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XI Congress of the Italian Society of Experimental Hematology

Turin, Italy, October 6-8, 2010

ABSTRACT BOOK

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Official Organ of the European Hematology Association

haematologica
the hematology journal

The origin of a name that reflects Europe's cultural roots.

Ancient Greek

αἷμα [haima] = blood
αἵματος [haimatos] = of blood
λόγος [logos]= reasoning

Scientific Latin

haematologicus (adjective) = related to blood

Scientific Latin

haematologica (adjective, plural and neuter, used as a noun) = hematological subjects

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XI Congress of the Italian Society of Experimental Hematology

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MAIN PROGRAM

GENETIC CONTROL OF HUMAN MYELOPOIESIS

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Human myelopoiesis initially takes place in the bone marrow microenvironment, in which can be distinguished two main compartments, bone and blood tissues.[1] Three different types of stem cells are present in this complex microenvironment, namely hemopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and the less defined Hemangioblasts.[2] HSCs are considered multipotent cells since can give rise to all blood cells and to osteoclasts;[3] MSCs too are multipotent cells that are able to differentiate into osteoblasts, chondrocytes, adipocytes and stromal cells,[4,5] while hemangioblast progenitors differentiate mainly into endothelial cells. Functionally, HSCs can interact via cell adhesion with various microenvironmental cell types, giving rise to different stem cell niches[6] such as the osteoblastic,[7] the vascular[8] and the stromal ones.[9] Depending on their cell cycle proliferative activity HSCs can also regulate gene expression in response to several cytokines and growth factors produced by microenvironmental cells.[10] Our studies clearly suggest that the kinetic model of hemopoiesis can explain the functional properties of HSCs such as self-renewal, commitment, differentiation and engraftment. When HSCs are quiescent cells out of cycle, they simply survive for a long period of time, and the vast majority of HSCs in the bone marrow is in G0 phase. When HSCs enter in the G1 phase of the cycle, their gene expression is modulated mainly by microenvironmental stimuli and cell adhesion, which activate specific signal transduction pathways. The study of different HSCs populations performed by gene expression profiling (GEPs) suggests that HSCs sub-population $CD34^{-}/Lin^{-}$ is quiescent until the expression of the CD34 adhesion molecule, when both Lin^{-} and Lin^{+} enter the cell cycle and become functionally active.[11,12] The mechanism that leads to self-renewal is not completely understood, even if several signal transduction pathways seem to be involved, different from those responsible for growth arrest and quiescence.[13] It has to be pointed out that the master regulators of HSCs lineages are transcription factors[14] able to activate different and specific genetic programs playing synergistic or antagonistic effects. The different myeloid precursors are selectively induced to proliferate by different hemopoietic growth factors capable to activate mytogenic pathways that expand specific precursor populations.[15] Other microenvironmental stimuli are necessary to activate the differentiation transitions leading to terminally differentiated and specialized cell types. The half-life of terminally differentiated cells is different and depends upon the cell type; anyway apoptosis is the final event. Hemopoietic tissue can be considered a rapidly renewing tissue whose physiological homeostasis is based on the balance among quiescence, proliferation, differentiation and apoptosis. Any alteration of this balance leads to different pathological abnormalities including hematopoietic malignancies. Myelopoiesis is a plastic biological system, therefore we decided for a systems biology approach interfering with HSCs properties by different methodological strategies such as gene silencing, gene overexpression, treatment with bioactive compounds or drugs that interfere with epigenetic mechanisms. We accurately monitored the biological consequences of the different interferences using DNA microarrays to obtain GEPs, i.e. the molecular phenotypes. Our study allowed the characterization of the function of several genes relevant for the genetic control of hemopoiesis.[16-25] Furthermore, using different bioinformatics tools,[26,27] we developed transcriptome maps for HSCs, precursors and terminally differentiated myeloid cells.[28-30] This genome wide approach identified in HSCs and precursor cells several chromatin domains that are silent, constitutively or differentially expressed. It has to be pointed out that silent chromatin domains contain genes that are important for non-myelopoietic functions such as development, visual perception, neurogenesis, muscle differentiation etc. Constitutively expressed chromatin domains contain mainly housekeeping genes, while differentially expressed chromatin domains contain genes specific for the various differentiation lineages. At present, we are studying the positional effect of gene expression, basing on the 3D model of chromatin organization in interphase nuclei of HSCs and myeloid precursors, using multi fish hybridization, chromosome painting and confocal microscopy to better define the topology of these chromatin domains in relationship with the characterization of chromosome territories.[31-34] Preliminary results indicate the importance of these approaches to characterize the cellular context in which chromosome translocations may occur in myeloid leukemias.[35-37]

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THE MOLECULAR BASIS OF MEGAKARYOCYTOPOIESIS

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Megakaryocytopoiesis is the process that leads to the production of platelets.[1-4] This process involves:

1) Commitment of multipotent hematopoietic stem cells or megakaryocyte (MK)/erythroid progenitors (MEP) toward MK progenitors;
2) Proliferation and differentiation of MK progenitors;
3) Maturation and polyploidization of MK precursors (megakaryoblasts);
4) Platelet production by mature MKs through proplatelet formation.

The entire system requires coordinated activation of MK-specific genes, cell cycle modulation, and active prevention of erythroid differentiation. Specifically, these programs result from upregulation of MK-selective transcription factors, downregulation of erythroid-selective transcription factors and ongoing mediation of common erythro-megakaryocytic transcription factors.[5-9]

A number of transcription factors that regulate MK development are well established:

1) *Commitment from hemopoietic stem cell to MEP.* MYB is a potent negative modulator of megakaryocytopoiesis, because a decrease in MYB activity augments the commitment of multipotent progenitors towards the MK lineage.[10,11] In addition, one of the earliest events distinguishing megakaryocytic and erythroid lineages consists of differential RUNX1 expression.[12-14] Numerous in vitro and in vivo studies have emphasized the centrality of GATA-1 and its cofactor FOG-1 in early MK development. Mice with reduced GATA-1 expression in MKs display thrombocytopenia and aberrant megakaryocytic maturation. Specifically, they show hyperproliferating abnormal small immature MKs and reduced expression of MK-related genes. Interestingly, these mice also display MEP abnormalities, implicating GATA-1 in development of a properly primed bipotent progenitor.[6,15,16] In humans, GATA-1 mutations have been associated with defective megakaryocytopoiesis;[17]
2) *Differentiation of MEP into MK progenitors.* Experimental evidence supports the involvement of FLI-1[18] and SCL/TAL1[19] in the commitment of MEP towards the megakaryocytic lineage. In addition, MEPs become committed MK progenitors under the regulation of FOG-1 and either GATA-1 or GATA-2.[20,21] In addition, biochemical studies have demonstrated physical interaction and functional cooperation of GATA-1 and RUNX1 in transcriptional activation of megakaryocytic promoters (12). The P-TEFb kinase complex mediates RUNX-GATA cooperation and megakaryocytic development.[7]
3) *Differentiation of MK progenitors into megakaryoblasts.* Committed MK progenitors turn into early-maturing MKs with positive regulation by RUNX1, TEL, GATA-1, FLI-1 and NF-E2.[5,22,23] Specifically, as MK undergo endomitosis and cytoplasmic maturation, GATA-1 again plays an essential role with its cofactor FOG-1. FLI-1 cooperates with GATA-1 and FOG-1 to activate the transcription of late MK genes such as Glycoprotein (GP)IX, GPIbalpha and Platelet Factor 4 (PF4).[24] FLI-1 is an ETS family member and FLI-1 knock-out is lethal in the embryo due to a defect in the vascular development and marked thrombocytopenia with an excess of small immature MKs undergoing apoptosis.[25] The same abnormalities were observed in the Paris Trusseau Thrombocytopenia and the Jacobsen Syndrome, where a deletion in chromosome 11q23 is always present and involves both ETS1 and FLI-1.[26]
4) *Regulation of terminal MK maturation and platelet release.* NF-E2 is a prime regulator of MK terminal differentiation and platelet release.[5,27] Mice lacking p45 NF-E2 show megakaryocytosis and severe thrombocytopenia leading to fatal hemorrhage. Loss of p45 NF-E2 interferes with MK maturation at an advanced stage and platelet release is severely hampered.[5,28,29] NF-E2 has been shown to regulate transcription of megakaryocytic genes, including cytoskeletal proteins (beta-tubulin), enzymes (thromboxane synthase), signaling factors (GPIIbIIIa;Rab 27b) and effectors of apoptosis (caspase 12). Recent data indicate that SCL/TAL1 plays a major role in platelet production during stress thrombopoiesis by regulating NF-E2 transcriptional activity.[30]

miRNAs are small non coding RNAs that suppress gene expression. Transcription factors and microRNAs (miRNAs) act in concert to regulate gene expression during hematopoietic differentiation.[31] miRNA expression is regulated during MK differentiation.[32] A discrete subset of miRNAs is downregulated during in vitro MK differentiation of human CD34$^+$ stem cells. Downregulation of miR-10a and miR-130a correlates inversely with expression of their target HOXA1 and MAFB, respectively.[32] miR150 is highly expressed in MKs and promotes MK differentiation at the expenses of the erythroid pathway, mostly by controlling the level of the transcriptional regulator MYB.[33] MYB expression during MK differentiation is also suppressed by miR34a.[34] miR155 and miR146a are highly expressed in CD34+ stem cells, where they repress genetic programs that promote MK differentiation.[31]

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NEW MECHANISMS OF BLOOD PLATELET PRODUCTION

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Blood platelets, tiny cells shed by megakaryocytes, circulate throughout blood vessels and survey the integrity of the vascular system. Each day, an adult produces approximately 100 billion platelets. While our understanding of thrombopoiesis has grown considerably since James Homer Wright's 1910 publication on "The Histogenesis of Blood Platelets," the mechanisms by which platelets are formed from megakaryocytes in situ remains to be defined. The development of megakaryocyte cultures that produce bona fide platelets has provided a means to study the intermediate structures called "proplatelets," long, thin extensions of the megakaryocyte cytoplasm that function as assembly lines for platelet production. Here, we discuss the mechanical events that lead to the extension and branching of proplatelets, and provide insights into the role of the spectrin-based membrane skeleton in proplatelet production. In addition, new mechanisms of the final stages of platelet production, individual platelet release, will be presented.

MEGAKARYOCYTOPOIESIS IN MYELOPROLIFERATIVE NEOPLASMS

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In the chronic myeloproliferative neoplasms (MPN) the involvement of a hematopoietic stem cell manifests itself with a global myeloproliferative process involving all three myeloid cell lines, that variably predominate in the different clinical entities.[1] A prominent proliferation of megakaryocytes is found in essential thrombocythemia (ET) and primary myelofibrosis (PMF), but while in ET megakaryocytes appear morphologically normal and maturing, dysmegakaryocytopoiesis occurs in PMF. In this disorder, megakaryocytes have a typical morphological appearance and topographical distribution that help making diagnosis, according to the WHO criteria.[2-5] Small to large megakaryocytes are present displaying an aberrant nuclear/cytoplasmic ratio with hyperchromatic, plump lobulated or irregularly folded nuclei, and are clustered tightly; the process of proplatelet formation is abnormal in terms of proplatelet density and size.[6] Clonal, dysplastic megakaryocytes are the source of growth factors locally released in abnormal quantities, which on turn stimulate polyclonal fibroblasts[7] to produce fibers contributing to the derangement of bone marrow microenvironment;[8] emperipolesis is one of the mechanisms for intramedullary megakaryocyte death.[9] However, stimulation of fibroblasts caused by megakaryocytic fibrogenic and inflammatory cytokines finally results in a pathological microenvironment that participates in the development of the hematopoietic clone.[10] The role of abnormal megakaryocytopoiesis in the pathogenesis of myelofibrosis is illustrated by murine models in which over-expression of TPO[11] or abnormalities of the transcription factor GATA-1[12] result in abnormal deposition of extracellular matrix proteins, neoangiogenesis[13] and osteogenesis, closely mimicking human disorder.[14] Transforming growth factor is probably the major cytokine involved in this process.[15,16] It is of interest that an N-terminal truncation of GATA-1 due to somatic mutations in Down syndrome children with the transient myeloproliferative disorder induces extensive proliferation of dysplastic murine fetal megakaryocytes, reinforcing the involvement of downstream GATA-1 targets;[17] however, until now, no genetic abnormality in GATA-1 has been demonstrated in PMF, although the content and cellular compartimentalization are defective.[18] On the contrary, GATA-1 mRNA was overexpressed in bone marrow aspirates of ET or polycythemia vera (PV) patients.[19]

The JAK2V617F mutation is a critical event in the pathogenesis of MPNs, occurring in a HSC which shows skewing towards the erythroid differentiation, at least in PV.[20] Retroviral transplant studies demonstrated that the JAK2V617F mutation can produce a myeloproliferative phenotype with erythrocytosis and variable leukocytosis, while thrombocytosis was not generally observed, notwithstanding progressive abnormalities of megakaryocyte maturation finally accompanied myelofibrosis.[21-24] On the other hand, transgenic mice displayed a variable phenotype that correlated with the transgene copy number; mice presenting relatively low number of transgenes manifested thrombocytosis while both erythrocytosis and thrombocytosis developed in the presence of high transgene copy number.[25,26] These observations suggest that different V617F allele burden correlates at least partially with MPN phenotypes;[27] according, most patients with PV harbor homozygous V617F clones compared to very few in ET,[28,29] and inverse correlation between V617F allele burden and platelet count exists.[30] To gain better insight into the role of JAK2V617F mutation in lineage decision, three knock-in mouse models have been recently described. In all the models, a MPN phenotype developed; it was characterized by erythrocytosis and thrombocytosis with evolution to myelofibrosis in two models expressing a mouse JAK2V617F allele,[31,32] and a ET-like phenotype with thrombocytosis and moderate polycythemia but not splenomegaly or myelofibrosis in the model expressing a conditional mutated human gene33. Quite unexpectedly, in one murine-JAK2 model, homozygosity for mutated allele resulted in further increase of platelet count.[31] Thus, these studies confirm the essential role of JAK/STAT pathway in the pathogenesis of MPNs, but additional factors must intervene in the preferential prolifer-

ation of maturing or dysplastic megakaryocytic lineage in ET and PMF, respectively, as compared to the expansion of erythroid lineage in PV. Signals originated from the PI3K/Akt[34] and/or downstream preferential activation of STAT335 versus STAT5[36] could be one of these variables. Finally, mice expressing MPLW515L mutation developed extensive proliferation of megakaryoytes and an acute myelofibrosis phenotype.[37] Insights into the abnormal regulation of megakaryocytopoiesis in ET derived from a whole-genome expression analysis highlighting a resistance to apoptosis with down-regulation of proapoptotic genes.[38] However, we must acknowledge that a comprehensive picture that could explain the complex and unique involvement of the megakaryocytic lineage in ET and PMF is still lacking.

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CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II (CDA II): FROM CLINICS TO GENE DISCOVERY

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CDA II represents the most frequent form of CDA with prevalence varying between European regions, with minimal values of 0.08 cases/million in Scandinavia and 2.60 cases/million in Italy.[1] CDA II was firstly described by H. Heimpel in 1967[2] whereas causative gene was identified approximately 40 years later. The main clinical findings are normocytic anaemia, jaundice and variable splenomegaly. Common complications are liver iron overload and gallstones. These features are also present in hereditary spherocytosis (HS) and it is possible to confuse these two conditions, as a matter of the fact several CDA II are truly diagnosed after splenectomy.[3]

This condition is associated with a well-defined morphological phenotype: bi- or multinucleated late precursors and flat vesicles of variable length have been visualised by electron microscopy (EM). Peripheral blood smears show distinct aniso-poikolocytosis with basophilic stippled red cells and a few (occasionally binucleated) mature erythroblasts.[3] On SDS-PAGE, Band 3 (anion exchange protein 1) and band 4.5 (glucose transporter 1) show a narrower band and faster migration due to hypoglycosylation. Equally specific is the detection by Western blotting (WB) of minor proteins derived from endoplasmic reticulum (ER) (GRP78, calreticulin, and PDI) and the decreased binding of tomato lectin.[4,5] For long time because of reduced glycosylation, MANII, MANA and GnTII were indicated as causative. After their cloning we are able to demonstrate that they are not primarily involved in CDA-II.[6] Using a genome wide study the gene was mapped on the long arm of chromosome 20.[8] Soon after a long walk searching for gene started and a large number of can-

didate genes located in this region were examined without results.[9] This localization help us to demonstrate that at least 10% of cases were not in linkage on chromosome 20 and that at least another gene must exist.

Using some cases from consanguineous families, K. Schwartz in collaboration with our group was able to redefine the region containing CDA II locus in a new interval, between D20S112-D20S106 markers: it contained 125 genes. Too much genes to solve this problem using the sequencing approach. In order to narrow down this region, we performed microarray analysis on RNA from CD34+ to orthochromatic normoblasts (14-21 days after erythroid differentiation by EPO) of healthy controls to identify differentially expressed genes during normal erythropoiesis. Supposing that CDA II was due to the absence or reduction of a protein, all up-regulated genes mapped in this area were examined. Based on this information, we selected 11 genes. With the assumption that the cis, median and trans N-glycan Golgi processing of erythroblasts glycoproteins was impaired, the SEC23B gene became a likely candidate. When we started to sequence it, immediately we demonstrated a lot of different mutations in Italian, French and German cases.[10]

To clarify the function of SEC23B gene in erythrocyte development, we examined its role in the zebrafish embryo by injection of antisense morpholinos. In morphants, erythrocytes look immature and a significant increase in immature, binucleated erythrocytes was detected.[10] In human cellular model we elucidated the expression of SEC23B in erythropoietic lineage during differentiation. During this period there is a clear up-load of SEC23B whereas the counterpart SEC23A is down-regulated[10] (Figure 1B). Gene SEC23B encodes the SEC23B component which is part of the cytoplasmic coat protein (COP)II complex[11] (Figure 1A) that controls the protein trafficking from RE and Golgi.

Up to now, 65 CDA II unrelated cases have been described,[10;12-14] with a lot of different causative mutations (Figure 1C). In a preliminary way we could conclude that any part of the gene could be mutated and that in the vast majority of countries there are sporadic mutations. The higher incidence of CDA II in Italy prompted us to search for the existence of a founder effect, particularly in southern Italy. There are at least four mutations appearing more frequent in southern Italy. This information could be relevant for the diagnosis of new cases.

