickle cell-related infarction and resultant abnormalities in the growth of bone; infection; and soft-tissue involvement. *Aims*. We aimed to evaluate the prevalence of skeletal manifestations in children with different hematological diseases and hematological malignancies and also to identify their nature, underlying factors, severity and Sequalae. *Design and Methods*. A retrospective analysis was conducted on a cohort of 500 randomly selected files of children at outpatient clinic of Pediatric hematology and oncology in Zagazig university hospital from 1998 to 2008. Two hundred patients with thalassemia major, one hundred patients with hemophilia, fifty patients with chronic ITP, thirty patients with thalassemia intermedia , Thirty cases with sickle cell disease, twenty cases with bone marrow failure, sixty cases with hematological malignancies and ten cases with gaucher disease. All available medical records were reviewed and data were collected with interest in collecting the following: medical history, physical examination, full laboratory and radiographic investigations, protocols of treatment, follow up data during treatment and after stopping it. Special concern was done for skeletal manifestations at initial presentation, during treatment as well as after stop of treatment. *Results*. Amongst 500 cases of our patients, Bone pain was the commonest complaint [59% of all patients].At initial presentation in leukemia [66%], during follow up in sickle cell anemia [72%] and ITP [40%]. The skeletal dysplasia was the least common presentation in our patients [5%], all of them with hereditary bone marrow failures [Fanconi anemia and Blackfan diamond syndrome].Arthritis occurred in 20% of all cases. *Conclusions*. Skeletal manifestations are highly prevalent in children with different hematological diseases and hematological malignancies. Bone diseases should be focus of interest during treatment and follow up of these patients.

1826

PARAMETERS AFFECTING COMPLIANCE OF THALASSAEMIA PATIENTS ON IRON CHELATION THERAPY STUDIED BY HAMILTON'S RATING SCALE FOR DEPRESSION

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Background. Compliance with iron chelation therapy in multitransfused thalassaemics is the predisposing factor for extended survival and improving quality of life. Although depression was found to affect compliance with Desferrioxamine (DFO), Deferiprone (DFP) or both, some depressed patients complied very well with the chelation regimen. Objective: To investigate the parameters of Hamilton rating scale (HAM-D) for depression potentially affecting compliance with different chelators in thalassaemia patients. *Design and Methods*. 45 thalassaemics were enrolled in the study, 19 males, 25 females, mean age 39 years. According to the chelation therapy patients divided into 4 groups. Group I: 9p on Deferasirox, Group II: 14p on DFO, Group III: 11p on DFP , Group IV: 11p on combination therapy with DFO and DFP. Every patient was privately interviewed and depression level was determined by Hamilton depression rating scale (HAM-RS) consisting of 24 parameters. HAM-D score >= 20 indicates depression. Compliance was estimated by compliance index. *Results*. Patients with HAM-D score >= 20 were found in overall groups. However depressed patients of groups I, II, III and IV had excellent compliance with chelation therapy in a ratio of 100%, 40%,50% and 22.2% respectively. Patients with increased rating in parameters 1,10,11,12,13 and 15 indicating depressed mood, anxiety and hypochondriasis showed poor compliance with chelation therapy. Although increased rating of the rest parameters leads to HAM-D score >=20, compliance was not affected. *Conclusions*. This study showed that depression generally affects compliance. However the prominent psychological parameters affecting compliance were depressed mood, psychic and somatic anxiety as well as hypochondriasis.

1827

MIDLINE CATHETERS AS ALTERNATIVE VASCULAR DEVICES IN PEDIATRIC THALASSEMIC PATIENTS WAITING FOR BONE MARROW TRANSPLANTATION

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Background. Thalassemia requires chronic long-life transfusions to treat the anemia caused by enhanced red blood cell destruction due to the disease. Midline catheters are emerging as a good alternative vascular devices, to ensure the best clinical practice, management and care also for pediatric patients waiting for bone marrow transplantation, that, to date, represents the only radical cure for thalassemia. *Design and Methods*. All patients in pre-transplant period who needed transfusion, intravenous drugs administration and blood samples collections were evaluated. Patients below 8 years old and with an inadequate basilic vein were excluded. From February to August 2008, patients on which it has been tried to insert a Midline catheter were 10: eight thalassemic and two microdrepanocytosis. Of these, two (1 microdrepanocytosis and 1 thalassemic) were excluded for the developing of an hematoma on the site of the insertion, and they have been submitted to a central vascular access in the surgical unit. None of the patients had piastrinopenia or clotting alterations before the insertion, and none of the patient was in a neutropenic condition. *Results*. For the 100% of the patients, the basilic vein was the best choice and none of them had any problem during the insertion. Midline catheters were used for maximum 48 days, with one episode of early removal (after 13 days) due to vein thrombosis, nevertheless antithrombotic treatment for previously splenectomy. Currently, two patients bring on this new type of catheter due to their pre-transplant treatment, three left the department because the end of treatments, and three switched to a tunnellized central access because started conditioning regimen and, due to their small size, they could not receive a peripherally inserted central access. *Conclusions*. In our experience, we can affirm that the use of these devices can carry on advantages to improve care, management and quality of life of the patients, supposing evidences could come from easy availability of ultrasound technique, easy possibility to perform the insertion bed-side, respecting all warnings in terms of patient's and operator's security, and easy acquisition of the specific required know-how, with the aim to reduce waiting time for the insertion. The only limit we faced is age, because children younger than 8 years old do not comply for the procedure, making mandatory the use of tunnellized central access. For correspondance: a.roveda@fondazioneime.org

1828

QUALITY OF LIFE IN BRAZILIAN PATIENTS WITH SICKLE CELL DISEASE: PRELIMINARY RELIABILITY REPORT OF THE SF-36 SURVEY

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Background. Sickle cell disease (SCD) is one of the most common genetic disorders in Brazil and deeply compromises the quality of life (QOL) of those who are afflicted. We have evaluated the reliability of the Short Form 36 survey (SF-36) in our patients with sickle cell disease. *Design and Methods*. A validated Brazilian Portuguese version of the SF-36 was administered to patients with sickle cell disease in the outpatient clinic of the University Hospital. The domains evaluated by the SF-36 questionnaire cover physical function, physical and emotional role function, bodily pain, vitality, social function, mental health, and general health. Reliability (internal consistency) of all scales and of each domain were evaluated with the Cronbach's α coefficient. We also assessed whether gender and genotype had any influence on the SF-36 subscale results. The Mann Whitney test was used to compare the scale and subscales between two groups. *Results*. Eighty-eight patients responded the SF-36. The median age was 23 years (13-60), 57% were female, and 80% were black. Genotypes were SS (78.4%), Sb thalassemia (11.4%) and SC (10.2%). The internal consistency of items in the SF-36 scale was 0.85, and the internal consistency of domains were 0.85 (physical function), 0.81 (physical role function), 0.82 (emotional role function), 0.85 (bodily pain), 0.79 (vitality), 0.60 (social function), 0.85 (mental health) and 0.78 (general health). SS patientes and women have significantly lower scales (481 vs. 572; p=0.028, and 463 vs. 550; p=0.015 respectively) and lower subscales on all domains. When SS patients were compared to non-SS patients, the subscales were significantly lower in social function (68 vs. 84; p=0.014), bodily pain (58 vs. 74; p=0.021), physical function (58 vs. 71; 0.026) and general health (44 vs. 62; p=0.030). In women, subscales were significantly lower in social function (66 vs. 79; p=0.011), mental health (60 vs. 77; p=0.005), physical function (54 vs. 69; p=0,04) and vitality (54 vs. 67; p=0.035). *Conclusions*. This prelimary report suggests that the Brazilian Portuguese version of the SF-36 has a good internal consistency in patients with sickle cell disease. Patients with the SS genotype and females appear to have a worse QOL.