Recently we identified an array of mutations in the SEC23B gene within a cohort of 42 unrelated CDA II patients originating mostly from Italy and from France, but also from other parts of the world.[13] Correlation between the mutations and various biological parameters suggested that the association of one missense mutation and one nonsense mutation was significantly more deleterious that the association of two missense mutations; or rather, there is a trend for patients carrying two missense mutations to be more mildly affected. Nevertheless, there remained an obvious overlap between the two groups. Homozygosity for two nonsense mutations was never encountered and must be fatal.[13]

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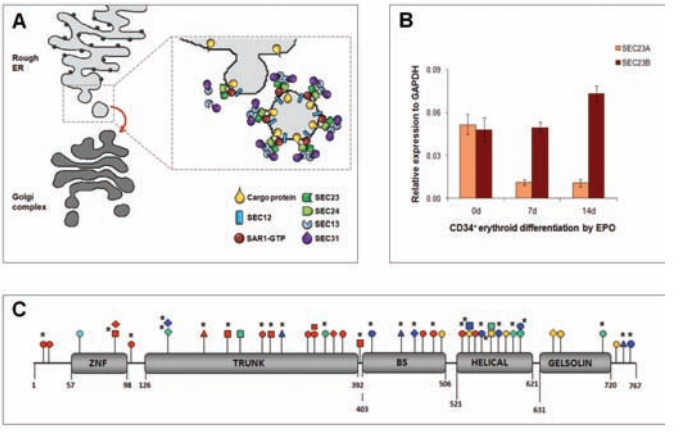

Figure 1. SEC23B and its role in CDA II disease. 3B is a component of the cytoplasmic coat protein (COP)II complex, a multi-subunit complex which mediates the anterograde transport of correctly folded secretory cargo from the ER towards the Golgi apparatus. Panel B. During erythroid differentiation of human CD34+ induced there is a clear up-load of SEC23B whereas the counterpart SEC23A is down-regulated. Panel C. Domains of the SEC23B protein are shown and delineated with numbered amino acids. ZNF: zinc finger; BS: β-sheet. Missense mutations are shown as circle, nonsense mutations as square, frameshift as rhombus and splicing mutations are triangles. The red symbols refer to the mutations firstly described in Schwarz *et al.,* 2009. The green shapes refer to the new mutations described in Bianchi, 2009, the yellow refer to those described in Iolascon *et al.,* 2010 and the only one mutation in light blue is described by Fermo *et al.,* 2010. Finally, the dark blue symbols refer to the new mutations identified in Italian patients. * Mutations identified in italian patients.

COMBINED GENETIC AND IMMUNOPHENOTYPIC CHARACTERIZATION TO IMPROVE PROGNOSTIC STRATIFICATION AND MINIMAL RESIDUAL DISEASE ASSESSMENT IN ACUTE MYELOID LEUKEMIA

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Over the past two decades, relevant insights into AML biologic and genetic characterization have allowed considerable progress in the management of this leukemia by better defining distinct prognostic groups for patient stratification and by identifying new targets for tailored therapies. This notwithstanding, the majority of AML patients still succumb to their disease due to leukemia progression or treatment-related toxicity such that the 5-year survival rates do not presently exceed 40% and 20% in adults and elderly patients, respectively (Dohner *et al.* 2009). In fact, while current chemotherapy produces CR rates of 80-90%, the emergency of chemotherapy-resistant clones occur in the vast majority of patients soon after remission. One of the main reasons underlying treatment failure relies on the fact that many patients receive inadequate therapy due to our limited knowledge of the biological features of the disease and of factors predictive of response to therapy.

Among clinical and biological features associated with AML, genetic aberrations represent most powerful predictors of prognosis and therefore the most reliable markers for tailoring treatment in the individual patient (Lo Coco *et al.*, 2008; Dohner *et al.*, 2009). In particular an integrated karyotypic and molecular approach to unravel cytogenetically silent lesions (such as mutation in FLT3r or NPM1 genes) significantly improves AML genetic characterization for better therapeutic stratification and routine screening for these abnormalities is now recommend-

ed for all newly diagnosed patients (Dohner *et al.*, 2009; Cheson *et al.*, 2003; Falini *et al.*, 2006; Alcalay *et al.*, 2005; Falini *et al.*, 2005). Some of the newly described genetic lesions (e.g. FLT3) may be targeted by specific inhibitors which have shown anti-leukemic efficacy in preliminary studies and are currently being evaluated in phase III clinical trials (Sanz *et al.*, 2009; Cheson *et al.*, 2003).

In addition to genetic aberrations, global changes of gene expression are constant features of the transformed AML phenotype and constitute molecular signatures with clinical relevance (Alcalay *et al.* 2005). Global epigenetic alterations, including alterations of DNA methylation and chromatin (epimutations), occur in all cancer types, are more frequent than gene mutations and can appear at different stages of tumor development (Minucci and Pelicci, 2006). Such epimutations might therefore have a significant impact on the selection of subpopulations of cells during tumor progression and the acquisition of resistance to anticancer drugs. Indeed, recent evidence suggests that changes in methylation of CpG islands, are crucial in determining tumor chemosensitivity (the DNA repair enzyme MGMT in gliomas; FancF – which activates the DNA repair complex containing BRCA1 and BRCA2 - in ovarian cancer) or tumor chemoresistence (mainly of pro-apoptotic genes such as Apaf-1; death receptors; mlh1). Moreover, epimutations require active mechanisms of maintenance and are, therefore, amenable to pharmacologic manipulation (Minucci and Pelicci, 2006; Zardo *et al.*, 2008; Petti *et al.*, 2002; Cimino *et al.*, 2006; Insinga *et al.*, 2005; Di Croce *et al.*, 2002).

Recent experimental evidence also indicates that specific microRNAs (miRNA), which act as endogenous modulators of target gene expression, are aberrantly expressed in AML (Fazi *et al.*, 2007; Fazi and Nervi, 2008). In addition, epigenetic/transcriptional regulation of microRNA genes and that of their targets or of the factors responsible for their expression is emerging as a unitary mechanistic model for hematopoietic cell fate decision whose de-regulation leads to leukemia (Felli *et al.*, 2005; Fontana *et al.*, 2007; Starnes *et al.*, 2009; Labbaye *et al.*, 2008; Fazi *et al.*, 2007; Rosa *et al.*, 2007; Fazi *et al.*, 2005). This offers suitable novel targets for diagnosis, prognosis and molecular therapeutic intervention (Bonci *et al.*, 2008).

Finally, some of the AML genetic alterations have been exploited to better assess response to therapy through the study of minimal residual disease (MRD) employing sensitive assays such as Q-PCR (Buccisano *et al.*, 2009; Gorello *et al.*, 2006; Cilloni *et al.*, 2009), However, while these molecular signatures certainly improved upfront prognostic evaluation in AML, their relevance in predicting response/resistance to chemotherapy is modest. Likewise, the clinical impact of detecting MRD through measuring these alterations in the post-remission phase is debated.

Multiparametric flow cytometry (MPFC) is an alternative method to quantify MRD. It consists of the combination of 4-5 surface markers (usually cross-lineage or asynchronous ones) and/or flow-cytometric physical abnormalities that characterize the leukemia blasts and are absent or very infrequent in normal bone marrow (leukemia associated phenotypes; LAIPs). We and others have shown that a specific LAIP can be found in up to 85-90% of AMLs patients. Using a cut-off value of 3.5x10-4 residual (LAIP-positive) leukemic cells to discriminate MRD-negative from -positive cases, we found that persistence of LAIP-positive MRD predicts clinical outcome in a consistent manner especially when measured at the post-consolidation time-point (Buccisano *et al.*, 2009; Maurillo *et al.*, 2008; Buccisano *et al.*, 2006).

Based on the above considerations, it is conceivable that an improved outcome evaluation in AML may emerge from the combination of upfront and delayed prognosticators. Moreover, the integration of MRD longitudinal characterization and molecular analysis of LAIPs might provide key novel insights on the tumor maintaining stem cell subpopulation (Viale *et al.*, 2009), and on molecular mechanisms and features associated to response to therapy and chemoresistance in AML. Thus a combined upfront and delayed characterization of LAIP and the bulk AML population on a large collection of clinical samples, will improve our understanding of mechanisms and features associated to disease progression, response to therapy and chemoresistance in this disease. This approach may in turn lead to: i) improved prognostic stratification and rationale use of treatment intensity (including SCT) and ii) therapeutic targeting of specific lesions and pathways associated to the disease chemoresistance.

We recently analysed clinical outcome in a large group of adult patients with newly diagnosed AML in whom upfront karyotype (K) and molecular genetics, and serial MRD measurements at distinct time points were available. The main finding of this study was that the conventional prognostic stratification based on 3 karyotypic risk categories (favorable, intermediate and poor K) might be considerably improved and simplified into 2 prognostically defined groups, i.e. a low-risk group including good K-MRDneg and intermediate K-MRDneg patients; and a high-risk group including good K-MRDpos, intermediate K-MRDpos, poor-risk cytogenetics and FLT3-ITD patients. In this scenario, MRD status at the end of consolidation appears to alter substantially the initial prognosis as dictated by the sole genetic allocation (Buccisano *et al.*, Blood 2010). Based on these findings the Italian multicenter cooperative group GIMEMA will start in 2010 a new phase II clinical trial focused on biological studies which is expected to enroll during the first 3 years at least 600 consecutive patients (aged 18-60 yrs) with newly diagnosed AML. All patients will be studied by karyotype, molecular genetics and immunophenotype (LAIP) at diagnosis in order to determine their upfront (by K, FLT3 and NPM1 status) and post-consolidation (by MRD status) risk category. After standard and uniform induction and consolidation chemotherapy, patients will be stratified for further treatments according to this upfront plus delayed prognostic categorization.

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UNRAVELING CLL PATHOGENESIS: NOVEL CLUES IN THE QUEST FOR DISEASE ORIGIN

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Over the last decade our understanding of CLL pathogenesis has been dramatically changed by a continuous flurry of information regarding its molecular and functional features.

The initial hypothesis that CLL represented a homogenous disease, derived from naïve B lymphocytes, has been definitely overcome by ever-increasing data originating from flow cytometric[1] and micro-arrays[2] studies that clearly showed that CLL cells share the same surface marker and gene expression profiles of activated B cells. Together with the evidence that more than 50% of CLL cases expressed somatically mutated IGHV genes,[3] these findings led to the common notion that CLL is a disease of antigen-experienced B lymphocytes. Accordingly, immunoglobulin heavy chain variable gene repertoire (IGHV) is highly restricted in CLL and it is noteworthy that many cases (>30% of CLL patients)[4] carry a very similar heavy chain complementarity-determining region 3 (VH CDR3), defined as VH CDR3 stereotypy.[5,6] Considering that the VH CDR3 labels the antigen specificity of the expressed immunoglobulin, BCR stereotypy suggests a potential role for the antigenic pressure in CLL development. Growing evidences advocate that the presence of stereotyped BCR might also influence clinical outcome, besides and independently of IGHV mutational status and gene usage.[7] In terms of antigenic specificity, CLL cells, in particular those from unmutated IGHV cases, can produce polyreactive antibodies that closely resemble natural autoantibodies, including anti-rheumatoid factor, anti-DNA or anti-cardiolipin; alternatively, some monoclonal antibodies obtained from CLL clones target molecular structures involved in eliminating and scavenging apoptotic cells and bacteria, such as vimentin, filamin B, PRAP-1, phosphorylcoline, oxidized LDL and bacterial polysaccharides.[8] These hints give rise to the quest for cognate "stereotyped" antigens, on the assumption that a chronic ongoing antigenic stimulation, along with alternative, non specific trigger (e.g. Toll-like receptors activation[9]) might represent the pathogenetic drive during both pre-leukemic and post-transformation phase, selecting the leukemic clone.

New insights into the pre-leukemic phase of CLL have been derived from a number of studies investigating the recently identified diagnostic category of monoclonal B-cell lymphocytosis (MBL).[10,11] This condition, characterised by the presence of clonal B-cell populations in the peripheral blood of otherwise healthy individuals, in most cases shares the same unique immunophenotypic profile of CLL (CLL-like MBL). Despite a reproducible phenotype, this category collects different biological entities carrying a variable risk of leukemia development, at least in part associated to the B cell count.

On the one hand, CLL-like MBL can be frequently detected in the general population[12-15] especially among aging individuals, where it seems to remain stable with a low if any risk of progression to a frank leukemia, though the follow-up studies are so far limited. On the other hand, MBL cases detected in a clinical setting (i.e. associated with lymphocytosis, now defined as Clinical MBL) demonstrate a sizeable 1.1-1.4% per year risk of progression into clinically overt disease.[16-19] The real nature of CLL-like MBL (preneoplastic condition or inevitable fate related to immunosenescence) is indeed a still debated question, attracting many research efforts.[20]

As no (neoplastic) cell is an island, the critical role of microenviroment in CLL pathogenesis cannot be overlooked. Within the so called proliferation centers (PCs), the histopathological hallmark of CLL,[21]located in lymph nodes (LN) and bone marrow (BM), leukemic lymphocytes are in close contact with stromal, nurse-like and T (mainly $CD4^+CD40L^+$) cells, supporting the growth and survival of CLL lymphocytes, through direct cell-to-cell interaction (e.g. CD40-CD40L, CD38-CD31) or secreting cytokines and chemokines (such as CXCL12/SDF-1).[22-24] It is now commonly recognized that LN and BM represent the actual sites where all relevant events in CLL survival, expansion and propagation happen and where leukemic cells can be triggered by auto or non-self antigen exposure and can be selected for clonal expansion.[25,26] A direct association between PC prominence and prognosis has recently emerged, proposing a detrimental effect of increased cellular generation on clinical outcome.[27] Recent findings are undoubtedly shedding light on CLL pathogenesis but our understanding of the interactions between CLL cells and microenviroment, the relationship between MBL and CLL and the Ig receptor biology as well, is far from being complete and deserve further research efforts that might help to reach a better biologically-oriented assessment of clinical prognosis and to develop novel therapeutic strategies.

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MICRORNAS IN MYELOPOIETIC DIFFERENTIATION

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Granulocytes (neutrophils) and monocytes/macrophages develop from progenitors that include common myeloid progenitors (CMPs) and, subsequently, granulocyte/macrophage progenitors (GMPs) in a process known as myelopoiesis.[1] The production of these two alternative lineages depends on the unique combination and relative expression levels of key transcription factors (TFs) promoting competing specific genetic programs.[1] CCAAT/enhancer binding proteins (C/EBPs), PU.1 and interferon-regulatory factors (IRFs) are among the master myelopoietic TFs. A remarkable feature of TFs in the myeloid system is that in human acute myeloid leukemia (AML) the majority of them are involved in chromosomal translocations or contain somatic mutations.[1] microRNAs provide an additional level of control beyond TFs.[2] They modulate a variety of developmental and physiological processes by fine-tuning the post-transcriptional expression of mRNAs. Beyond its mere presence or absence, the concentration at which a given protein is expressed may influence lineage choice and differentiation.[1,2] For this reason, miRNAs are crucial players in hematopoietic cell lineage specification by controlling factor concentration.[3] Indeed, several miRNAs are highly expressed in specific hematopoietic lineages and manipulation of their levels has been correlated with changes in cellular properties or differentiation.[3] Moreover, similarly to transcription factors, they have been found mutated or altered by chromosomal translocations associated with leukemia.[4]

Our group identified one of the first case of such control, the granulocyte-specific miR-223. miR-223 was shown to act as a positive regulator of ATRA-induced granulocytic differentiation of acute promyelocitic leukemia (APL) cells and to be transcriptionally controlled by the key transcription factor C/EBPα.[5] Later, a second regulatory element in the miR-223 genomic region was described to be controlled by PU.1 and C/EBPβ.[6] NFI-A was initially found as a relevant target of miR-223 in APL differentiation.[5] More recently, it has been shown that NFI-A restrains myeloid potential by conveying precursor cells to the erythroid lineage by activating β-globin transcription in erythroblasts while repressing G-CSF receptor expression.[7] Downregulation of miR-223 has been shown to occur selectively in AML patients carrying the t(8;21) chromosome translocation, responsible for the production of the fusion AML1-ETO protein; moreover, the AML1-ETO protein was found to inhibit miR-223 expression through binding to its promoter.[8] This indicates that the deregulation of miR-223 may indeed contribute to the differentiation block underlying myeloid leukemia pathogenesis. More recently, an independent study confirmed that C/EBPα is able to up-modulate miR-223 during granulopoiesis of AML cells, through translational repression of E2F1 and repression of cell proliferation.[9] Conversely to what it has been found in human cells, miR-223 knock-out mice display expansion of the granulocytic progenitor compartment leading to neutrophilia, and altered granulocyte immunological function.[10]

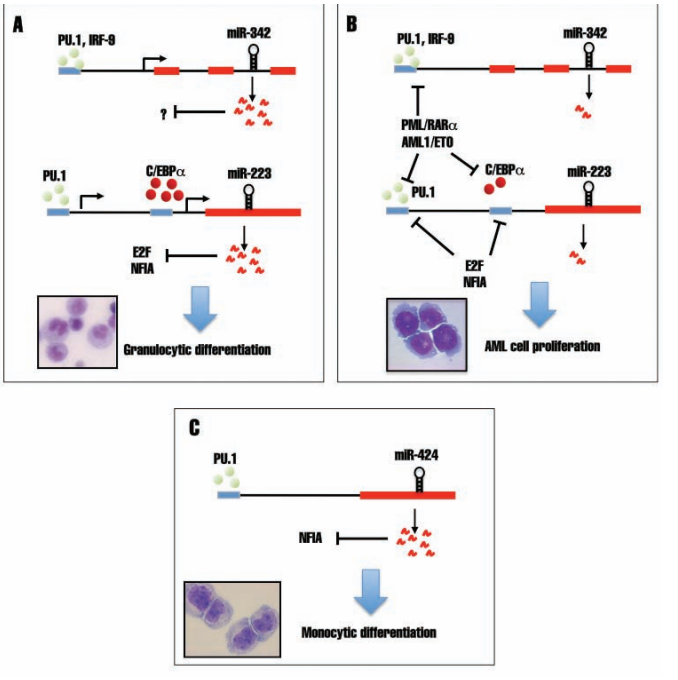

Figure 1. During granulopoiesis (A), C/EBPα and PU.1 bind and transactivate the miR-223 promoter regions, which in turn leads to E2F1 and NFIA repression, while PU.1 and IRF-9 are responsible for miR-342 transactivation resulting in myeloid differentiation.[5,9,12] When C/EBPα and PU.1 are deregulated by various mechanisms in AML (B), transactivation of miR-223 and miR-342 is inhibited, which results in accumulation of E2F1 and NFIA.[5,9,13] Fusion proteins inhibit miR-223 transcription through a negative feedback loop resulting in myeloid cell-cycle progression and block of differentiation.[5,9] During monocytic differentiation (C), PU.1 binds and transactivates the miR-424 promoter region, which in turn leads to NFIA repression, resulting again in myeloid differentiation.[11]

The transcription factor Mef2c has been identified as a crucial target of miR-223 in mouse myeloid precursors and indeed conditional knock-out of Mef2c within the miR-223 knock-out mouse rescued the proliferation abnormality but not the differentiation defect and the functionality of granulocytes.[10] These apparently contradictory findings might be due to the extreme diversity of experimental models used or the cellular stage which miR-223 is over-expressed, deleted or knocked down.

In the direction of studying the molecular circuitries regulated by miRNAs and involved in the control of myelopoiesis, we also described a new pathway driving human monocytopoiesis involving a PU.1-regulated non-coding RNA, miR-424, and again NFI-A.[11] These components are interlinked in a temporally regulated circuitry, where PU.1 increasingly activates miR-424 transcription during differentiation, and stimulates monocytopoiesis through miR-424-dependent translational repression of NFI-A.[11] Our group also found that PU.1, in association with IRF-9, induces the transcription of miR-342 during RA-induced granulopoiesis of APL cells, and this is responsible for stimulation of differentiation.[12] Recently, miR-342 was shown to be down-regulated in APL

blasts and repressed by the APL-associated oncogene PML/RARα, indicating that miR-342 de-regulation may contribute to leukemogenesis.[13] However, miR-342 relevant molecular targets in myelopoiesis have not been yet identified.

In conclusion, miRNAs play relevant roles in the molecular pathogenesis of AML interfering with pathways essential for myeloid differentiation and it is now clear that they represent a class of genes with a great potential for use in diagnosis, prognosis and therapy of AML.