1829

ASSESSMENT OF QUALITY OF LIFE IN IRANIAN CHILDREN AND ADOLESCENTS WITH SEVERE HEMOPHILIA A AND B BY HAEMO-QOL QUESTIONNAIRE,A SINGLE INSTITUTE REPORT(SARVAR CLINIC, MASHHAD, IRAN)

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Background. Assessment of quantitative parameters is easier than qualitative ones.But it should not be forgotten that it is important to know quality of life (QOL) as a qualitative parameter in medicine to evaluate and if necessary to improve the patients care. Severe hemophilia A and B can lead to recurrent bleeding in joints, detoriate joints functions and significantly affect the QOL. *Aims*. The aim of this study is to evaluate the quality of life in children (8-12 y) and adolecens (13-16 y) with severe hemophilia A and B, by standard Hamo-Qol questionnaire. *Design and Methods*. 20 registerd patients with severe hemophilia A or B in two age groups (8-12 y and 13-16 y) and their parents were asked to fill out the standard Haemo-Qol questionnaire.This questionnaire was translated to persian language by authors ,pediatric psychologist and an epidemiologist (all together). According to patients self-report and parent-report and on the base of scoring list of standard Haemo-Qol questionnaire the QOL was assessed for each case. Results: In group A (8-12y) according to self-reports they had lower QOL in friends subscale [mean 62/50 (SD: 30/83)] and supports subscale [mean 62/50 (SD:26/27). Parents report showed that their children had lower QOL in friend subscale [mean 74/11(SD:23/78)] and physical health [mean 61/22 (SD:19/82)]. In group B (13-16 y) according to self-reports they had lower QOL in supports subscale [mean

51/28(SD:10/20)] and family subscale [mean 50 (SD:20/05)]. Parents report showed that their children had lower QOL in family subscale [mean 66/85(SD:13/85)] . In both age groups in comparison with patients self-reports, parents think that their children have lower QOL. *Conclusions*. According to results it seems that most of our patients and their parents experience a bad QOL. Further efforts are necessary to examine other aspects of QOL in these patients. And considering therapies that reduce bleeding events, like prophylaxis treatment, warrant more attention.

1830

A PHYSIOTHERAPEUTIC APPROACH TO REDUCE FATIGUE IN PEDIATRIC ONCOLOGY

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Background. 2007 we closed the gap between the presence of Cancer-related-fatigue (CRF) and the missing of an adequate assessment in Germany. Now we use the first german version of the PedsQL 3.0 Multidimensional Fatigue Scale to measure CRF in pediatric oncology. Aims: Now, after we made sure, that this most bothersome side effect is present in children, we`re searching for a strategy to reduce this phenomenon. According to the efforts made with adults, we developed a training program. We start this sports-program after bone-narrow- or stem-cell transplantation as a standard concept. *Design and Methods*. Additional to the PedsQL questionnaire we want to demonstrate the increase of physical strength (or feeling of well-being). Therefore we evaluate handcraft once a week, doing a lactate-measurement and examine the QoL with a questionnaire. *Results*: First impressions of the children will be demonstrated and a review of the last 6 months will be given.

1831

WITHDRAWN

1832

ANALYSIS OF A DAY-HOSPITAL THROUGH CALCULATION OF ITS PERFORMANCE INDICATORS

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Background. The day-hospital (DH) is one of the main alternatives to conventional hospitalization. The optimal use of its resources improves hospital management with reduction of hospital admissions and economic cost. *Aims*. To analyze the efficiency and the efficacy of a medical DH through calculation of its performance indicators (PI) and asses whether changes in organizational model improves the PI. *Design and Methods*. The DH is open from 7:30 am to 17 pm, from Monday to Friday and has 22 treatment places. Three nurses work in the DH with an overlapped schedule, so that from 7.30 am to 10 am there are available 270 minutes of nurse, from 10 am to 12 pm: 360 minutes and from 12 pm to 17 pm: 720 minutes. We have collected, during 61 consecutive days of 2007, the data of all patients (Pts) attended and all procedures performed. Also, we have collected the time spent by the nurse in performing a procedure and the time of place occupation for each procedure. *Results*. 1.286 scheduled Pts and 229 (15%) urgent Pts were attended. The scheduled Pts generated 1.352 procedures. 61% of procedures were of medium duration (between 1 - 2 h), 23% were of long duration (> 2 h) and 16% were of short duration (< 1 h). The nursing occupancy rate (NOR) was highly variable among days with a median of 76% (range 51 - 116%). The place occupancy rate (POR) was 24% with a turnover rate of 1.1. Also, the scheduled Pts and procedures generated different minutes of activity throughout the day, being from 7:30 am to 10 am when there is highly activity. Moreover, the NOR and the POR varies throughout the day, being 137% and 45% from 7:30 am to 10 am, 78% and 37% from 10 am to 12 pm and 45% and 11% from 12 pm to 17 pm, respectively. If we change the nursing work schedule and the time that the DH remains open the NOR increases to 90% and the POR to 32%. *Conclusions*. The DH studied has a low efficacy and efficiency. The analysis of its PI suggests mismanagement in the scheduling of patients and procedures as well as poor management of nursing work schedule. The activity is highly variable throughout the day and there is less time of nurse during the hours of the day with major activity in the DH. These disparities can cause a reduction of equity and quality of care. To optimize the resources of the DH, we propose the implementation of a reengineering organizational process.