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MICRORNA EXPRESSION IN MULTIPLE MYELOMA

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In the last decade, important advances in molecular cytogenetics and global genomic studies of myeloma cells and their normal counterparts have allowed a significant progress in understanding MM pathogenesis, providing the basis for a molecular prognostic classification and the identification of novel potential therapeutic targets.[1-3] MM is characterized by a profound genomic instability that involves both ploidy and structural rearrangements.[4] Nearly half of MM tumors are defined as hyperdiploid associated with trisomies of odd chromosomes and a low prevalence of chromosomal translocations involving the immunoglobulin heavy chain locus (*IGH*) on chromosome 14q32.[4] The remaining tumors are referred as nonhyperdiploid and are frequently associated with the constitutive activation of *CCND1*(11q13), *CCND3*(6p21), *MAF*(16q23), *MAFB*(20q11), or *FGFR3/MMSET*(4p16.3) genes as a result of IGH translocations.[5-8] The mechanisms underlying this dichotomic pattern have not been elucidated but hyperdiploid patients have a generally better prognosis. In addition, gains or losses of specific genomic regions including 13q, 17p13, 1p, 16q, 14q losses and 1q gains are adversely linked to prognosis in MM. Furthermore, gene expression profiling (GEP) studies have support the high biological heterogeneity of MM providing further insights into the pathophysiology and treatment of myeloma, including response to specific agents.[9-12]

The discovery of different classes of small noncoding RNAs (ncRNAs) has recently added a further level of complexity in normal and cancer cell biology. A large class of ncRNAs is represented by the microRNAs (miRNAs) which exert important regulatory roles in cell cycle, survival and differentiation programs at both transcriptional and post-transcriptional levels. It has been reported that the combination of nonrandom chromosomal abnormalities and other types of genetic alterations or epigenetic events may contribute to the deregulation of miRNA in many types of tumors including hematologic disorders.13,14 Data concerning the miRNAs involvement in MM have been reported only recently by our and other groups. Loffler et al. have shown that IL-6 regulates *miR-21* transcription in IL-6-dependent MM cell lines through a STAT-3-related mechanism, and that ectopic *miR-21* expression can sustain their growth in the absence of IL-6.[15] Pichiorri *et al.* have reported a set of miRNAs that can be associated with neoplastic transformation and progression in MM.[16] Roccaro *et al.* focused on the down-regulation of miR-15a and miR-16 detected in a small cohort of relapsed-refractory MM patients, demonstrating that they may play a role in the proliferation and growth of myeloma cells.[17] Our group identified a set of deregulated miRNAs correlated with copy number (CN) alterations or gene expression patterns[18,19] in human myeloma cell lines (HMCLs). More recently we found the presence of distinct patterns of deregulated miRNA in specific molecular types of MM patients, mainly associated with the major *IGH* translocations or distinct amplified/deleted chromosomal regions:[20] in particular, the overexpression of *miR*-221/222 and the *let-7e, miR-125a-5p* and *miR-99b* (belonging to a cluster at 19q13.33) in t(4;14) patients and the *miR-155, miR-133, miR-150, miR-1* overexpression in patients with the t(14;16). Furthermore, the occurrence of loss-of-heterozygosity (LOH) was found significantly associated with the altered expression of miRNAs located in the involved regions, such as *let-7b* at 22q13.31 and *miR-140-3*p at 16q22. Finally, in an attempt to define the consequences of deregulated miRNA expression, we performed integrative analyses based on computational target prediction, miRNA and mRNA profiling. Specifically, we searched for putative functional targeting relationships in MM cells supported by expression data, i.e. anti-correlations between miRNA and target mRNA expression profiles, and thus defined a global miRNAs/mRNAs regulatory network. From the general MM miRNAs-genes network, different sub-networks were derived according to the specific patterns of miRNA expression found to be associated with distinct genetic subtypes, in particular patients with t(4;14) or translocated *MAF* genes. Finally, the comparison of genome-wide miRNA expression between MM cases and patients affected by primary plasma cell leukemia (pPCL), an aggressive, rare variant of myeloma characterized by poor prognosis, revealed the differential expression of several miRNAs, some of which may have a particular importance in the context of MM and PCL dyscrasias as already know to be involved in human cancer and B-cell malignancies in particular. Overall, these data demonstrate that distinct signatures are associated with clinical phases and specific molecular types of MM suggesting that distinct miRNAs may play an important role in neoplastic transformation and progression of the disease as well as in prognostic risk group stratification.

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MICRORNA IN ACUTE LYMPHOBLASTIC LEUKEMIA

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MicroRNAs (miRs), a class of small RNAs of approximately 22 nt, regulate gene expression at the post-transcriptional level.[1] So far, roughly 700 miRs have been cloned in human and are annotated in miRBase (www.microrna.sanger.ac.uk); each miR is predicted to regulate the expression of about 1000 mRNAs.

Several studies have shown that miRs play an important role in different processes, such as cell cycle, differentiation and apoptosis.[2] Similarly, they are involved in oncogenesis.[3]

Numerous miRs participate in controlling physiologic lymphoid development. In fact, Bartel's group[4] showed that miR-181 overexpression in lymphoid progenitors induced an increase of the B-lineage cells both *in vitro* and *in vivo*; a work by Chen *et al.*[5] proved that miR-181 is also required to modulate the T-cell receptor signaling strength, to develop and select T-lymphocytes. Overall, these two studies highlight the importance of miR-181 in both B- and T-cell development.

miR-150 also plays a role in lymphoid lineage differentiation: Zhou *et al.* have reported that miR-150 is highly expressed in mature B-cell stages and, in mice, enforced expression of miR-150 in lymphoid progenitors blocks B-cell development without affecting T-cell development.[6] Furthermore, it has been shown that this miR regulates B-cell differentiation by targeting c-Myb.[7]

The miR 17-92 cluster also exerts an important role in lymphoid development: indeed, its deletion leads to enhanced levels of the apoptotic protein Bim and inhibition of transition from a pro-B to pre-B cell.[8]

Finally, miR-155 can be involved in both leukemic transformation, as well as physiologic immune response: in fact, Croce's group[9] previously reported that its overexpression in transgenic mice leads to a preleukemic pre-B-cell proliferation syndrome, followed by an overt leukemia, whereas two papers have shown that miR-155 regulates germinal center reaction and T-helper cell differentiation,[10] and the function of lymphocytes and dendritic cells.[11]

The most important functions of miRs in lymphocyte development are summarized in Table 1.

These tiny transcripts also appear to play a role in acute lymphoblastic leukemia (ALL). Lu *et al.*[12] have shown that miR expression profiles cluster ALL samples into distinct subtypes that reflect the developmental lineage. More recently, Mi *et al.*[13] have compared ALL with acute myeloid leukemia (AML) cases and identified 4 discriminative miRs: miR-128a and miR-128b are more highly expressed in ALL. In adult ALL, our group showed that miR unsupervised hierarchical clustering largely recapitulates ALL subgroups. Furthermore, it was possible to identify a small set of miRs discriminative of T- vs B-ALL and 6 miRs specific of B-lineage ALL subgroups harboring specific molecular lesions.[14] Furthermore, we identified a subgroup of adults with T-ALL with a "myeloid-like" profile, that displayed high levels of miR-223, comparable to those observed in AML.[15]

Table 1. MiRs involved in lymphoid development

MiR	*Function*	*Reference*
miR-181	Involved in B-lineage commitment; regulates T-cell	Chen *et al.*[4] Li *et al.*[5]
MiR-150	Regulates B-cell differentiation; highly expressed in mature B cells; regulates c-Myb	Zhou *et al.*,[6] Xiao *et al.*[7]
MiR 17-92	Regulates transition from pro-B to pre-B cells; regulates Bim expression.	Ventura *et al.*[8]
MiR 155	Induces a pre-leukemic syndrome/leukemia, regulates germinal center reaction; regulates T cells and dendritic cell functions.	Costinean *et al.*, Thai *et al.*, Rodriguez *et al.*[9-11]

Table 2. MiRs involved in acute lymphoblastic leukemia.

References	Main findings
Lu *et al.*[12]	MiRs reflect developmental linage.
Mi *et al.*[13]	Identification of a set of miRs that discriminate ALL from AML.
Fulci *et al.*[14]	Identification of a set of miRs discriminative of T- vs B-ALL and 6 miRs specific of B-lineage ALL subgroups harboring specific molecular lesions.
Chiaretti *et al.*[15]	MiR-223 is overexpressed in a subset of T-ALL patients with "myeloid-like" gene expression features.
Ju *et al.*[16]	Identification of miRs differentially expressed between leukemic and normal $CD19^+$ cells.
Schotte *et al.*[17]	Identification of 19 miRs and 8 miRs differentially expressed between pediatric ALL and $CD34^+$ precursors and between MLL-rearranged infants and other ALLs, respectively.
Popovic *et al.*[18]	Evidence that *MLL* regulates the expression of miR-196b during embryonic stem (ES) cell differentiation, while leukemogenic MLL cause its overexpression.
Schotte *et al.*[19]	Overexpression of 196-b occurs in leukemias with HOXA-cluster aberrant activation.
Coskun *et al.*[20]	MiR-196b and miR-196a are suggested as ERG regulator.
Mi *et al.*[21]	MiR 17-92 cluster is highly expressed in MLL^+ cases and induces increased proliferation and reduced proliferation.
Wong *et al.*[22]	MiR-17-19b constructs increase LSCs frequency, induce a differentiation block and enhance proliferation; p21 is a target of miR-17.
Usvasalo *et al.*[23]	Deletions of 9p lead to loss of miR-31.
Chim *et al.*[24]	MiR-34a can be methylated in lymphoid disorders.
Kotani *et al.*[25]	Detection of miR-128b mutations.

In children, Ju *et al.* reported the differential expression of a set of miRs between ALL and $CD19^+$ cells from healthy donors.[16] Schotte *et al.* described 19 miRs differentially expressed between pediatric ALL and $CD34^+$ precursors, and 8 miRs that differed between MLL-rearranged infants and other ALLs. Among those, particular attention was given to miR-196b, highly expressed in MLL^+ cases.[17].

The role of miR-196b is particularly intriguing: in fact, *MLL* normally regulates the expression of miR-196b during embryonic stem (ES) cell differentiation and leukemogenic *MLL* fusion proteins cause its overexpression.[18] Interestingly, further studies from Schotte *et al.* demonstrated the overexpression of this miR, that is located within the HOXA cluster, also in other leukemias with HOXA-cluster aberrant activation, suggesting its involvement in the biology of all HOXA-activated leukemias.[19] Finally, miR-196b, together with miR-196a, has been suggested as ERG regulator.[20] Another group of miR of particular interest is the miR17-92 cluster: in fact, in MLL^+ cases this cluster is highly expressed, induces increased proliferation and reduces proliferation;[21] furthermore, increased levels of the miR-17-19b constructs lead to a higher frequency of LSCs, a differ-

entiation block, and enhanced proliferation, the latter associated with reduced expression of p21, that is therefore considered as a target of miR-17.[22] Finally, recent evidences suggest that in ALL miRs can be affected by: i) deletions, as for miR-31, a regulator of *IKZF1*;[23] ii) methylation, as for miR-34a, a transcriptional target of p53;[24] iii), mutations, as for miR-128b,[25] thus making transcription regulation a complex and puzzling scenario. The main results are summarized in Table 2. In summary, miRs play critical roles in almost every cell function[26,27] and the same miRs can be involved in physiologic and malignant scenarios: specific examples include miR-155, miR-181, miR-223 and the miR 17-92 cluster. Their potential use as therapeutic agents or targets is under investigation.

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MINIMAL RESIDUAL DISEASE BY PCR-BASED APPROACH: IMPACT ON THERAPEUTIC PARADIGMS IN MATURE LYMPHOPROLIFERATIVE DISORDERS

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The application of new drugs and therapeutical paradigms resulted in major outcome advances in several hematological malignancies. Nevertheless a considerable proportion of patients still experiences disease relapse, even after a long period of clinical remission. Early identification of patients at high-risk of relapse is a major target of current translational research in onco-hematology as it could allow devising tailored treatments for high-risk patients and a more rapid outcome evaluation in clinical trials.[1-2] Minimal residual disease (MRD) detection is defined as any approach aimed at detecting and possibly quantifying residual tumor cells beyond the sensitivity level of routine imaging and laboratory techniques. Whenever a patient achieves complete clinical remission a number of different scenarios might take place including full eradication of the neoplastic clone, long-term persistence of quiescent or non-clonogenic or immunologically regulated tumor cells or persistence of clonogenic cells capable of giving rise to a full clinical relapse within months or years. Thus MRD analysis can lead to a better understanding of tumor clone kinetics, and to the early identification of patients at high-risk of relapse. Although several techniques could be adapted to this aim, including flow cytometry and fluorescent-in-situ-hybridization (FISH), polimerase chain reaction (PCR) has shown the highest sensibility and specificity, particularly in non-Hodgkin lymphoma (NHL) but also in multiple myeloma (MM).[3] From a clinical point of view the prognostic value of MRD detection by PCR-based methods in mature lymphoid tumors has been debated over nearly two decades. Following the seminal works of Gribben *et al.*[4] in autografted follicular lymphoma (FL) patients, the prognostic role of MRD has been demonstrated in many different mature lymphoid neoplasms and is now well established at least in FL[5] mantle cell lymphoma (MCL)[6] and MM.[7] In many of this tumors MRD is widely adopted as secondary endpoint of clinical trials by several cooperative groups, particularly in Europe. Over the long period in which MRD detection has been employed in clinical studies methodological approaches have considerably evolved as exemplified by the fact that qualitative PCR has been implemented and often substituted by real-time quantitative PCR. Among different disease entities, FL is the first mature B-cell tumor in which MRD was applied and still remains the disease in which MRD detection has been more frequently employed. Most studies so far reported showed a major and often impressive predictive value of MRD detection in this neoplasm.[4,5,8] Only a minority of studies failed to see the prognostic value of PCR detection,[9] which might suggest the presence of some limitations associated to sample timing, nature of treatment and technical performances of MRD laboratory. In MCL instead the earliest reports on MRD were published much later compared to FL because of greater technical complexity and therapeutic skepticism. Indeed the first report by Andersen *et al.*[10] emphasized the typical chemoresistance of this disease as shown by the extremely low rate of patients achieving molecular remission (MR). The introduction of real-time quantitative PCR as well as the availability of more effective anti-lymphoma therapies have allowed a wider and more useful employment of MRD in this neoplasm.6 Currently the predictive value of MRD analysis in patients with MCL is well-established and evidence of the therapeutic value of pre-emptive treatment of molecular relapse has been recently demonstrated.[11] In MM technical complexity and therapeutic skepticism have had a negative impact on the development of MRD studies that was even superior to that observed in MCL.[12,13] Nevertheless therapeutic progress has started to boost the interest for MRD evaluation in MM: in fact MRs have been observed in a significant proportion of patients undergoing allogeneic transplantation[14] and have been recently documented also in autografted patients consolidated with therapeutic regimens based on new drugs.[7] Also real time quantitative PCR proved to be highly successful for prognostic discrimination in MM.[7,15] Besides it should be noted that in MM flow-cytometry has also been successfully and more easily employed for MRD detection:[16] thus in the future a combination of PCR and flow cytometry will probably represent the most effective approach to MRD monitoring in MM.[3] As MRD becomes critical in the clinical management of NHL and MM patients, the issue of standardization and adoption of consensus defi-

nitions is becoming of major importance. Expert consensus has been established on a number of technical requirements and definitions suitable for clinical use. Moreover several MRD laboratories have started to perform quality control rounds on a national and European basis with the ultimate aim of achieving full standardization of PCR techniques in mature lymphoid disorders as already achieved in the acute lymphoblastic leukemia field.[17] In conclusion, MRD represent an attractive tool in onco-hematology, which might provide direct benefit to optimize the clinical management of patients with NHL and MM. The success of this approach is indeed demonstrated by the growing number of clinical trials that will consider MRD monitoring and even MRD-tailored treatment in both these disease entities.

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MINIMAL RESIDUAL DISEASE DETECTION IN ACUTE MYELOID LEUKEMIA

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In spite of recent advances in deciphering biologic complexity of acute myeloid leukemia (AML), the current management of adult patients is still largely based on the "one fits all" principle. Moreover, pre-treatment prognostic parameters including age, white blood cell count, immunophenotypic pattern, chromosome and genetic abnormalities cannot always reliably predict individual patient outcome. As a matter of fact, despite complete remission rates (CR) of 50-80%, approximately 35-40% of young and less than 20% of elderly patients are cured of their disease. Beside baseline prognosticators, it has become ever more evident that the kinetic of response to initial therapy may provide an additional prognostic marker. In this context, monitoring of minimal residual disease (MRD) has been recognized as a crucial prognostic tool that can be used to assess the magnitude of disease eradication and to refine the risk of relapse on an individual basis. Polimerase chain reaction (PCR) or multiparametric flow cytometry (MPFC) are the preferred methods for investigating MRD. PCR can detect at high sensitivity and specificity fusion transcripts or gene mutations and over-expression. Fusion transcripts originating from non random genetic abnormalities, such as RUNX1-RUNX1T1 (or AML1-ETO), CBFβ/MYH11, and MLL gene rearrangements, can be used as targets for reverse-trascriptase (RT) or real-time quantitative (RQ) PCR-based studies of MRD. Fusion transcripts are present in approximately 30% of patients and allow for a sensitivity of MRD detection of about 0.01%. Recognition of gene mutations in fusion gene negative AML has potentially increased to 60-70% the proportion of AML cases suitable for PCR-based MRD monitoring. Concerns about the use of these genes as candidates for MRD detection regard their stability over the course of disease. FLT3-ITD mutation is relatively common among cytogenetically normal AML but, differently from NPM1 mutations that appear to be stable at relapse, it is reportedly unstable and prone to cause false-negative results. WT1 is overexpressed in more than 70% of AML and very recently an experts consensus has been formalized on standardization of laboratory procedures to quantify it. Retrospective clinical analysis indicates that WT1 levels after induction therapy are associated with outcome, with the lowest predicting long term remission. MPFC is applied to detect combinations of cell antigens that are expressed in the leukemic cells but not in normal bone marrow cells. The interest about this technique is due to its wide applicability (≥90% of AML cases), quickness, specificity and ability to distinguish viable cells from the bone marrow background. On the other hand, such an approach still suffers from lack of a sufficient degree of standardization among different laboratories. The results published by many groups have clearly established that MPFC is a useful method for predicting relapse, however, which level and when a given level of MRD is of prognostic significance still represent a subject of dispute. Discrepant experiences have been published with some authors advocating the importance of an early determination soon after induction and others supporting the hypothesis that delayed time-points (post-consolidation) may be even more informative. Considering the early or delayed option makes us recognize different therapeutic scenarios. The early time-point option may prove useful to identify as soon as possible high-risk patients for whom a fast allocation to very intensive treatments is required. On the contrary, opponents to this hypothesis raise concerns of situations of over-treatment for patients showing a slow blast clearance which can cause MRD to be still positive after induction and negative after consolidation. Therefore, although the delayed time-point option implies deferral of the therapeutic decision, it might offers a more realistic risk-stratification avoiding complications due to over-treatment. In conclusion, whatever the method, quantification of MRD provides opportunity to implement AML risk-stratification and to develop strategies of risk-adapted therapy. Prospective studies are needed to better define pending technical issues and features such as choice of time-points. These prospective studies should also provide the opportunity to generate comprehensive prognostic algorithms which take into account conventional parameters, such as cytogenetic and genetic profile, and those strictly inherent in the quality of response, such as determination of residual leukemia.

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MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Monitoring of minimal residual disease (MRD) provides a direct measurement of the degree of response to chemotherapy and allows to estimate the amount of residual tumor in addition to establishing its presence (or absence).[1-5] This information can be used to improve strategies of risk assessment and treatment selection in the management of acute

lymphoblastic leukemia (ALL) patients, since one of the most important challenges in leukemia treatment is to dissect between patients who require more intensive (and potentially more toxic) therapy and those for whom cure rates may be achieved with less intensive therapy.[6-15] The conventional criteria for remission in patients with acute leukemia are based on the morphologic examination of bone marrow (BM) samples and patients are considered to be in complete remission (CR) when BM aspirates contain less than 5% blasts. In the presence of a morphologic CR, however, the extent of MRD varies considerably. The distinction between leukemic and normal cells is exceedingly difficult in bone marrow samples recovering after cessation of chemotherapy; this is particularly true in patients with ALL because the morphology of ALL blast cells is often indistinguishable from that of lymphoid precursors or activated lymphocytes. ALL cells can thus be potentially distinguished from normal hematopoietic progenitors on the basis of morphology, immunophenotype,[3,16,17] karyotypic or genetic abnormalities and Ig/TCR gene rearrangements.[18,19] These different characteristics have been exploited in an attempt to detect small numbers of blasts within normal cells; consequently, the methods for MDR analysis include immunophenotype, conventional cytogenetics and FISH, PCR techniques. On the other hand, the greatest obstacle to the routine use of MRD studies in ALL therapy protocols is that none of the techniques currently available for MRD detection can be applied to all patients. All MRD methods described hereby are being used to monitor MRD by various groups, the selection depending primarily on existing expertise and strength of preclinical studies within the different groups. Immunophenotyping techniques using multicolor-gated flow cytometry are based on the aberrant expression of antigens by the leukemic cell population and on the identification of markers that may be found on malignant cells in combinations that are normally not observed in normal BM and peripheral blood (PB) cells.[16,17,20-25] Overall, flow cytometry can be utilized to monitor MRD in about 85-90% of cases. The use of this approach requires a deep understanding of the immunophenotypes expressed by normal hematopoietic cells, not only during steady-state conditions, but also during chemotherapy and active regeneration. Polymerase-chain-reaction (PCR) amplification of antigen-receptor genes is a reliable and accurate method for monitoring MRD; two main categories of targets can be used to distinguish leukemic cells from normal cells with PCR. One is represented by gene fusions, such as BCR-ABL1, MLL-AF4, TCF3-PBX1, and ETV6-RUNX1, which result in the expression of aberrant mRNA transcripts in leukemic cells.[26-29] The second category of PCR targets for MRD studies in ALL is composed of the clonal rearrangement of the Ig and/ot TCR genes whose junctional regions are unique to the leukemic clone. The Ig heavy chain genes undergo rearrangement in 90-95% of patients with B-lineage ALL. TCR gene rearrangements occur in 95% of T-lineage ALL (TCR delta, TCR gamma and TCR beta) and in 50-70% of B-lineage ALL (TCR delta and TCR gamma).[30-32] Flow cytometry, gene fusions and Ig/TCR gene rearrangements show advantages and disadvantages in the monitoring of MRD (Table1); for example, flow cytometry results can be obtained within a few hours of sample collection, while the development of a patient-specific PCR assay is time consuming (often more than 2 weeks) . Since PCR may detect residual leukemic cells in cases not amenable to flow cytometric investigation, and vice-versa, it is possible to apply the two techniques in tandem.[33-35] A commonly used cut-off level to define MRD positivity is 0.01% of bone marrow mononuclear cells. The selection of this level is due to the fact that this is the typical limit of detection for routine flow cytometric and molecular assays, and it has been shown to discriminate between patients with different risks of relapse. One of the most immediately obvious applications of MRD testing is its use in measuring early treatment response and identifying patients who achieve morphologic remission but still harbor considerable levels of disease.[36] Also, there is an increasing need for standardization of methodologies and harmonization of terminology.[37] Current studies incorporating MRD to guide treatment decisions will clarify whether this approach produces significantly higher cure rates and/or lower toxicities. There are numerous ways to include MRD studies in clinical trials, depending on treatment schedule, intensity and previous experience. In addition to their capacity to predict outcome on the basis of early response to therapy, MRD methods can also be used to recognize leukemia relapse before it is morphologically overt, to determine the leukemia burden before hemopoietic stem cell transplant, to investigate the presence of residual disease in $CD34^+$ cells collected for autografting procedures and to measure the effectiveness of a treatment regimen compared with a previous one.[7,9-15,38-43] Numerous studies have demonstrated conclusively that MRD is a powerful prognostic indicator in childhood ALL and protocols are accordingly tailored. Although the clinical significance of MRD has been studied less extensively in adult ALL, there is considerable evidence supporting its potential usefulness and in most multicenter studies MRD monitoring is currently incorporate in the clinical trials and guides treatment decisions.

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METHODOLOGICAL APPROACHES AND PRECLINICAL MODELS TO BRING A DRUG FROM THE BENCH TO THE BEDSIDE

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Cancer is increasingly recognized as "miscommunication" disease, in which inter- and intra-cellular signals are aberrantly sent and/or received, resulting in the uncontrolled proliferation, survival, and invasiveness of the cancer cell. Indeed, many of the genetic and epigenetic aberrations underlying neoplastic transformation and progression, ultimately impinge on the inappropriate activation/inactivation of intracellular signaling pathways. Such signaling cascades usually proceed from the cell surface, where growth factors interact with their specific receptors, to cytoplasmic signaling intermediates, where different signals are integrated and both positive and negative feedback circuitry are in place to ensure signal fidelity and transduction accuracy, to nuclear transcription factors/complexes, that ultimately lead to the transcription/translation of effector genes and proteins involved in specific cellular functions. While the signal may be inappropriately transduced at several, and usually multiple, levels, one interesting feature of aberrant cancer signaling is that cancer cells may become "addicted" to specific signals and hence exquisitely sensitive to their modulation.[1] In rare cases, such as CML, a single, 'apical', genetic lesion (the t(9;22) chromosomal translocation that gives rise to the BCR-ABL fusion protein) can be identified that drives the activation of an array of diverse signaling pathways, including NF-kB, AKT and STAT5 among others.[2] In such cases, pharmacological interference with the "causative" genetic alteration severely impairs the ability of transformed cells to proliferate and survive and dramatically alters the natural history of the disease, leading to arguably the most impressive "success story" in the field of cancer therapy over the past 20 years.[3] At the other end of the spectrum lies perhaps the deadliest of human cancers, pancreatic cancer, in which an average of 63 genetic alterations per case were recently detected by comprehensive genetic analysis. These alterations defined a core set of 12 different cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors.[4] Although most of human cancers lie between these two extremes, a single genetic alteration necessary and sufficient to drive the array of phenotypic hallmarks of malignancy is the exception rather than the rule and the malignant behavior is usually driven by the accumulation of several genetic and epigenetic aberrations. An additional level of complexity comes from the fact that our knowledge of signal transduction pathways has evolved, over the past 20 years, from the classical notion of "linear" signaling pathways, whereby a single receptor would transduce signals through specific "intermediates" to a limited number of final "effectors", to the much more complex vision of "signaling networks", in which every single component is closely intertwined with an array of different players, thereby creating an extremely complex scheme of vertical and parallel signaling pathways regulated by positive and negative feedback loops.[5] In this context, even the most specific interference with a single signaling component may actually lead to unexpected, and sometimes "undesired" from a therapeutic perspective, functional outputs. Such new level of complexity obviously requires completely novel strategies to both pathway investigation (for example the use of high throughput technologies and "omics" approaches) and interpretation of the results.[6-9] This may help explain why, in addition to a handful of success stories (such as the development of imatinib for the treatment of CML and GIST or that of trastuzumab for breast cancer), the clinical development of other compounds that specifically target protein kinases has been more troublesome, especially with regard to their combination with classical cytotoxic agents.[10] In addition to the inherent complexity of cancer signaling as a therapeutic target, these setbacks reflect a variety of other factors specifically related to the inadequacy of classical drug development paradigms when applied to "targeted" therapy, including a rush to get compounds into the clinic, a lack of validated biomarkers, insufficient characterization of patient populations appropriate for treatment, and oversight of pharmacodynamic and scheduling issues. Finally, as most cancers display complex genetic/epigenetic aberrations and since major signalling pathways extensively crosstalk to each other through 'vertical' and 'lateral' feedback loops operating at multiple levels along the signaling cascades, effective cancer therapy will most likely rely on the mechanism-based combined targeting of multiple signaling pathways, in an attempt to achieve synergistic therapeutic effects;[11] thus, how to develop rational drug combinations in the clinical setting will be a high-priority issue for scientists, methodologists, and clinicians alike.

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MOLECULAR TARGETING OF MTOR SIGNALING AND NEW PROSPECTS FOR COMBINATION THERAPIES IN ACUTE LEUKEMIAS

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mTOR (mammalian target of rapamycin) was originally identified in the yeast Saccharomyces Cerevisiae as the target of rapamycin, a macrolide antibiotic produced by the bacterium Streptomyces hygroscopicus. mTOR is a component of two distinct multiprotein complexes, mTORC1 and mTORC2[1] (Figure 1). mTORC1 is sensitive to rapamycin and its analogs (rapalogs: RAD001, CCI779, AP23753). mTORC2 is generally described as being insensitive to rapamycin/rapalogs. However, long-term (>24 hours) treatment of about 20% of cancer cell lines with rapamycin/rapalogs leads to dissociation of Rictor and SIN1 from mTORC2, resulting in its inhibition. Rapamycin/rapalogs are allosteric mTORC1 inhibitors and do not directly affect the mTOR catalytic site. There are mTORC1 functions, such as 4E-BP1 phosphorylation, which are not blocked by rapamycin/rapalogs. mTORC1 integrates environmental clues (nutrients, oxygen levels, growth factors) and information from the cell metabolic status.[2] Thus, mTORC1 controls anabolic processes to promote translation, ribosome biogenesis, and lipid synthesis. Furthermore, mTORC1 inhibits autophagy, a starvation-induced catabolic process wherein organelles are enclosed in a membrane structure and delivered to lysosomes. Several cell signaling pathways impinge on mTORC1, including PI3K/Akt, MEK/ERK, Wnt/GSK3beta, and LKB1/AMPK1.[3] mTORC1 is activated in the major part of patients with acute leukemias, of both myelogenous and lymphoid origin.[3,4] The regulation of mTORC2 function is less understood, however its activation requires PI3K, but is largely insensitive to either nutrients or energy conditions. mTORC2 phosphorylates Akt on Ser 473 and SGK1 on Ser 422.[3] At present rapamycin/rapalogs are in clinical development for the treatment of a variety of malignancies, including acute leukemias. Some promising activity has been reported in patients with advanced renal clear cell carcinoma, endometrial carcinoma, and mantle cell lymphoma.[2] Overall, rapamycin/rapalogs are well tolerated. However, the use of these drugs as broad based monotherapies does not appear as effective as expected, as documented by trials in acute myelogenous leukemia patients. Therefore, they are now being tested in clinical trials in combination with other drugs, including classical chemotherapeutic agents, EGFR and VEGFR inhibitors, MEK inhibitors.[2]

The modest efficacy of rapamycin/rapalogs has been attributed to their inability to inhibit 4E-BP1 phosphorylation which controls the synthesis of oncogenetic proteins. This phenomenon occurs in acute leukemias,[5,6] and could be circumvented by the use of dual PI3K/mTOR inhibitors (PI-103, NVP-BEZ235) which target the ATP-binding site of both kinases.[5] NVP-BEZ235 is now being clinically tested in solid tumors.[3]

Recently, a new generation of mTOR inhibitors (PP242, WYE354, AZD8055), which target the mTOR active site, has been released. These compounds suppress both mTORC1 and mTORC2 activity with significant selectivity over PI3K, and are much more effective than rapamycin/rapalogs on cell growth, survival, and proliferation. Thus, active site mTOR inhibitors have the potential to be potent anticancer agents, as documented by initial in vitro and in vivo investigations.[7]

Despite recent advances in the understanding of both mTORC1 and mTORC2 structure/function, our knowledge is still limited. Active site mTOR inhibitors will likely reveal new mTOR targets and further our understanding of mTOR signaling in health and disease. This valuable information could then be translated into the clinic, to improve future therapeutic strategies based on mTOR inhibition.

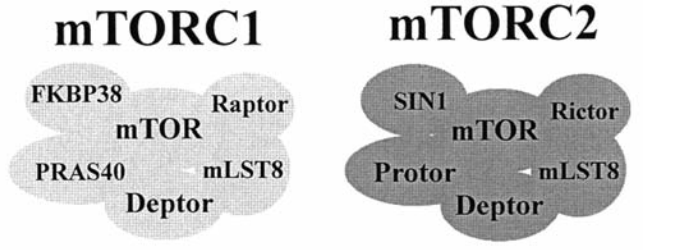

Figure 1. Components of mTORC1 and mTORC2.

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MOLECULAR TARGETS FOR LYMPHOMA THERAPY

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Knowledge of the molecular pathways sustaining lymphoma growth are a prerequisite for target therapy. The molecular heterogeneity of diffuse large B cell lymphoma (DLBCL), the most frequent lymphoma type in adults, provides a useful model for envisaging different strategies of target therapy. Targets exploited for rational therapeutic strategies of DLBCL in pre-clinical models or in early phase clinical trials include (Table 1): i) protein kinase Cβ; ii) the BCL6 proto-oncogene; iii) histone deacetylase; iv) the B-cell receptor (BCR) cascade; v) the NF-κB system.

Protein kinase Cβ (PKCβ) associates with poor prognosis and reduced survival in DLBCL. Enzastaurin is a selective inhibitor of PKCβ that induces apoptosis and inhibits the proliferation of DLBCL. In a phase II multicenter trial of oral enzastaurin conducted in relapsed/refractory DLBCL, the drug was well tolerated and associated with prolonged FFP in a subset of patients. These pilot data prompted the development of multicenter phase III trials of standard induction therapies (rituximab-CHOP) with or without enzastaurin as initial therapy in DLBCL.

The BCL6 gene is a master regulator of germinal center development (Figure 1). In DLBCL, the common functional consequence of BCL6 translocations is the juxtaposition of heterologous promoters to the BCL6 coding domain, causing deregulated BCL6 expression. Thus, BCL-6 rearrangements may prevent downregulation of BCL-6 and, in turn, block the differentiation of germinal center B cells toward the plasma cell stage. Another way whereby BCL6 contributes to lymphomagenesis is functional inactivation of TP53.

The fact that BCL6 is lymphomagenic and is frequently activated in *de novo* DLBCL represent optimal prerequisites for its exploitation as a therapeutic target. Recently, a peptomimetic inhibitor of BCL6 has been shown to display potent anti-lymphoma activity both *in vitro* and in animal models. This peptide inhibitor, known as RI-BPI (for retroinverso BCL6 peptide inhibitor), selectively kills DLBCL cells associated with the B cell receptor (BCR) gene expression profile. Acetylation plays a major role in down regulating BCL6, with histone deacetylase (HDAC) being required to lift this repression. Pharmacologic inhibition of HDAC in lymphomas expressing BCL6 may lead to tonic acetylation and inhibition of this pathway. This deacetylation pathway interferes with the TP53 pathway, providing further rationale for inhibiting deacetylation in DLBCL. Several HDAC inhibitors are under investigation in DLBCL. A subset of DLBCL have a transcriptional profile characterized by increased expression of multiple components of the BCR signaling cascade including the SYK tyrosine kinase. BCR engagement recruits and activates SYK and downstream pathways. Although BCR signaling is triggered by antigen binding, emerging data highlight the role of "tonic" BCR survival signals in the absence of receptor engagement. SYK plays a critical role in tonic BCR signaling, transmitting downstream events and amplifying the original signal. The SYK inhibitor R406 induces apoptosis in the majority of DLBCL cell lines and primary tumors and specifically inhibits tonic BCR signaling. A phase I/II trial of an oral version of the SYK inhibitor, R788/fostamatimib disodium (FOS D) has documented some activity in DLBCL.

Sustained activity of NF-κB signaling leads to aberrant expression of NF-κB target genes involved in cell survival, cell proliferation, cell adhesion, and inflammation (Figure 2). Gene expression studies have shown that the most aggressive biological type of DLBCL, i.e. activated B cell-like (ABC) DLBCL, associates with constitutive activation of the NF-κB transcription complex, frequently due to molecular lesions of NF-κB genes. Activation of NF-κB may provide a suitable target for rational therapy by drugs interfering with NF-κB. IMIDs may interfere with NF-κB among several other effects of this class of drugs. Studies of lenalidomide combined with CHOP are in progress in the context of DLBCL.

Beside the biology of tumor cells, also the genetic background of the host may be relevant for lymphoma prognostication and tailored therapy. Pharmacogenetic studies have documented that host single nucleotide polymorphisms (SNPs) affecting genes involved in drug metabolism, detoxification, and transport are responsible, at least in part, for the inter-individual variability in efficacy and toxicity of a given pharmacologic treatment. In DLBCL treated with R-CHOP21, a recent study documented that host SNPs affecting alkylating agent detoxification and doxorubicin pharmacodynamics are independent predictors of event free survival and toxicity.

Overall, although target therapy is frequently looked upon as an "all problem-solving" strategy, it may not be so in such a molecularly heterogeneous disease as DLBCL. The genetic asset of DLBCL is far more complex than that, say, of chronic myeloid leukemia, and the exploitation of target therapies in the single patient requires a detailed biological analysis of the lymphoma sample.

Table 1. Molecular targets for rational therapeutic strategies of DLBCL.

Target	Function	Drug(s)	Reference
PKC	Serine/threonine kinase phosphorylating the scaffolding protein CARD11	Enzastaurin	7
BCL6	Transcriptional repressor	RI-BPI	2
SYK	BCR signaling	Fostamatimib	3
NFκB pathway	Regulates cell survival, cell proliferation, and cell adhesion	Bortezomib, Lenalidomide, other NF-κB inhibitors	5
HDAC	Deacetylation of histones and of BCL6	HDAC inhibitors	6

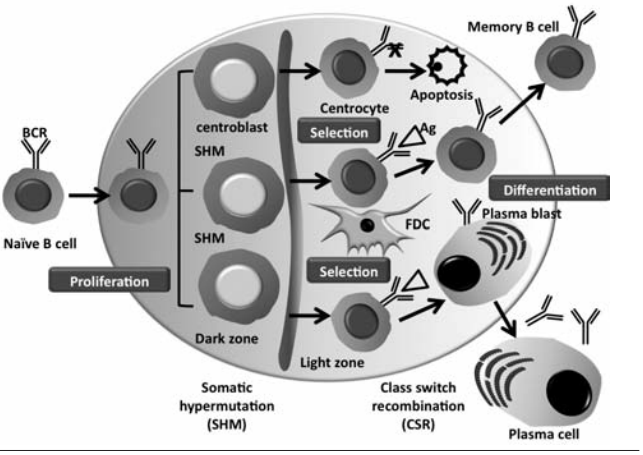

Figure 1. The germinal centre reaction. Naïve B cells differentiate into centroblasts and undergo clonal expansion in the dark zone of the germinal centre. During this process, somatic hypermutation (SHM) targets the IGV regions of the B cell receptor (BCR); some of these mutations may change the aminoacid sequence and increase, or decrease, the BCR affinity for antigen (Ag). Centroblasts subsequently differentiate into centrocytes, which reside in the germinal centre light zone. With help from T cells (not shown) and follicular dendritic cells (FDC), the mutated BCR is selected for antigen binding. Newly generated centrocytes whose mutations decreased BCR affinity for antigen undergo apoptosis and are removed. A subset of centrocytes undergoes class switch recombination. Antigen selected centrocytes eventually differentiate into memory B cells or plasma cells. Expression of BCL6 is restricted to GC centroblasts and centrocytes.