1833

EVALUATION OF A NEW POINT OF CARE AUTOMATED COMPLETE BLOOD COUNT (CBC) ANALYZER IN NEWBORN BABIES

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Background. Hemoglobin, WBC counts and differentials are currently performed on complex and very expansive hardware in central laboratories not available in certain settings. Chempaq XBC®(Chempaq A/S, Denmark) is a rapid and easy point of care (POC) device with low volume samples requirement that can benefit certain patients with special clinical characteristics like newborn babies particularly in outreach facilities. This device needs a low volume sample that is analysed in a disposable cassette. Leukocytes, lymphocytes, monocytes and granulocytes are counted and their size is determined by impedance in a cell. Hemoglobin is measured photometrically at two wavelengths. Aims. To evaluate the precision, accuracy and linearity of the new POC device Chempaq XBC® for hemoglobin, leukocyte counts and 3-part differentials compared with an established laboratory based analyzer: Advia® 2120 (Bayer AG, Germany) in a cohort of 100 consecutive newborn babies. *Design and Methods*. A fresh 20 uL venous blood sample was drawn from each individual and simultaneously tested for hemoglobin (g/dL), leukocyte counts and 3-part differentials ($x10^9$/L) in the POC device and the established laboratory analyzer. Linear regression techniques were used to compare results. *Conclusions*. Chempaq XBC® is a reliable POC analyzer that provides accurate results for Hb, WBC and 3-part differentials for newborn babies which is clinically relevant for certain clinical settings where complex and costly central laboratory analyzers are not available.

Table 1. Linear regression for Chempaq XBC vs Advia

Assay	Slope	Y-Int	r
Hb (g/dL)	0.87	1.47	0.96
WBC ($x10^9$/L)	0.97	-0.15	0.99
Neutrophils ($x10^9$/L)	0.88	1.25	0.93
Lymphocytes ($x10^9$/L)	0.91	1.62	0.81
Monocytes ($x10^9$/L)	0.63	0.20	0.71

1834

MOBILIZATION WITH CHEMOTHERAPY COMPARED TO G-CSF ALONE: AN ANALYSIS OF RESOURCE USE AND COST IN AUTOLOGOUS STEM CELL TRANSPLANT PATIENTS

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Background. The most common mobilization regimens for autologous stem cell transplant (ASCT) are granulocyte-colony stimulating factor (G-CSF) alone or in combination with chemotherapy. Chemotherapy plus G-CSF has been shown to mobilize more cells than G-CSF alone but often at the expense of costly side effects. *Aims*. The purpose of this study was to evaluate resource use and cost during mobilization for patients mobilized with chemotherapy plus G-CSF vs. G-CSF alone. *Design and Methods*. Patients 18 years of age or older with evidence of ASCT between January 1, 2000 and December 31, 2006 were identified from a nationally-representative database of private payer medical and pharmacy claims. Patients had to receive apheresis within 60 days prior to ASCT and have a history of multiple myeloma, non-Hodgkin's lymphoma, or Hodgkin's disease; patients also were required to be continuously enrolled in one health plan for at least 90 days pre-ASCT and have no evidence of a prior ASCT. Using claims data, patients were classified into one of two mobilization regimens: G-CSF alone or chemotherapy with G-CSF.