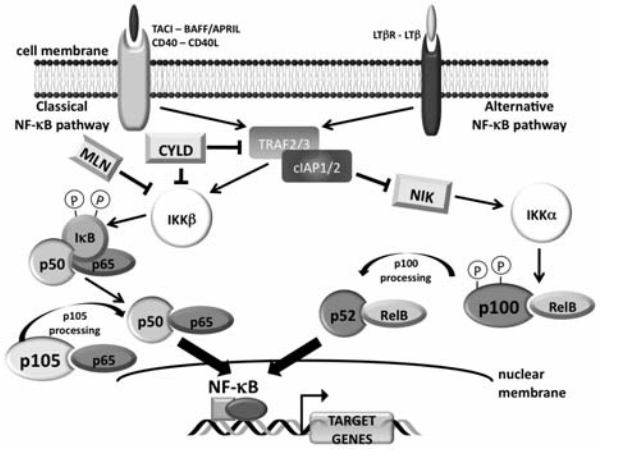

Figure 2. The NF-κB pathway and its involvement in DLBCL. Five subunits combine into hetero- and homodimers to create the NF-κB transcription factor family (p50, p52, c-Rel, p65/RelA, and RelB). Such dimers are inactive in the cytoplasm in most normal cells, due to the interaction of NF-κB dimers with IκB inhibitors. Activation of NF-κB signaling may follow two general pathways. In the classical (or canonical) pathway, IKKβ phosphorylates the inhibitory IκB molecule, leading to degradation (not shown) in the proteosome. As a result, the NF-κB heterodimers p50/p65 and c-rel/p65 accumulate in the nucleus. In the alternative (or non-canonical) pathway, IKKα phosphorylates p100/NFKB2, resulting in proteasomal removal of an inhibitory C-terminal domain and generating the NF-κB p52 subunit. As a consequence, the p52/RelB heterodimers preferentially accumulate in the nucleus. Following translocation to the nucleus, p50/p65, c-rel/p65 and p52/RelB activate transcription of target genes. In DLBCL, the NF-κB pathway is altered by several structural alterations, whose common effect is to activate the NF-κB cascade.

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CLINICAL MANAGEMENT OF "POOR MOBILIZERS"

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Patients with advanced or treatment-refractory Hodgkin Disease (HD) and non-Hodgkin Lymphoma (NHL) and patients with Multiple Myeloma (MM), may be successfully treated with high-dose chemotherapy followed by autologous transplantation of peripheral blood stem cells (PBSCs).[1-3] Successful engraftment of PBSCs is well correlated with the number of $CD34^+$ stem cells infused.[4] PBSCs are mobilized in the PB by treating patients with granulocyte colony-stimulating factor (G-CSF) for 5 to 7 days before leukapheresis. Circulating stem cells can also be increased with disease-specific, non-myeloablative chemotherapy, often in combination with G-CSF (reviewed in 5). The minimum number of $CD34^+$ cells required for a single autologous stem cell transplantation (ASCT) is generally considered to be $2\text{-}2.5\times10^6$ $CD34^+$ cells/Kg.[4,5] However, a significant proportion of MM or lymphoma patients fail to mobilize adequate numbers of PBSCs after conventional mobilization strategies (i.e. G-CSF with or without chemotherapy) and can not proceed to the planned ASCT. In fact, depending on prior treatment with stem cell toxic drugs, underlying disease, age, prior radiotherapy and bone marrow involvement, the failure rate with current strategies is estimated to be between 5% and 40%.[5,6] In Bologna transplant center database, for instance, 25% and 36% of NHL and MM patients, respectively, had been classified as poor mobilizers and failed to undergo subsequent single or double ASCT, respectively (Lemoli RM, unpublished data).

In MM, most retrospective studies addressing mobilization have identified the number of prior regimens, the time to mobilization and prior exposure to alkylating agents like melphalan and/or radiotherapy as predictors of mobilization failure. Moreover, novel anti-myeloma therapies such as lenalidomide have been demonstrated to impair PBSC mobilization.[7-11]

Similarly, in lymphoma patients, prior chemotherapy with drugs like fludarabine, nitrogen mustard, carmustine, lomustine, chlorambucil and busulphan has been shown to cause damage to the stem cell pool and affect stem cell mobilization whereas cytarabine and vinblastine have been shown to cause limited damages.[12,13] Moreover, the use of the radioimmunoconjugate Ibritumomab tiuxetano as induction-consolidation therapy may impair subsequent PBSC mobilization.

It is not clear from the literature whether patients who do not yield the optimal number of cells (generally considered to be between 4 and 6×10^6 $CD34^+$ cells/Kg recipient body weight for a single transplant) should be qualified as partial failures/successes. Depending on the therapeutic goal and the definition of "poor mobilizer", failure rates are expected to be higher than those historically reported in the literature. Mobilization failures leave patients with suboptimal alternative treatment strategies (e.g., bone marrow collection, allogeneic transplantation, further mobilization attempts, etc).

Plerixafor (formerly AMD3100) is a CXCR4 chemokine antagonist that has been shown to increase the number of circulating CD34+ cells in healthy volunteers and cancer patients when administered alone or with G-CSF. Of note, the combination of plerixafor and G-CSF successfully rescued 66% of MM and lymphoma patients who had previously failed at least one round of mobilization with G-CSF with or without chemotherapy.[14] Two Phase III, multi-centre, randomized, double-blinded, placebo-controlled studies were recently conducted to evaluate the safety and efficacy of plerixafor plus G-CSF versus placebo plus G-CSF to mobilize, front line, $CD34^+$ stem cells in patients with NHL and MM, respectively.[15,16] In both studies, the combination of plerixafor plus G-CSF was safe and well tolerated and the efficacy results demonstrated that plerixafor and G-CSF mobilized significantly higher numbers of hematopoietic stem cells than G-CSF alone.

Very recently, we reported[17] 13 MM and lymphoma patients, candidates to autologous PBSC transplantation who were classified as "poor mobilizers" or "no mobilizers" at all based on daily monitoring of $CD34^+$ cell count during the recovery phase after chemotherapy and G-CSF. Poor mobilization was defined when the concentration of PB $CD34^+$ cells was always lower than 10 cells/μL during the recovery phase after chemotherapy and/or the collection of PBSCs was predicted to be inadequate to perform single or double ASCT. Following plerixafor treatment, 13/13 patients were able to collect the minimum required cell dose (2×10^6 $CD34^+$ cells/Kg for single ASCT) in 3 or less apheresis. We observed a remarkable fold-increase (median value= 4.7) in the number of circulating $CD34^+$ cells after plerixafor administration.[17]

Noteworthy, in this group of patients injection of plerixafor was shown to be safe. We did not observe any clinical adverse events or any variation in laboratory values even in 3 patients older than 60 years and in one patient undergoing dialysis during mobilization. We believe that the results presented here may provide a useful information for transplanters as many patients may benefit from the pre-emptive administration of plerixafor during their mobilization course, resulting in saving of unnecessary apheresis procedures, days of hospitalization and improving patients quality of life. Moreover, the identification of proven "poor mobilizers" during mobilization, based on daily assessment of $CD34^+$ cell count, may provide a potent tool for early intervention. To this end, future studies should prospectively test well defined algorithms, perhaps based on WBC and $CD34^+$ cell count and/or the results of first day collection, to optimize the use of plerixafor after chemotherapy.

Five/13 patients have already undergone ASCT. Plerixafor-mobilized PBSC engrafted rapidly and the hematological recovery remained stable after 3, 6, 6, 6 and 11 months of follow-up respectively. Accordingly, the infection rate, red blood cell and platelet transfusion requirement or supportive care was minimal.

In summary, our results suggest that the addition of plerixafor to G-CSF after chemotherapy was safe and provide some evidence of efficacy in patients planned to undergo ASCT who were failing PBSC mobilization.

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EXPERTS PANEL GITMO: ASCT PROCEDURES IN ACUTE MYELOID LEUKEMIA

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Background. The role of autologous stem cell transplantation (ASCT) in patients with acute myeloid leukemia (AML) remains unclear. Although allogeneic stem cell transplantation (allo-SCT) is considered the most effective anti-leukemic treatment for young patients with intermediate-high-risk AML, there is still controversy on the best therapeutic approach for other patient populations. *Methods.* Three Consensus Conferences were held in order to implement recommendations, according to the Nominal Group Technique. Recommendations elaborated by national and international scientific societies were integrated with the consensus statements elaborated by the experts. The Panel agreed on 9 major topics: 1) eligibility to ASCT; 2) stem cell (SC) source; 3) management of poor mobilizers; 4) management of super-mobilizers; 5) use of myeloid growth factors; 6) enumeration of $CD34^+$ cells and harvest quality control (including SC procurement and processing); 7) supportive therapy; 8) conditioning regimen; 9) evaluation of minimal residual disease (MRD). The Board reviewed literature in order to retrieve high-quality evidence, i.e. randomized clinical trials and meta-analyses. Retrival was extended to national and international guidelines dedicated to either disease-management or transplant procedures. Harmonization with evidence-based guidelines was attempted whenever possible, in order to provide GITMO Centers with coherent recommendations. *Results*. A number of trials have attempted to compare ASCT with allogeneic SCT. Using this so-called 'donor versus no donor comparison', ASCT was associated with a higher RI (relapse incidence) when compared with allogeneic SCT. However, because of a higher transplant related mortality (TRM) following allogeneic SCT, no difference in overall survival (OS) was demonstrated in any of these trials. To date, seven studies compared ASCT with non-myeloablative chemotherapy or no further therapy in first CR. One reported a significantly better DFS for the patient group treated with ASCT, while none showed a statistically significant difference in OS between the two treatment arms. Three meta-analyses, comparing ASCT with conventional consolidation showed similar OS , however in these studies only a minority (34%) of patients attaining CR were randomised between ASCT and chemotherapy. The most recent analysis to further assess the value of ASCT using bone marrow (BM) as SC source, did not show better OS for patients treated with ASCT. However, a significantly improvement of DFS for the ASCT group was found. Two reasons may explain discrepancies between improved DFS and no better OS. First, the meta-analysis revealed a significantly higher TRM in the ASCT group. Secondly, although the RI was greater in the non-ASCT group (58% versus 47% of ASCT group), a smaller proportion of relapsing ASCT patients could be salvaged. SC Source.

Table 1. Expert Panel GITMO Recommendations for ASCT in AML.

Code	Statements
1	In order to evaluate patient eligibility to consolidation autologous SCT, the following issues should be assessed after consolidation: performance status, comorbidities, minimal residual disease (MRD), active infectious diseases.
2	The following tests should be performed 30 days after consolidation chemotherapy:
·	an internally validated system for measurement of MRD
·	transthoracic echocardiography assessing percent ejection fraction
·	respiratory functional test assessing DLCO, and, possibly, FEV
·	estimated creatinine clearance
·	AST, ALT and bilirubin
·	serology for HBV-HCV-HIV and HBV-DNA or HCV-RNA titers in positive patients
·	liver biopsy, in patient showing high HBV-DNA or HCV-RNA titers
3	Patients should not proceed to autologous SCT if they show:
·	a poor performance status (ECOG≥2)
·	EF<45-50%
·	DLCO <60%
·	Creatinine >2.5 or creatinine clearance <30 mL/min
·	Bilirubin>2
·	AST>3 times the upper normal limit
·	Active uncontrolled infectious disease
4	A previous mycotic infections does not contraindicate autologous SCT if the patient has been treated for at least one month and a secondary prophylaxys is adopted
5	All the patients are candidate for peripheral stem cell mobilization.
6	There is no consensus on the number of consolidation cycles needed before mobilization, which should be done after the best *in vivo* purging has been achieved.
7	Patients who show minimal residual disease (after induction) or who mobilized too many C D34+ stem cells (i.e. CD34+ harvest<2.5x10^6/kg) should undergo a second consolidation cycle or undergo alternative strategies.
8	Chemo-free mobilization can be performed with G-CSF alone in patients who failed mobilization with chemotherapy plus growth factor.
9	Patients are defined "proven poor mobilizers" if the cumulative CD34+ harvested cells are less than 2.5x10^6/Kg or if if they show a CD34+ peak < 10/mcl during mobilization.
10	Poor mobilization in this setting can also be predicted according to negative factors such as baseline CD34 count, FUO during hematopoietic recovery, low platelet count before the start of mobilization, less than 3 overall chemotherapy cycles, prior treatments with fludarabine.
11	Proven poor mobilizers should undergo bone marrow biopsy one month after the last chemotherapy cycle: if bone marrow cellularity is higher than 30%, platelet count higher than 100 and neutrophil count higher than 1500, they can undergo harvesting of bone marrow stem cells (after or not G-CSF priming); alternatively patients can try remobilization with G-CSF alone 10 mcg/kg/day for 5-7 days.
12	Bone marrow harvest should be higher than 0.5x20^8/Kg mononuclear cells, as assessed after freezing. Patients should receive all harvested stem cells, both bone marrow and peripheral stem cells.
13	After mobilization, blood count should be performed daily; CD34+ cells should be assessed daily and at the same hour, starting from the day neutrophil count increases over 1000. Apheresis should start when peripheral CD34+ count increases over 10/mcl and allow a cumulative harvest higher than 2.0-2.5x10^6/Kg. Large volume ahereses (with adequate prophylaxis of citrate-related adverse events) should be preferred in this setting rather than multiple procedures
14	The count should be quantified by ISCT standard method: single-color staining analysis, in association with a 3-color method. Single-platform assay by the addition of fluorescent counting beads, and the addition of the viability dye 7-amino-actinomycin D to allow the exclusion of dead cells from the analysis CD34 count needs to be performed by an accreditated laboratory, which joined an external quality assessment program.

Autologous mobilized peripheral blood stem cell (PBSC) transplantation offers a much faster hematopoietic recovery. Both Italian Society of Hematology (SIE) and American Society of Bone Marrow Transplantation (ASBMT) guidelines recommend the collection of mobilized PBSCs after consolidation chemotherapy. Regardless the SC source, the best *in vivo* purging before mobilization has been recommended, since MRD

predicts the relapse rate after ASCT. However, this recommendation cannot be translated into a specific guideline since no specific chemotherapy regimen proved to be superior to the others. Moreover, whereas ≥2 consolidation courses may provide better control of MRD, prolonged chemotherapy prior PBSC collection may impair SC mobilization. AML patients are hard-to mobilize: approximately 35% of them do not achieve a $CD34^+$ cell count ≥20/mcL, after mobilization; adopting the threshold of $2.5\times10^6 CD34^+$ cells/Kg, 10-40% of AML patients in first CR fail to collect an adequate amount of PBSC. "Super-mobilizers" were defined as those AML patients showing ≥0.8% $CD34^+$ stem cells in the harvests or those achieving a cumulative $CD34^+$ harvest $>7\times10^6$/kg. Such patients represent about 25% of the AML mobilized patients and they have higher RI, irrespectively of their cytogenetic risk. Therefore the Panel agreed that both the presence of MRD and over-mobilization were valuable criteria for recommending better in vitro purging, rather than ex vivo purging or alternative strategies(Table I). ASCT represents a therapeutic option also for the fit elderly AML patients; in Italy one-third of patients transplanted after 1998 were older than 55 years, compared to 11% of those transplanted before 1998. In a recent GITMO survey AML patients autotransplanted in last decade showed significantly improved OS, despite the relevant increase of median age. At the multivariate analysis TRM was significantly reduced by: younger age, CR1 or CR2 status before ASCT and conditioning without TBI; RI was increased by advanced age/disease status, PBSC source and by conditioning regimens not including Busulfan. Conclusions. The replacement of the BM source with the PB source has been generally considered one of the main factors which reduced the costs and morbidity, but it has not clearly established if TRM has been lowered thanks to the PBSC coming. While this seems true for Lymphoma or Myeloma patients receiving ASCT, TRM is still a concern for older people receiving ASCT for AML. Extensive use of PBSC should not be encouraged if it is not associated with strong *in vivo* purging before PBSC collection; conditioning regimens should be Busulfan-based, taking care to avoid TBI and other regimens; ASCT in CR2 remains an option in patients <55 years old; finally the benefit of ASCT for patients over 55 years old should be carefully evaluated as these patients have still high TRM and very high RI.

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THE DISPOSAL OF CRYOPRESERVED HEMATOPOIETIC STEM CELLS

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In recent years, the use of hematopoietic stem cells has shown an exponential increase, which can explained by the affinity of transplantation techniques and by a broadening of clinical indications. Currently most transplant protocols require the infusion of an optimal dose of cells, which can vary depending on the type of transplant (autologous or allogeneic) or on the underlying disease.

In both cases however, we can verify that the cells are harvested and stored in liquid nitrogen prior to infusion, and if the cells are not used (for example due to loss of clinical indication, failure to reach the minimum target or the patient has deceased) it can be possible, in the absence of specific strategies, to check the critical state related to their accumulation with the risk of a complete depletion of the available cold areas. This situation, most common in cases of autologous transplantation, could also occur even in case of an allogeneic transplantation, if it were necessary to freeze the cells because the fresh cells could not be infused or in the case where there were excess collected cells.

For this reason, it is necessary for each Transplantation Program to establish appropriate strategies that can protect the patient but also to ensure the possibility of having sufficient cold areas with an efficacious system. The Italian law on cells and tissues (Legislative Decree No 16/2010[1] - Annex IV, 2.5a), European legislation (DE 17/2006[2]) and the international accreditation standards (Standard Jacie[3], D11) require defined procedures with established criteria for which the Transplant Centre can authorize the disposal of unused or surplus cells. These procedures must provide adequate information concerning pre-collection, the patient and / or donor (in the informed consent), it must define the storage duration of cells, the circumstances for their disposal and the possibility of their transfer to other structures. In the absence of an agreement pre-collection or where the patient and / or donor is not traceable a procedure must be available.

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3. FACT-JACIE International Standards - Fourth Edition

ROLE OF GLYCOPROTEIN IB-VON WILLEBRAND FACTOR COMPLEX AND OF OTHER MODULATORS IN PLATELET FORMATION

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Von Willebrand Factor (vWF) is a well-known mediator of platelet adhesion to the vessel wall and of platelet-platelet interactions under high shear-stress conditions.[1] It is synthesized both in endothelial cells and megakaryocytes (MK) where it is stored in secretory organelles as large multimers.[2] vWF has also been identified as a sensitive and distinct marker for early MKpoiesis[3] and, more recently, exposure of human MK to vWF at high shear rates was reported to accelerate platelet production.[4] Type 2B von Willebrand disease (vWD2B) results from a gain-of-function of vWF that has an increased affinity for platelet glycoprotein (GP) Ib alpha.[5,6] Thrombocytopenia is often present in vWD2B patients and the presence of giant platelets has been reported.[7-10]

We recently demonstrated that primary CD34$^+$ cells from healthy donors (HD) differentiated for 7 days in the presence of TPO and vWF released a higher number of platelets in a dose dependent manner, when compared to TPO alone.[11] The promoting effects of vWF on platelet production are selective and related to GPIb α, as demonstrated by experiments performed with specific inhibitors. Figure 1A shows that the use of the antibody against GPIbalpha (LJIb1) can block vWF-dependent accelerated platelet production while LJIb10 MoAb against the thrombin-binding site of GPIb alpha was not effective. The lack of inhibition with tirofiban further shows that vWF is not mediating its promoting effect through GPIIbIIIa; in fact, fibronectin and fibrinogen, other ligands of GPIIbIIIa are not able to increase platelet production. vWF boosts CD34-derived platelet production from vWD2B patients as well, even if the total amount of platelet production remains low if compared to HD (Figure 1B). This observation suggests that interaction of exogenous vWF to membrane GPIb alpha is able to promote MK differentiation and that an increased vWF/GPIb interaction can accelerate the final step of platelet release. Such a difference might be due to the activation of specific signalling pathways downstream GPIb alpha. To this regard, it has been demonstrated that GPIb alpha, during MK differentiation, binds the signal transduction protein 14-3-3xi, down-regulating the Akt phosphorylation levels downstream TPO/c-mpl signalling.[12]

It has been established that PKCepsilon interacts with 14-3-3xi[13] and we have demonstrated that it is a key molecule in the signalling pathways downstream TPO, being finely regulated during MK differentiation.[14] Figure 2A shows that PKCepsilon is induced in the early phases of TPO-mediated MK differentiation, while it is down-regulated from day 6 onward. As reported in Figure 2B-C, the over-expression of PKCepsilon in TPO-induced primary CD34 cells from day 8 onward – using as negative control an inactive mutated PKCepsilon (PKCepsilon-m)[15] – impairs: i) the platelet production (Figura 2B); ii) the expression of CD61, CD41 and CD42b surface markers (Figure 2C); iii) the expression of CD62p cytoplasmic maturation marker (Figure 2C).

This is in agreement with the notion that PKCepsilon is not expressed in human platelets,[16,17] although Crosby *et al.*[18] once reported its presence. On the contrary, it is now well established that mouse platelets express PKCepsilon, where it plays a role in GPVI-mediated aggregation and dense granule release.[16] To this respect it is worth noting that in human

monocytes the adhesion to endothelial cells involves PKCepsilon signaling.[19] Figure 3 shows that human platelet adhesion to collagen under controlled physiological shear rates is selectively increased by the transfection of the human recombinant PKCepsilon (rhPKCepsilon) protein.

It would be important to unravel the crosstalk between GPIb alpha/vWF complex and PKCepsilon. To this respect, one could speculate that a slightly anticipated down-regulation of PKCepsilon might promote MK differentiation and platelet release while, on the contrary, an ectopic expression of PKCε in mature platelets might promote their activatory status.

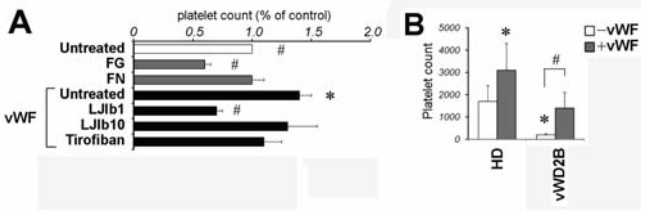

Figure 1. vWF increases platelet production. Panel A. Effects of fibrinogen (FG), fibronectin (FN) and vWF on platelet production of CD34+ cultures differentiated for 7 days with TPO. LJIb1, LJIb10 and Tirofiban are used as antagonist of GPIb alpha vWF binding domain, GPIb alpha thrombin binding domain and GPIIbIIIa, respectively. Means ± SD are reported. (*P<0.05 vs TPO treatment; #P<0.05 versus TPO and vWF treatment). Panel B. Platelet production from cultured CD34+ MK obtained from 3 different healthy donors (HD) used as controls and from 3 patients with vWD2B. Means ± SD are reported. (*P<0.05 vs TPO treatment of HD; # P<0.05).

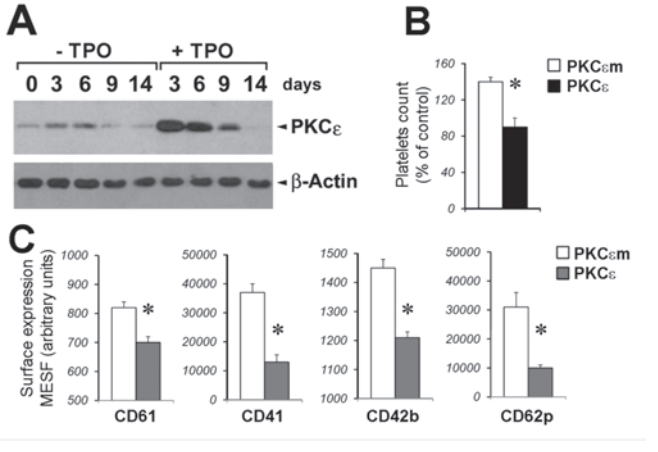

Figure 2. Expression and effects of PKCepsilon in MK differentiation. Panel A: Western blot detection of total PKCepsilon protein expression in CD34 cells cultured in serum free medium with IL3 + SCF in the presence (+TPO) or absence (-TPO) of thrombopoietin. Beta-Actin was monitored for protein loading. Panel B: PKCepsilon over-expression impairs the release of platelets in the culture. CD34 cell cultures from 3 independent healthy donors at day 8 of culture with TPO were transfected with PKCepsilon (black histogram) or mutated PKCepsilon (empty histogram) plasmids. Transfected cells were then cultured for a further 5 days in the presence of TPO. Data are expressed as means ± S.D. as percentage of control (untransfected cell culture). (*P<0.05). Panel C: PKCepsilon over-expression impairs MK differentiation. CD34 cell cultures from 3 independent healthy donors at day 8 of culture with TPO were transfected with PKCepsilon (grey histogram) or mutated PKCepsilon (empty histogram) plasmids. Transfected cells were then cultured for a further 5 days in the presence of TPO. Data are expressed as means ± S.D. of absolute numbers of surface antigens expressed/cell. (*P<0.05).

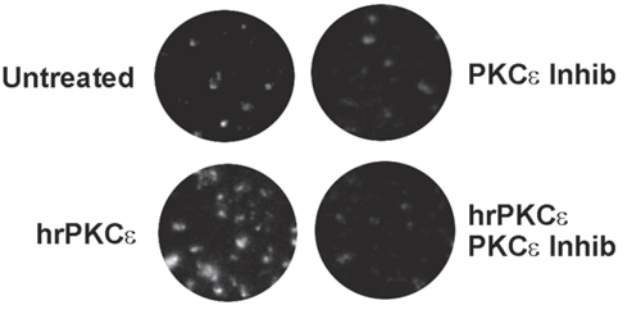

Figure 3. PKCepsilon selectively enhances platelet adhesion under flow condition. Platelets, transfected with human recombinant PKCepsilon (hrPKCe and/or PKCepsilon inhibitor (PKCe inhib), were reconstituted in whole blood, previously deprived from PRP, and tested for their adhesion capacity under flow. Mepacrine-loaded platelets (5-7x10^8/mL) and washed erythrocytes (hematocrit 42-45%) suspended in plasma, were perfused (600 s^{-1}) for 3 minutes over immobilized fibrillar type I collagen. Representative single-frame images of each surface are also shown.

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F8 GENE MUTATIONS AS AN INDEPENDENT RISK FACTOR FOR INHIBITOR DEVELOPMENT AND TREATMENT IN HEMOPHILIA A

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The pathogenesis of inhibitor formation is only partly understood. Several factors have been suggested to modulate inhibitor formation. The incidence of inhibitors depends on both genetic (severity of hemophilia, type of mutation, ethnicity, family history of inhibitors, the HLA genotype) and non-genetic factors (age at first treatment, intensity of treatment, continuous infusion, and multiple product switches).[1] The development of inhibitory antibodies following replacement treatment in hemophilic patients has been clearly shown to correlate with the type and location of F8 gene mutations[2-9] and algorithms or scores for predicting inhibitor development in newly diagnosed patients have been also proposed.[3,10] In the AICE Hemophilia A Registry, the type of mutation is one of the most important risk factor predisposing to inhibitor development. In keeping with previous results,[4,11] a high proportion of patients presenting with mutations leading to a null allele developed inhibitors (24-71%) (Table 1). Replacement therapy in patients completely lacking FVIII protein may lead to immunization with a foreign protein and cause the development of antibodies. On the other hand, the incidence of inhibitor development in patients with missense mutations was smaller (5%). These findings support the hypothesis that some traces of protein, although non-functional, that are synthesized are sufficient to induce immune tolerance in most of these patients.

Patients with small insertions and deletions in a hot spot consensus sequence carried a similar risk (6% and 12%, respectively), lower than that recorded in patients with a mutation occurring outside these consensus sequences (23% and 26%, respectively). An endogenous restoration of the reading frame for mutations occurring in short repeats or homonucleotide traits, such as poly-A runs, may take place allowing for the formation of few protein molecules that protect against inhibitor development.

A higher likelihood of inhibitor formation was found in patients with missense mutations occurring in the light chain, especially in the C1 domain. These findings support the conclusion that some regions within the FVIII protein play a major role for its antigenic integrity.[12] Actually, data from the HAMSTeRS database suggest that the inhibitor prevalence in missense mutations depends on the location of the mutation (Table 1). The C1 domain significantly contributes to the three-dimensional structure of the light chain and is likely that amino acid replacements in this region would perturb more markedly the folding of the molecule and, in turn, affect protein stability and functionality, with the ultimate result of an absolute lacking of the mature protein in the bloodstream. Likewise, patients with a nonsense mutation occurring in the light chain appear to carry a higher likelihood to develop inhibitors than patients presenting with mutations in the heavy chain or in the B-domain. These findings are in agreement with previous results[12] and with data available from the HAMSTeRS database.

The Italian Immune Tolerance Induction (ITI) Registry for the first time provides data suggesting a relationship between F8 gene mutations and ITI outcome.[13] Actually the mutations associated with high inhibitor risk were related to a 8-fold higher likelihood of lack of ITI success (Table 2). On the other hand, small insertions and deletions and missense mutations predicted a better outcome both in inhibitor development and in ITI outcome. Presently, few studies specifically analyzed the relationship between F8 genotype and ITI outcome.[8,14-16] The Italian ITI Registry showed a strong association between F8 gene classes of mutations and ITI outcome, irrespective of patients' clinical features and FVIII dose administered.

Genetic counselling is an important part of the care of hemophiliacs and their families. In each family with HA, identification of the F8 gene mutation allows for the knowledge of the disease-causing defect, would provide an effective genetic counselling and a rapid carrier and antenatal diagnosis within the family, and may serve to have a measure of the risk for inhibitor development.

Table 1. Proportion of severe HA patients with different F8 gene mutations developing inhibitors.

	Italy (n=940)		*Germany[4] (n=753)*	*HAMSTeRS (n=845)*
Large deletions	10/15	(67 %)	41 %	46%
Nonsense mutations	32/80	(40 %)	31 %	35%
IVS-1 inversions	6/21	(29 %)	17 % (26%)°[12]	n.a.
IVS-22 inversions	123/484	(25 %)	21 %	n.a.
Splicing-site mutations	8/36	(22 %)	17 %	8%
Small deletions	15/90	(17 %)	16 %[§]	17%
Small insertions	9/60	(15 %)	19%	
Missense mutations	8/147	(5 %)	5 %	10%
Other	3/7	(43 %)	n.a.	n.a.

n.a.: not available. [§]small deletion plus small insertion. °1127 patients investigated.

Table 2. Identified FVIII gene mutations* and ITI outcome in patients registered into the PROFIT Study.

ITI Outcome	*F8 genotype*						
	Large del (n=6)	*Translocations (n=50)*	*Nonsense (n=11)*	*Splice site (n=3)*	*Small ins (n=8)*	*Small del (n=7)*	*Missense (n=1)*
Success, n	1	24	7	1	7	5	1
Partial success, n	1	8	1	1			
Failure, n	4	18	3	1	1	2	
Success rate, %	17	48	64	33	87	71	100
	High risk mutations: 33/70 (47%)				Low risk mutations: 13/16 (81%)		
Lack of success°, %		37/70 (53.%)^				3/16 (19%)^	

del: deletion; inv: inversion; ins: insertion. *Among patients with F8 translocations, 49 subjects had the intron 22 inversion and the remaining patient the intron 1 inversion. The latter achieved successful ITI. ^P = 0.01, high-risk vs. low-risk mutations, risk ratio 1.7 [95% confidence interval (CI 1.1–2.1]; on multivariate analysis, adjusted odds ratio was 6.2 (95% CI 1.1–36.0), P=0.04.

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THROMBIN GENERATION BY TUMOR CELLS

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Patients with cancer are characterized by a hypercoagulable state and a high risk of venous thromboembolism. Abnormalities of the hemostatic system described in cancer patients include elevated levels of coagulation factors, such as fibrinogen, FV, FVIII, FIX, and FXI, and elevated levels of markers of prothrombin activation (i.e. F1+2 fragments) and thrombin formation (Thrombin-antithrombin complex, TAT), as well as fibrinogen degradation products (i.e. D-Dimer).

The pathogenesis of the thrombophilic state in these patients is multifactorial. An important role is attributed to the capacity of tumor cells to activate the hemostatic system and generate thrombin by means of the expression of procoagulants molecules (i.e. Tissue Factor, TF, and Cancer Procoagulant, CP). It is now well accepted that the tumor cell exploit the blood coagulation system to support its own growth and spread. Among blood coagulation proteases, thrombin has shown a crucial role in tumor progression by both in vitro and *in vivo* studies. Many of the thrombin effect are mediated by the activation of intracellular signalling pathways through the interaction of the protease with transmembrane domain G protein- coupled receptors, known as protease-activated receptors (i.e. PAR-1, PAR-3, and PAR-4).

On the basis of the pleiotropic activity of thrombin, we are characterizing the thrombin generation capacity of different tumor cell lines by using the Calibrated Automated Thrombogram (CAT) assay. In this system the formation of thrombin in plasma is induced by calcium in the presence of tumor cell lysates, and is monitored by measuring the fluorescence intensity generated by the cleavage of a thrombin substrate in real time. Specific software converts the fluorescent signal to a thrombin concentration by continuous comparison with the signal generated by a thrombin calibrator. The resulting thrombin generation curve (Figure 1) is described by 4 parameters: 1) lagtime (time needed to detect the beginning of the curve); 2) ETP (endogenous thrombin potential, calculated as the area under curve), 3) Peak (the maximum height reached by the curve), and 4) ttPeak (the time to reach the peak of the curve).

We have currently evaluated the following tumor cell lines: i.e. MDA. MB231 and MCF7 (breast), H69 (small cell lung), and NB4, HEL, and K562 (all myelogenous leukemia). The endothelial HMEC-1 and embryonic kidney HEK-293 cell lines are used as non-tumor cells. To better characterize the contribution of the different cell procoagulants, the CAT assay is performed in normal pool plasma (NPP), FVII-, FXII- and FIX-deficient plasma, and in the presence of anti-TF antibody.

The results of these experiments show that different tumor cell lines possess the capacity of inducing thrombin generation in plasma, however this capacity may differ in the mechanisms involved (i.e.TF, CP, or anionic phospholipids expression). The specific characteristics of each cell type to inducing thrombin generation may be useful to select specific agents to block the cellular procoagulant activity.

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BEST ABSTRACTS

BEST-01

CLINICAL OUTCOMES PREDICTION FOR NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS TREATED WITH THALIDOMIDE-DEXAMETHASONE AND AUTOLOGOUS STEM CELL TRANSPLANTATION BY 8-GENE SIGNATURE OF CD138+ PLASMA CELLS

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Background. Efficacy of Thalidomide-dexamethasone (TD) as induction therapy in preparation for autologous stem cell transplantation (ASCT) in multiple myeloma provided the basis for the design of the phase II "Bologna 2002" study incorporating TD into double autotransplantation as up-front therapy for younger patients (pts) with newly diagnosed disease. *Aim.* We performed a molecular study aimed at identifying a gene expression profile (GEP) signature predictive of attainment of at least near complete response (nCR) to TD and subsequent autotransplantation. *Methods.* For this purpose, we analyzed bone marrow samples obtained at diagnosis from 112 pts who received TD before double ASCT. The differential gene expression of CD138+ enriched plasma cells was evaluated by means of expression microarray using the Affymetrix platform. Significant expression results were validated by Real-time PCR. *Results.* Two subsequent study phases were planned. Firstly, a GEP supervised analysis was performed on a training set of 32 pts, allowing to identify 157 probe sets differentially expressed ($P<0.05$) in pts with at least nCR (e.g. responders) versus those who failed at least nCR (e.g. non responders) to TD induction therapy. Most of the 157 genes resulted down expressed in responder pts and were mainly involved in cell cycle and apoptosis regulation. In particular, signaling pathways which might be affected by the de-regulated expression of genes in responding pts, are the MAPK signaling pathway (Ppp3r1, PRKY, PRKX, FAS, ATF2, MAP4K3 and DUSP4), the Wnt signaling pathway (Ppp3r1, PRKY, PRKX and CCND2) and the p53 pathway (CCND2, FAS, CCNDE and MDM2). In the second phase of the study, we generated an 8-gene GEP signature which predicted at diagnosis the probability to achieve at least a nCR after TD induction therapy. The performance of this assay was subsequently validated by Real-time PCR in a training set of 80 pts: 36 pts were predicted as responders to TD , whereas 44 as non responders. The post autotransplantation outcome was analyzed according to Real-time expression results. On an intention-to-treat basis, the rate of CR, either immunofixation negative or positive, was 51.4% among CR-predicted ps and 23% (P=0-001) in the subgroup of 44 NR-predicted pts. The 65-months probability of OS for CR-predicted pts was 72% as compared to 41% for those who were predicted to be NR (P=0.03). The projected rates of TTP and PFS at 55 months for CR-predicted and NR-predicted pts were 69% *vs.* 34% (P=0.003) and 55% *vs.* 19% (P=0.01), respectively. *Conclusions.* These results can be an important first step to identify at diagnosis those pts who are more likely to respond favorably to a particular treatment strategy.

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BEST-02

TIMP-1 DEFICIENCY SUBVERTS CELL CYCLE DYNAMICS IN LONG-TERM HSCS

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Introduction. Although TIMP-1 was initially described as a mere inhibitor of Metalloproteinases (MMPs), recent findings have offered a different perspective on its biological role, contributing to unveil its multifaceted nature. In addition to inhibiting MMP activity, TIMP-1 has been proven to play MMP-independent, cytokine-like roles and to be involved in the regulation of numerous biological functions, including cell proliferation and survival. We therefore hypothesized that TIMP-1 might be involved in the homeostatic regulation of hematopoietic stem cells (HSCs), whose biological behavior is the synthesis of both microenvironmental and intrinsic cues. *Methods.* Bone marrow hematopoietic stem cells (HSCs) were isolated from TIMP-1-/- mice based on the phenotype Side Population c-Kit+Lin-Sca-1+ (SPKLS). *In vitro* cultural assays as well as *in vivo* transplantation assays were employed to investigate how TIMP-1 obliteration affects murine hematopoiesis. Cell-cycle dynamics in KO SPKLS HSCs were characterized by Pyronin Y/Hoechst staining, Ki-67 staining, as well as evaluation of RNA expression of cell cycle inhibitors, such as p53, p57, and p21. *Results.* We found that TIMP-1-/- mice have decreased HSC numbers and, consistent with this finding, TIMP-1-/- HSCs display reduced capability of long-term repopulation. Interestingly, the cell cycle distribution of TIMP-1-/- LT-HSCs is profoundly distorted, with a consistent proportion of the stem cell pool arrested in the G1 phase, suggesting that TIMP-1 is intrinsically involved in the regulation of the HSC proliferation dynamics. Of note, TIMP-1-/- HSCs present decreased levels of CD44 glycoprotein, whose expression has been proven to be controlled by p53, the master regulator of the G1/S transition. Interestingly, p53 RNA levels are indeed increased in TIMP-1-/- SPKLS HSCs compared to controls. Likewise, the expression level of other cell-cycle inhibitors, such as p57 and p21, were found to be higher in KO SPKLS HSCs, indicating a disregulation of cell-cycle dynamics. *Conclusions.* Our study highlights a novel biological role of TIMP-1 in the regulation of the HSC compartment and suggests a novel mechanism presiding over stem cell quiescence in the framework of the BM milieu.