Total resource use and direct medical costs were calculated from the time of administration of the mobilization regimen to the first apheresis day; paid claims were used as a proxy for costs and expressed in 2006 US$. *Results.* 235 ASCT patients were identified; 172 (73%) were mobilized with G-CSF alone and 63 (27%) with chemotherapy and G-CSF. Total mobilization costs were 27% higher among patients mobilized with chemotherapy versus those mobilized with G-CSF alone ($39,686 *vs.* $31,251, p=0.020). Patients mobilized with chemotherapy had higher inpatient hospital costs ($8,225 vs. $5,854, p=0.056), more use of growth factor ($9,217 *vs.* $6,948, p=0.003), and higher pharmacy costs ($5,343 *vs.* $1,484, p<0.001) than patients mobilized with G-CSF alone. Rituximab comprised 48% of pharmacy costs in the chemotherapy and G-CSF group, while the remaining costs were due to chemotherapy drugs, MESNA, and anti-emetics. *Conclusions*. ASCT patients mobilized with chemotherapy had higher total mobilization costs than patients mobilized with G-CSF alone. This significant difference in costs was due to more costly hospitalizations and greater use of G-CSF and other pharmaceuticals in patients mobilized with chemotherapy.

Table 1.

	Chemotherapy and G-CSF	G-CSF Alone	% Difference	*P*-value
Total Mobilization Costs	**$39,686 (31,188)**	**$31,251 (31,750)**	**27%**	**0.020**
Inpatient	$8,225 (19,214)	$5,854 (18,312)	41%	0.056
Outpatient	$16,901 (17,085)	$16,965 (19,772)	0%	0.971
Pharmacy	$14,560 (12,067)	$8,432 (13,502)	73%	<0.001
G-CSF	$9,217 (7,069)	$6,948 (10,514)	33%	0.003
Rituximab	$2,547 (6,228)	$332 (1,625)	667%	<0.001
Other retail pharmacy	$2,797 (2,923)	$1,152 (2,644)	143%	<0.001

Values represented as Mean (SD)

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ELECTRONIC HEALTH (EHR) FOR HEMATOLOGY AND ONCOLOGY PATIENTS

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Background. In 2008 ASCO developed 'Practical Guide for Selecting and Implementing Electronic Health Records' *www.cancer.net/ASCO/Practice+Resources*. It not only marks recognition of the necessity of the EHR, but also the importance of their specialization. Analysis of disease progression and treatment outcomes requires large amounts of time-sensitive data and causal connections. Most of these data exist in electronic form, but there are few instruments that allow extraction information from different data bases, compression, and collection on the single screen. *Design and Methods*. A National Standard «The Electronic Health Record», has been operating in Russia since 2008. It is similar with ASCO EHR Guide in many respects. We have developed this Standard based on the experience acquired by creation and implementation an EHR system for the National Center for Hematology. This system allows integrated data presentations on a uniform axis of time and direct access to all information concerning the patient. This approach is important for an estimation of correlations in dynamic changes in clinical parameters. The system has been already working more than 10 years. Now we have 400 physician-users and about 2 million of personal medical records on over 30 thousands patients treated in our center. The system has decreased a number of medical errors, proved to be an effective tool of clinical decision support, and allowed economizing time and resources. In the National Standard we have put in pawn recommendations to use new Internet technologies. The new system unites Internet-based life-long personal health records of patients with all clinical and administrative instruments of data presentation and processing. The Internet system promises to be cost-efficient in the field of remote consultation and telemedicine, but its efficiency will depend on a degree of standardization for different medical institutions. *Conclusions*. The world community of hematologists and oncologists and there patients will greatly benefit (efficiency of therapy, telemedicine, referrals and recruiting patients for clinical trails) from uniform approaches to EHR.