BEST-03

EPHA3 IS ABNORMALLY EXPRESSED IN CHRONIC MYELOPROLIPHERATIVE DISORDERS AND COULD REPRESENT A NEW MOLECULAR THERAPEUTICAL TARGET

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Introduction. Eph receptors tyrosine kinases (RTKs) are involved in many key developmental processes. Although Eph RTKs are undetectable in adult tissues, they are overexpressed in many tumors, suggesting their possible role in oncogenesis. Activation of TKs is a common finding in the pathogenesis of chronic myeloproliferative disorders (MPN The aim of the study was to investigate to role of EphA3 in MPN and to explore the possibility of targeting EphA3 with a monoclonal antibody (mAb). *Methods.* EphA3 mRNA was analyzed using Real Time PCR in 298 samples obtained from 244 MPN patients (244 BM and 54 PB) and in 38 healthy donors (18 PB and 20 BM). 73 were PV, 65 ET, 24 IM, 24 CMML, 8 HES, 50 CML. In 10 patients and 5 healthy subjects $CD34^+$ cells were selected and analyzed by FACS for the presence of EphA3. In addition the expression of EphA3 ligands were analyzed (Ephrine A3, A4, A5, B2) in both BM cells and stromal cells from MPN and healthy donors. Protein expression and localization were analysed by Western Blot, immunofluorescence and by FACS. The biological effects of EphA3 overexpression were studied by transfecting EphA3 plasmid in COS cell line negative for EphA3 expression. Finally samples were incubated with a specific mAb able to block the EphA3 receptor. Proliferation, apoptosis and colony growth were then evaluated. *Results.* We found that normal BM, PB and $CD34^+$ cells are negative for EphA3 expression. By contrast, EphA3 is significantly increased in 45% of PV, in 55% of ET in 90% of CMML, 15% of HES and in all PMF. Finally 30% of CML overexpressed EphA3 mainly during accelerated phase and blast crisis. In all the $CD34^+$ samples from MPN we found high levels of expression compared to the corresponding unfractioned samples (P=0.001). We never observed differences in the ligands expression levels when compared to healthy subjects. Western Blot and immunofluorescence demonstrated an increased phosphorylation of the EphA3 receptor in MPN. EphA3 transfection into normal cells results in loss of adhesion, cell rounding and increased proliferation. The incubation of $EphA3^+$ cells or cells transfected with EphA3 with a specific mAb against EphA3 resulted in a significant reduction of proliferation (31±4% and 45±9% respectively), increased apoptosis and colony growth inhibition (median value of 34.2 *vs.* 76.5) and finally, it changed the adhesion properties. All these effects were not observed in EphA3- cells and normal controls. *Conclusion.* EphA3 is abnormally expressed and activated in different MPN patients. The inhibition of EphA3 phosphorylation results in growth arrest and apoptosis of EphA3 overexpressing cells. Therefore, basing on these data, EphA3 is now exploited as a new molecular target in chronic myeloproliferative disorders and clinical trials using a monoclonal antibody against EphA3 are ongoing.

BEST-04

A GENETIC VARIANT OF MLH1, A GENE INVOLVED IN DNA MISMATCH REPAIR, IS AN INDEPENDENT PREDICTOR OF OVERALL SURVIVAL IN DIFFUSE LARGE B-CELL LYMPHOMA TREATED WITH R-CHOP

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Introduction. Several drugs utilized in diffuse large B cell lymphoma (DLBCL) treatment rely on DNA damage for tumor killing. Host genetic variability in genes repairing DNA damage may affect response to drugs and prognosis. Aims. We verified the impact of SNPs of DNA repair genes on prognosis of R-CHOP treated DLBCL. *Methods.* The study utilized a training-validation design and was based on a training (n=163) and a validation series (n=156) of R-CHOP treated DLBCL. Clinical characteristics at diagnosis, follow up length and outcome were comparable for the training and the validation series. Thirty-five SNPs from 18 genes were analyzed on patients germline DNA. These included SNPs affecting: i) mismatch repair genes (MLH1); ii) base excision repair genes (XRCC1, OGG1); iii) nucleotide excision repair genes (ERCC1, ERCC2, ERCC4, ERCC5, ERCC6, XPA, XPC); iv) double strand break repair genes (BRCA1, BRCA2, LIG4, XRCC2, XRCC3, XRCC4, XRCC6); and v) direct reversal genes (MGMT). Clinical endpoints were overall survival (OS) and progression free survival (PFS). *Results.* In the validation series, univariate analysis controlled for multiple comparisons identified MLH1rs1799977 as the sole SNP predicting OS in R-CHOP treated DLBCL. Patients carrying MLH1rs1799977 AG/GG genotype displayed an increased risk of death (HR:3.23; 4-years OS:55.5%) compared to AA carriers (4-years OS:80.9%) (P=.0002; q=.009; Figure1A). Multivariate analysis selected MLH1rs1799977 (HR:3.14; P=.0004) as an independent predictor of OS, along with IPI (P=.0377) and bulky (P=.0044). The poor prognosis heralded by MLH1rs1799977 AG/GG genotype in DLBCL is due to both first and second line treatment failure. In fact, patients carrying the MLH1rs1799977 AG/GG genotype displayed an increased risk of progression after R-CHOP (HR:2.02; 4-years PFS:47.5%) compared to AA carriers (4-years PFS:65.6%) (P=.0073), and multivariate analysis identified MLH1rs1799977 (HR:1.96; P=.0104) as an independent predictor of PFS, along with IPI (P=.0026) and bulky (P=.0125). Also, patients carrying the MLH1rs1799977 AG/GG genotype displayed an increased risk of failing second line platinum-based regimens (HR:3.04; 2-year OS from salvage:16.0%) compared to AA carriers (2-year OS from salvage:57.3%) (P=.0074). By bivariate analysis, MLH1rs1799977 predicted OS from salvage (HR:2.96; P=.0156) independent of having or having not consolidated with SCT. The prognostic value of MLH1rs1799977 as predictor of OS was validated in the independent series of 156 DLBCL. Survival analysis in the validation series confirmed that MLH1rs1799977 is a prognostic factor in DLBCL treated with R-CHOP. First, by univariate analysis, MLH1rs1799977 AG/GG genotype was associated with poor OS (HR:3.22; 4-years OS:67.4%) compared to AA genotype (4-years OS:84.8%) (P=.0013; Figure 1B), and multivariate analysis selected MLH1rs1799977 as an independent predictor of OS (HR:3.15; P=.0016), after adjusting for IPI (P=.0001) and bulky (P=.1302). Second, in the validation series MLH1rs1799977 AG/GG genotype was associated with poor PFS (HR:1.98; 4-years PFS:55.8%) compared to AA genotype (4-years PFS:69.8%) (P=.0170), and multivariate analysis selected MLH1rs1799977 as an independent predictor of PFS (HR:1.86; P=.0188), along with IPI (P=.0001) and bulky (P=.697). Third, MLH1rs1799977 AG/GG genotype predicted poor OS from salvage with second line platinum-based regimens in the validation series (HR:2.95; 2-years OS from salvage:25.3%) compared to AA genotype (2-years OS from salvage:66.6%; P=.0270). *Conclusions.* MLH1rs1799977 is an independent predictor of survival in DLBCL treated with R-CHOP. The biologic plau-

sibility of this association is supported by four lines evidence: i) MLH1rs1799977 is a nonsynonymous SNP causing the I219V amino acidic substitution on MLH1, a gene of the mismatch repair pathway; ii) in silico, MLH1rs1799977 is predicted to have deleterious consequences; iii) *in vitro*, the G variant allele of MLH1rs1799977 associates with reduced MLH1 protein expression; iv) loss of MLH1 in tumor cells is known to induce refractoriness to doxorubicin and platinum compounds. Consistently, DLBCL carriers of the MLH1rs1799977 AG/GG genotypes displayed poor OS possibly due to altered MLH1 expression.

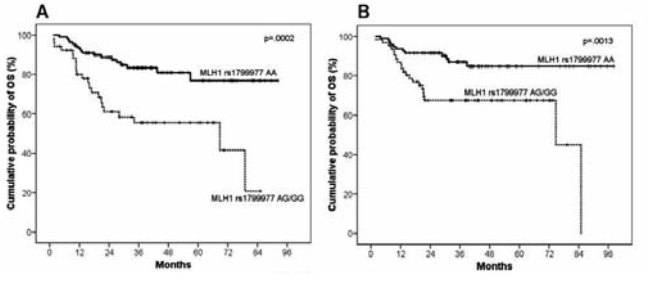

Figure 1.

BEST-05

CD38 IS PART OF A NETWORK OF MOLECULES REGULATING THE HOMING OF CLL CELLS

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Introduction. Recirculation of leukemic cells from blood to favorable growth niches in the lymphoid organs is an essential step in the maintenance and progression of chronic lymphocytic leukemia (CLL). It is regulated by an intricate network of signals, including chemokines and chemokine receptors, adhesion molecules and proteases to digest the extracellular matrix. Among these, the CXCL12/CXCR4 axis is believed to play an essential role in re-directing CLL cells to bone marrow and the lymph nodes. The CD49d integrin mediates adhesion to other cells via the VCAM-1 ligand or to the extracellular matrix via the CS-1 fibronectin fragment. Lastly, extra-vasation is mainly controlled by MMP-9, the only gelatinases expressed by CLL cells. The working hypothesis is that CD38 bridges these three aspects of leukocyte homing, through a physical association and a functional synergy with CXCR4, CD49d and MMP-9. *Methods.* CLL cells from a molecularly and clinically characterized cohort of patients were used in the study. Functional responses to CXCL12 were studied by immunoblot and chemotaxis. Genetic manipulation of CLL cells or MEC-1 cell line was carried out using a lentiviral techinique. The activity of MMP-9 in CLL cells was analyzed by gelatin zymography in basal condition and after co-culture with different stimuli. *Results.* Results indicate that CD38 expression marks i) CLL cells that are highly sensitive to the actions of the CXCL12 chemokine, including ERK1/2 activation and chemotaxis. Furthermore, ii) CD38 expression is associated with CD49d, confirming previous results and with iii) the presence of the active form of MMP-9. These data suggest that the CD38+ component of a CLL clone is enriched in cells that have the molecular machinery to migrate from the blood to the lymphoid organs. A direct proof of the involvement of CD38 in the homing process was obtained using a lentiviral technique that allows genetic modification of freshly purified CLL cells or derived cell lines. De novo expression of CD38 was followed by an increased sensitivity to the CXCL12 chemokine as well as by an increased adhesion to the VCAM-1 ligand as compared to the same cells infected with an empty virus. Activation of CD38 using agonistic antibodies was followed by increased chemotaxis and increased MMP-9 activation, while blocking anti-CD38 mAbs were highly effective in preventing CLL homing in an *in vivo* mouse model. *Conclusions.* These results indicate that CD38 is part of a complex network of molecules/signals that fine tune homing of leukemic cells to growth-permissive microenvironment.

ORAL COMMUNICATIONS

Acute Leukemias I

C001

WHOLE TRANSCRIPTOME DEEP-SEQUENCING IDENTIFIES NOVEL POINT MUTATIONS, GENE EXPRESSION AND ALTERNATIVE SPLICING PROFILES IN BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. The BCR-ABL1$^+$ ALL is the most frequent and prognostically unfavorable subtype of ALL in adults. In order to define the full repertoire of leukemia-related mutations, changes in expression profiles and alternative splicing (AS) events, the leukemia transcriptome of a BCR-ABL1$^+$ ALL patient at diagnosis and relapse was sequenced using a Whole Transcriptome Sequencing (RNA-Seq) approach. The selected cases had previously been profiled by high-resolution SNP and gene expression arrays and candidate gene re-sequencing. *Methods.* Poly(A) RNA from blast cells was used to prepare cDNA libraries for Illumina/Solexa Genome Analyzer. Obtained sequence reads were mapped to the human genome reference sequence (UCSC hg18) to identify single nucleotide variants (SNVs). Reads that showed no match were mapped to a dataset of all possible splice junctions created in silico to identify AS events. The number of reads corresponding to RNA from known exons was also estimated and a normalized measure of gene expression level (RPKM) was computed. *Results.* RNA-seq generated 13.9 and 15.8 million reads from de novo and relapsed ALL samples, most of which successfully mapped to the reference sequence of the human genome. With the exclusion of the T315I BCR-ABL mutation, 7 novel missense mutations were detected after applying stringent criteria to reduce the SNV discovery false positive rate: 4 were exclusively found in the primary ALL sample and affected genes involved in metabolic processes (DPEP1, ZC3H12D, TMEM46) or transport (MVP); 3 relapse-related mutations affected genes involved in cell cycle regulation (CDC2L1) and catalytic activity (CTSZ, CXorf21). Differences in mutational patterns suggest that the leukemia clone from which relapsed cells have been developed was not the predominant one at diagnosis and that relapse specific variants were mutations probably acquired during progression. Moreover, 4,334 and 3,651 primary and relapse isoforms with at least one AS event were identified. An average of 1.5 and 1.3 AS per isoform was estimated. Finally, a detailed gene expression profile was obtained indicating that more than 60% of annotated human genes were transcribed in leukemia cells in both diagnosis and relapse phases. Approximately 23% of genes were up-regulated at relapse compared to diagnosis, and most of them affected cell cycle progression (AURORA A, SURVIVIN, PLK1, CDK1, Cyclin A, Cyclin B), suggesting that the loss of cell cycle control may play a role in disease progression. Conversely, only 9% of active genes in both samples were down-regulated at relapse compared to diagnosis. *Conclusions.* Discovery of novel missense mutations, as well as exhaustive alternative splicing and gene expression profiles were achieved for the first time for a BCR-ABL1+ positive ALL demonstrating that RNA-Seq is a suitable approach for identifying a wide spectrum of genetic alterations.

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C002

BONE MARROW EMERGENCE OF CYTOTOXIC AUTOLOGOUS BCR-ABL-SPECIFIC T CELLS IN PH$^+$ ALL PATIENTS DURING THERAPY WITH TYROSINE-KINASE INHIBITORS

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Introduction. Recently, Imatinib mesylate (IM) was demonstrated to be permissive for the emergence of anti-CML T cells, thus supporting the notion that anti-tumor immunity may effectively participate in the control of Philadelphia-positive (Ph$^+$) leukemia. However, immunological surveys are still lacking in the setting of Ph$^+$ acute lymphoblastic leukemia (Ph+ALL). *Methods.* We performed a long-term immunological monitoring (range 2-65 months) to assess the occurrence of BCR-ABL-specific T cells in the bone marrow (BM) and peripheral blood (PB) from 11 Ph+ALL patients (pts) during IM maintenance therapy and from 5 Ph+ALL pts on 2nd generation tyrosine-kinase inhibitors (TKIs), using, as antigenic stimulation, different pools of short and long peptides (9-20mers), deriving from the complete spanning of p190BCR-ABL fusion region, as described in Riva G, *et al*. Blood 2010;115:1512-8. *Results.* p190BCR-ABL-specific T cells were detected by IFNg-ELISPOT in all 11 pts under IM. Robust anti-leukemic immune responses (20-350 SFCs/10^6 cells) were more frequently detectable in BM than in PB samples (67% *vs.* 25%, P 0.01), and resulted significantly associated with lower MRD values (P 0.001), while absent at leukemia relapse. Cytokine and memory profiling by antigen-specific Cytokine Secretion Assay (CSA) mainly identified Effector Memory (EM) CD8$^+$ and CD4$^+$ T cells, producing IFNg, TNFa and IL-2 (median % positive cells: 3.3, 3.1, 3.6, respectively). Moreover, p190BCR-ABL-specific cytotoxic CD8$^+$ and CD4$^+$ T cell subsets were readily expandable *ex-vivo* from pts' BM samples in short-term coltures, and were directly demonstrated to be able to lyse p190BCR-ABL-positive leukemic blasts (or target cells pulsed with p190BCR-ABL-derived peptides), using standard 51chromium-release cytotoxicity assays (median lysis 1600 LU10/10^6, range 0-3300). In addition, to address whether BCR-ABL-specific BM T cells were allowed to spontaneously surface also during long-term therapy with 2nd generation tyrosine-kinase inhibitors (TKIs), we have further analyzed 5 Ph+ALL pts treated with Nilotinib or Dasatinib. Interestingly, specific cytotoxic BM immune responses were maintained or recovered in 2/2 pts after the switch from IM to 2nd generation TKIs (either Nilotinib or Dasatinib), as well as 3 other pts, treated with Dasatinib as first-line therapy, similarly showed the early emergence of p190BCR-ABL-specific EM T cells (20-200 SFCs/10^6 BM cells). *Conclusions.* Our investigations disclose, for the first time, that BM-homing BCR-ABL-specific autologous T cells are allowed to spontaneously develop in Ph+ALL pts, possibly cooperating with TKIs, and may represent a novel factor for disease control and prognostic evaluations. In perspective, our data also suggest that these autologous BCR-ABL-specific T cells may be successfully expanded ex vivo and possibly exploited as adjuvant immunotherapy for Ph+ALL pts, undergoing maintenance treatment with IM or with 2nd generation TKIs.

C003

NG2 PROTEOGLYCAN CONFERS CHEMORESISTANCE TO LEUKEMIC CELLS THROUGH UP-REGULATION AND FUNCTIONAL MODULATION OF DRUG TRANSPORTERS

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Introduction. NG2 (Neuron-Glial antigen 2), a unique chondroitin sulfate transmembrane proteoglycan, is prognostic cell surface antigen with unknown function expressed by certain AML and ALL carrying MLL (Mixed Lineage Leukemia) rearrangements and chromosomal (4;11) translocations. NG2 expression is associated with bad prognosis leukemias, very refractory to present therapies: from here the aim of investigating its role as chemio-resistance marker. *Methods.* We proceeded to a wide screening by RT-PCR for the expression of different drug transporters in both NG2$^+$ and NG2$^-$ wild type leukemic cell lines and performed *in vitro* transfection for ectopic expression in NG2- cells. FACS

analysis was conducted for the evaluation of NG2 surface expression and western blotting experiments to verify the efficiency of apoptosis apparatus. Functional drug transporter assays, such as Rhodamine 123 and Calcein efflux assay, have been exploited to study the influence of NG2 in pharmacoresistance. *Results.* We demonstrate that endogenously up-regulated NG2 expression in cells lines with MLL rearrangement and t(4;11) translocation, or forced expression of NG2 in leukemic lacking these chromosomal aberrations, induce reduced susceptibility of the cells to doxorubicine, metothrexate, aracytadine C, etoposide, calapin inhibitor, and berberine and analogously show superior efflux abilities of $Ca2^{+}$-dependent calcein AM, rhodamine 123 and fluorescent doxorubicine transport across the membrane. This effect was associated with two phenomena: an up-regulated, or de novo induced, transcription and surface expression of BCRP, MDR1, MRP1 and MRP2, and a modulation of the relative efficacy of the MRP1 and MRP2, as determined by combined fluorescent efflux assays and the use of specific antagonists. Modulation of the functional activity of the drug trasporters seem to involve a putative interaction between the proteoglycan and the molecule, and preliminary evidence also suggest that NG2 may physically interact with one or more of these transporters. *Conclusions.* We conclude that NG2 confers resistance to diverse classes of drugs currently employed in the treatment of AML and ALL, and that this effect is mediated by activation of signaling cascades that up-regulate cell surface expression of drug transporters, as well as by a direct modulation of their functional activity. We foresee that the outcome of this study will highlight novel ways to combine diagnostic/prognostic traits with individualized treatment regimes, such as immunotherapy direct to NG2, since this proteoglycan is not present on the surface of normal hematopoietic precursor cells or normal blood cells.