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BEHAVIOUR OF HOMOCYSTEINE PLASMATIC IN LONGEVOUS PATIENTS

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Introduction 158 male longevous patients were studied. They were all residents in old people´s homes and abandoned by their relatives. At the start of this case study the patients showed general neglect, not only about personal hygiene, but also about the building and fittings and they had difficulty in moving about. As regard the blood values, they were badly altered, showing severe anemia, macrocitocis accompanied with severe trombocitocis, also the homocysteine plasmatic was rather high. In the first place it was decided to move the old people to another home where they could have better hygienic and building conditions. The nourishing diet was also radically changed leaving out the former one which consisted of carbohydrates the seven days of the week and changing it for the ingestion of protein, vegetables and diary products nutriments. *Design and Methods*. During the first 40 days they were helped with folic acid to raise the hemoglobin level which was between 7 and 8,20 g/dl and with a minimum dose of acenocoumarol to prevent thrombosis events. At the next blood control it was clear sighted that slowly and gradually the blood values were encouragingly changing and basically the homocysteine plasmatic value which fell almost at a normal value. That´s why it was decided to suspend the folic acid but not the acenocoumarol, which was used for a time to allow the new diet to perform the corresponding supply. *Results*. After 90 days, when the corresponding hematological blood control was done, the patients had achieved normal analytical values: the homocysteine plasmatic had fallen to normal values, the hemoglobin values were between 12 and 13 g/dL accompanied with normocitic elements and values of count of thrombocytes between 300000 to 400000 x mm^3. *Conclusions.* These patients, by means of the diet and a period when they had folic acid, recovered their health, vitality and basically their rights to enjoy completely a better quality of life, thus achieving psycho-social stability. The authors provided free attention as regards treatment, medicine supplies, control and following of every part of the treatment, taking the place of the State in the care of the people´s health.

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DAPTOMICIN VS TEICOPLANIN, WITH OR WITHOUT IV CALCIUM CHLORIDE SUPPORT, IN POST CHEMOTHERAPY FEBRILE NEUTROPENIA: MONOCENTRIC PHARMACOECONOMIC PROSPECTIC RANDOMIZED STUDY

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Background. Daptomicin is a bactericidal antibiotic highly effective in gram positive infection. *in vitro* experiments seem to show that calcium ione might henance bactericidal activity of Daptomicin. Moreover it's well known that empirical use of glycopeptides in febrile neutropenia is of scarce utility. Data regarding use of Daptomicin in febrile neutropenia, in terms of safety, feasibility, clinical efficacy and pharmacoeconomy are actually lacking. *Aims.* Aim of this study is to evaluate efficacy of Daptomicin respect to Teicoplanin in febrile neutropenia in terms of: -days of fever, -days of hospitalization, -cost of antibiotic and supportive therapy per day of hospitalization, -days of fever in patients receiving Daptomicin and Teicoplanin if supported or not with intravenous calcium. *Design and Methods*. This is a monocentric, prospectic randomized study. Patients with post chemotherapy febrile neutropenia were randomized to receive empiric therapy with Piperacillin-Tazobactam 4.5 g tid iv, Amikacin 15 mg/kg/day iv and Teicoplanin 6 mg/kg/day iv (arm A) or Daptomicin 6 mg/kg/day iv (arm B). In each arm, patients were randomized 1:1 to receive iv support of Calcium Chloride up to achieve 10 mEq/l of blood calcium. Daily costs of antibiotic and supportive therapy for each patient was calculed dividing the global cost of entire period of hospitalization for the days of hospitalization. In 8 months 34 patients were enrolled. 32

patients were evaluable (16 in each arm). In arm A all patients were male. Median age was 44 years (R 30-72). 7 patients had NHL, 8 AML and 1 received autologous bone marrow transplantation. In arm B M/F was 11/5. Median age was 54 years (R 30-75). 9 patients had NHL and 7 AML. *Results*. In arm A median of days of fever was 10.5 days (R 6-12) and median of hospitalization was 35.5 days (R 10-40). Median cost of antibiotic and supportive therapy was 750 euros/day (R 329-1250). No differences in days of fever were noted between patient supported with calcium or not (10.5 days in two groups). In arm B median of days of fever was 10.5 days (R 4-17) and median of hospitalization was 24 days (R 19-27). Median cost of antibiotic and supportive therapy was 453 euros/day (R 178-807). Patient supported with calcium showed 5.5 days of fever (R 4-7) vs 15.5 days (R 14-17) in patients not supported. No adverse effects in patients receiving daptomicin were registered. *Conclusions*. Daptomicin seems to be safe and feasible in patients with post chemotherapy febrile neutropenia. Data regarding days of fever, hospitalization, cost of antibiotic and supportive therapy per day of hospitalization are difficult to evaluate because of eterogeneity in two treatment groups. In arm B the group receiving calcium showed similar characteristics than other group. This suggest that calcium support might really potentiate *in vivo* Daptomicin efficacy, shortening number of days of fever. These data need confirmation in a larger cohort of patients.

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