C004

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

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Introduction. Ph^{+} ALL is observed in about 30% of adult ALL and is associated with a very poor prognosis and early relapse. Tyrosine kinase inhibitors have improved overall treatment results, with a rapid response and a complete remission (CR) rate ranging 90%. However most patients experienced hematological relapse in a short time. Molecular analysis based on quantitative assays (i.e. quantitative polymerase chain reaction, qPCR) provides detection of residual leukemic cells measuring BCR-ABL1 transcript level and becomes necessary in the monitoring of minimal residual disease to confirm molecular CR or to detect early relapse. We investigated the efficacy of a high sensitive method based on nanofluidic platform (Fluidigm Corporation, South San Francisco, CA) to detect and quantify residual and rare BCR-ABL1 copies in Ph^{+} ALL patients who obtained molecular remission as assessed by conventional qPCR. *Methods.* The 12.765 Digital array (Fluidigm) is a nanofluidic biochip that consists in twelve panels, each containing 765 individual reaction chambers where samples are portioned prior to qPCR; as fluorescent signal is produced only in chambers containing copies of the target sequence, digital array provides an absolute quantification by counting the number of positive reactions. Digital raw data are then processed by the BioMark Digital PCR Analysis software (Fluidigm), that estimates the true number of molecules per panel using the Poisson probabilistic distribution. We analyzed 22 Ph^{+} ALL samples expressing the P190 (11) or P210 (11) BCR-ABL1 isoform in complete (87%) or major (13%) molecular response (BCR-ABL1/ABL ratio $\leq$0.001 or <0.1, respectively) as assessed by conventional qPCR; RNA integrity was evaluated using the control gene ABL. *Results.* First, we assessed the sensitivity and reproducibility of the assay using six serial dilutions of plasmids (Ipsogen) expressing known copy number of BCR-ABL1 P190 transcript (10000; 1000; 100; 50; 10; 1 copies). A 2 μL volume of input cDNA was loaded and two panel for each dilution were used. Results showed a detection rate until a copy of target sequence and a pairing significantly effective between replicates (P=0.0014, Paired t TEST analysis). We then analyzed duplicates of Ph^{+} ALL samples with a positive control for each chip: digital array resulted positive in 58% of complete molecular response samples, with 5.5 as median number of copies detected (range 0.5-11). *Conclusions.* The Fluidigm nanofluidic platform provides a high sensitive assay, able to detect until a single copy of BCR-ABL1 transcript with greater accuracy than conventional qPCR, as demonstrated for samples in molecular remission, and could provide an accurate monitoring method for Ph^{+} ALL CR patients. Further studies to confirm these results are actually ongoing.

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C005

FROM DIAGNOSIS TO RELAPSE: AN HIGH-RESOLUTION MOLECULAR ALLELOKARYOTYPING ANALYSIS OF PAIRED DIAGNOSIS-RELAPSE SAMPLES IN BCR-ABL1-POSITIVE ALL INDICATES INVOLVEMENT OF ALTERATIONS TARGETING KEY REGULATORS OF TUMOR SUPPRESSION, CELL CYCLE CONTROL, AND LYMPHOID/B CELL DEVELOPMENT

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Introduction. Although treatment with tyrosine kinase inhibitors has revolutionized the management of adult patients with BCR-ABL1-positive ALL and significantly improved response rates, relapse is still an expected and early event in the majority of them. It is usually attributed to the emergence of resistant clones with mutations in BCR-ABL1 kinase domain or to BCR-ABL1-independent pathways but many questions remain unresolved about the genetic abnormalities responsible for relapse. *Patients and methods.* In an attempt to better understand the genetic mechanisms responsible for this phenomenon, we have analyzed matched diagnosis-relapse samples from 20 adult BCR-ABL1-positive ALL patients using high resolution Affymetrix single nucleotide polymorphism (SNP) arrays (GeneChip® Human Mapping 250K NspI, n=15 pairs and Genome-Wide Human SNP 6.0, n=5 pairs). Genetic differences were analyzed in terms of copy number changes and loss of heterozygosity (LOH) events. Patients were enrolled in clinical trials of GIMEMA AL Working Party and treated with imatinib alone or in combination with conventional chemotherapy (40%) or dasatinib as frontline therapy (60%). The median age at diagnosis was 54 years (range 23-74) and the median blast cell count was 97% (range 60-99). The median time to relapse was 27 months (range, 9-104). *Results.* In 2/20 (10%) patients no genomic differences between diagnosis and relapse samples were found ("stable group"), suggesting that only BCR-ABL1 mutations or extra-leukemic factors (e.g., drug distribution, drug metabolism, compliance) contributed to the relapse. In the remaining cases (90%), new acquired copy number alterations (CNAs) were detected at relapse ("unstable group"). Acquired macroscopic alterations (>1.5 MB) were detected in 6 (30%) cases and included gain of chromosome 1q, 16q22, 9q and 22q (regions flanking the ABL and BCR genes), 19p13.3 (ABCA7, APC2, ARID3A) and 11q12.1-11q25; macroscopic losses affected the locus 1p36 in 2 cases. The gene most frequently affected by microscopic CNAs (<1.5 Mb) was the tumor suppressor CDKN2A/B (20%). Other common acquired CNAs included gains of ABC transporter genes, such as ABCC1, ABCC6 (1q41) and BCL8 (15q11); losses affected EBF1 (5q33) and IGLL3 (22q11) genes involved in B-cell development, BTG1 (12q21) involved in cell cycle regulation and CHEK2 (22q12) involved in DNA repair. The majority (92%) of relapse samples harbored at least some of the CNAs present in the matched diagnosis sample, indicating a common clonal origin. *Conclusions.* Genomic alterations evolving from diagnosis to relapse have been identified demonstrating that a diversity of alterations contributes to relapse and with the most common alterations targeting key regulators of tumor suppression, cell cycle control, and lymphoid/B cell development.

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C006

PAX5/TEL HAS AN OPPOSITE DOMINANT EFFECT ON ENDOGENOUS PAX5, AFFECTING B CELL ADHESION AND SIGNALING, IN PRE-BI CELLS

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Introduction. PAX5 is a transcription factor essential for B-cell development. Recently, it has been found as frequent target of abnormalities in B-cell precursor ALL cases, showing point mutations, deletions or chromosomal translocations. The functional role of these lesions is poorly understood. In previous experiments, in mouse pre-BI cells, we showed that PAX5/TEL protein acts as an aberrant transcription factor with repressor function, causing a block on B-cell differentiation, short-term IL-7 independence and resistance to the anti-proliferative and pro-apoptotic effects of TGFb1. Moreover, PAX5/TEL enhances cell migration towards CXCL12, with the over expression of CXCR4. The aim of the present study was to comprehensively understand how PAX5/TEL affects the transcription process and eventually interferes with PAX5 and TEL pathways, in order to identify cellular processes related to its expression. *Methods.* We analyzed gene expression profile in pre-BI cells transduced either by MIGR-PAX5/TEL-IRES-GFP or by MIGR-GFP (Affymetrix Gene Chip technology, Mouse array 430A 2.0). Validation of Differentially Expressed Genes (DEGs) has been performed by quantitative RQ-PCR. *in vitro* adhesion assays have been performed on VCAM1-coated slides. *Results.* In pre-BI cells, PAX5/TEL significantly affected the transcription process; indeed, gene expression profile analysis indicated the presence of 340 DEGs and, among them, we observed 61% of down-regulated genes whereas 39% up-regulated. In both groups of genes, we identified the presence of numerous PAX5-target genes; in particular, genes which are normally activated by PAX5 were repressed by PAX5/TEL and, vice versa, genes which are physiologically repressed by PAX5 were activated by PAX5/TEL. Moreover, gene functional classification analyses suggested that PAX5/TEL modulates molecules which are related to fundamental cellular processes, such as phosphorylation, transcription, B cell receptor signaling, as well as adhesion. In particular, we demonstrated the modulation both of surface antigens responsible of extra cellular binding and of intracellular molecules, important for signaling involved in adhesion regulation. Moreover, *in vitro* adhesion assays on VCAM1-coated slides showed a significant reduction of adhesion capacities in PAX5/TEL positive cells. *Conclusions.* These analyses further sustain the role of PAX5/TEL as an aberrant transcription factor, with an effect on endogenous PAX5 that does not represent a classical dominant negative role, indeed we defined this effect as an 'opposite dominance', since PAX5/TEL caused the up-regulation of PAX5-repressed genes and, vice versa, the down-regulation of PAX5-activated targets. The biological consequences of this aberrant transcriptional activity are the impairment of B cell receptor signaling and the reduced adhesion capacity, both fundamental processes in B-cells, potentially involved in tumor transformation and in leukemia.

C007

CLINICAL OUTCOME AND MONITORING OF MINIMAL RESIDUAL DISEASE (MRD) IN PATIENTS WITH THE MLL-ENL GENETICALLY DEFINED ACUTE LYMPHOBLASTIC LEUKEMIA SUBTYPE

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Background. MLL-ENL fusion represents one of the most frequent abnormalities of ALL with the mixed-lineage leukemia (MLL) gene alteration. Due to the rarity and prevalence of this alteration in infants, the outcome of MLL-ENL positive ALL still deserve to be established. Therefore we analyzed 12 MLL-ENL positive ALL patients (4 children and 8 adults), consecutively diagnosed between April 1999 and June 2009. *Methods.* At diagnosis MLL-ENL was detected by a multiplex RT-PCR, while we used the same individual primers and conditions as the multiplex system for the MLL-ENL RT-PCR monitoring of MRD, at a sensitive level of 10^{-4}. In 4 cases, Ig/TCR gene rearrangements were detected at diagnosis and patient specific primers were designed to obtain at least two sensitive markers ($\geq 1\times10^{-4}$) and used to PCR-MRD. *Results.* The MLL-ENL fusion was identified in 4 (2.6%), 8 (0.6%) and 0 (0 %) of the 150 pediatric, 1215 adult and 70 elderly ALL tested patients, respectively. Four were females, 8 males. Median age was 26 years (range: 0.2-59 years). Eight patients had a WBC count $>50\times10^9$/L (median WBC = 74.5×10^9/L; range 2.9-707.0×10^9/L). A T-immunophenotype was detected in 3 of the 10 evaluable cases, while the 7 remaining had a B-precursor ALL (pro-B = 3; pre-pre B = 3 and pre-B = 1). Eleven out of twelve patients (92%) achieved CR; one died of infection. At 48 months 73.3% of patients is projected to be survivor and 66.7% to be event free survivor. At response evaluation, two of the 11 CR patients tested MLL-ENL negative and six positive. However, the MLL-ENL status did not correlate with outcome. In fact, the three relapses occurred in both the two RT-PCR negative cases and in one of the 6 positive patients, while the remaining 5 RT-PCR positive cases were in CR at 8, 9, 3, 3 and 5 years, respectively. In addition, MLL-ENL expression, not preceding a relapse, was detected several times during the follow-up of 5 long-survivors. In 4 cases (three children and 1 adult) therapeutic response was also determined by the parallel assessment of Ig/TCR markers. All the 4 cases remained MLL-ENL positive, but the 3 children achieved a Ig/TCR negativity, becoming long-survivors at 8, 9 and 6 years, respectively. The adult persisted Ig/TCR positive and underwent an allogeneic HSCT that induced a long-term continuous CR lasting for 5 years. Once again this case showed the MLL-ENL expression for several times during the follow-up. *Summary/Conclusions.* Present data suggests that: 1) the MLL-ENL fusion identifies a rare genetically determined leukemic entity with an extremely favourable prognosis also in adult ALL; 2) the inconsistency between the clinical cure and the presence of detectable MLL-ENL transcript, both at therapeutic response evaluation and during follow-up of long-term CR patients. This latter finding suggests the presence of a MLL-ENL-expressing "pre-leukemia" stem cells, similar to what demonstrated for the AML1-ETO positive leukemia setting.

C008

IMPLEMENTATION OF ARRAY BASED WHOLE-GENOME HIGH RESOLUTION TECHNOLOGIES CONFIRMS THE ABSENCE OF SECONDARY COPY NUMBER ALTERATIONS IN MLL-AF4 POSITIVE INFANT ALL PATIENTS

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Introduction. Human tumors are the results of the progressive accumulation of subsequent multiple genetic lesions. However, leukemia with very short latency, such as infant ALL with MLL gene rearrangement, might have a different patogenetic mechanism. By applying the Affymetrix® Human Mapping SNP 100K or 500K array, we and others have recently reported that in MLL-AF4 positive infant ALL with, an exceptionally limited number of copy number alterations (CNAs), compared to other leukemias occurring in older patients. It can be argued that, as a major limitation of these studies, submicroscopic lesions might have potentially been missed at the sensitivity of the method applied. *Methods.* We had the opportunity to apply for the first time the Affymetrix® cytogenetics whole-genome 2.7M array to ten diagnostic-remission paired MLL-AF4 positive infant ALL patients previously analysed by SNP 100K, with the aim to implement and validate our published data. Through 2.7 million markers across the entire genome (including 400K SNPs and 2.3M non-polymorphic copy number markers) this new technology provides a higher resolution enabling the detection of even smaller regions. *Results.* We confirmed that MLL-AF4 positive infant ALL patients lack additional tumor-associated structural chromosomal abnormalities (deletions and amplifications). Numerous constitutive loss of heterozygosity (LOH) regions were also observed. Interestingly, the accurate reanalysis of three out of four patients in which we had observed by SNP 100K the LOH(14)(q21.2), including the FANCM gene, confirmed the presence and the constitutive nature of this aberration, which is the most recurrent in infant ALL patients. *Conclusions.* Our results, together with the evidences of a prenatal origin of the rearrangement, the exceptionally high concordance rate in monochorionic twins and the short latency, suggest that, unlike other form of leukemia occurring in children, adolescent and adults, infant ALL could be a single-hit leukemia, as MLL fusions gene, per se, has a major role in driving and hastening the leukemogenetic process without the need of secondary cooperating mutations, at the best available sensitivity. High throughput genomic sequence could reveal whether lesions (i.e. point mutations) below the detection level applied might contribute to the pathogenesis of this unique leukemia.

Myelodysplastic Syndromes

C009

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

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

C010

PROLIFERATIVE AND APOPTOTIC SIGNALLING IN BONE MARROW CELL SUBPOPULATIONS OF MYELODYSPLASTIC SYNDROMES PATIENTS USING FLOW-CYTOMETRY TECHNIQUE

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Introduction. Myelodysplastic syndromes (MDS) are heterogeneous clonal diseases characterised by cytopenias as a result of ineffective hemopoiesis. Development of effective treatments has been mainly impaired by limited insights into MDS pathogenesis. Moreover, little is known about signal transduction pathways altered MDS cells. We have devised a multiparameter flow-cytometry method of signal transduction pathways analysis allowing rapid and specific separate evaluation in specific cellular subpopulations of MDS bone marrow cells. *Methods.* Cells were

fixed with formaldehyde and permeabilized with methanol, then stained with APC anti-human CD34, PE anti-human CD71, PerCP anti-human CD45 and Alexa-Fluor488 anti-STAT5 (pY694), Alexa-Fluor488 anti-ERK1/2 (pT202/pY204), Alexa-Fluor488 anti-p38 (pT180/pY182) and Alexa-Fluor488 anti-cleaved caspase-3 (D175). Samples were analysed on a cytometer with 6 color laser. We studied in 60 MDS cases phosphorylation of ERK1/2 and p38 MAP kinases, phosphorylation of signal transducer and activator of trascription (STAT)5 and proteolitic activation of caspase-3 in bone marrow mononuclear cell subpopulations $CD34^+$, $CD45^+$ and $CD71^+CD45^-$ and compared with normal bone marrow cells. We performed the analysis before and after erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) stimulation. *Results.* Baseline activation of MAPK phospho-proteins, STAT5 and caspase-3 was variable among MDS cases and among distinct cellular subpopulations. Non-parametric Wilcoxon test indicated that STAT5 was significantly activated in all $CD34^+$, $CD45^+$ and $CD71^+CD45^-$ MDS cells compared with cells from normal controls while caspase-3 was activated in $CD34^+$ and $CD45^+$ MDS cells. By the Kruskal-Wallis test we observed that refractory anemia (RA) and refractory anemia with excess of blasts-1 (RAEB-1) were more prevalently affected by this phenomenon. G-CSF stimulation activated STAT5 in $CD34^+$ from MDS and normal control, without significant differences. EPO stimulation failed to induce STAT5 activation in $CD71^+CD45^-$ cells subpopulation of 22/36 MDS cases while it was effective in normal cells and in 14/36 $CD71^+CD45^-$ MDS cells. Non-parametric Wilcoxon test showed that STAT5 activation induced by EPO in $CD71^+CD45^-$ MDS cells was significantly lower than in normal cells. Evaluation of EPO response *in vivo* (Hb increase >2 g/dL without transfusions after 8 weeks of EPO treatment) has shown that in 20/22 cases it correlated with *in vitro* EPO dependent STAT5 activation (Spearman's rho=0.62 and P=0.002). *Conclusions.* Signal transduction pathways may be analysed separately in specific subpopulation of MDS marrow cells. Our study indicates that different subpopulation ($CD34^+$, $CD45^+$, $CD71^+$CD45-) show diverse pathway activations and diverse apoptosis pattern. Response to growth factors is also restricted to specific cellular subpopulations.

C011

EFFECT OF COMBINATION OF VALPROIC ACID AND AZACITIDINE ON PHOSPHOLIPASE C BETA1 SIGNALLING IN HIGH-RISK MDS

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Introduction. MDS are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis in one or more of the lineages of the bone marrow. The disease can result in a slow decrease in blood cell counts, but it may also have a more aggressive evolution, that is a worsening severe cytopenia or, in about 30% of all the patients, transformation into AML. Azacitidine (AZA), a DNA methyltransferase inhibitor, can prolong survival and delay MDS evolution into AML, alone or in combination with the histone deacetylase inhibitor valproic acid (VPA), which improves the clinical response induced by azacitidine alone. Nuclear inositides are essential co-factors for several nuclear processes, including DNA repair, transcription regulation and RNA dynamics in normal and neoplastic conditions. Namely, nuclear phosphoinositide-phospholipase C (PI-PLC) β1 appears to play a fundamental role in cell cycle regulation, mainly targeting cyclin D3. Moreover, PI-PLCβ1 has been demonstrated to be a marker for monitoring the effect of AZA on high-risk MDS patients. *Methods.* The effect of AZA and VPA on inositide signaling pathways was studied on MDS patients (IPSS risk: intermediate-2 or high) receiving the combination of azacitidine (75 mg^2/d SC for 7 days/28 days) with VPA (600-1.500 mg/die orally). Some of them also received All-Trans Retinoic Acid (ATRA), if refractory to the combination of AZA and VPA. The results of the MDS samples were compared with patients receiving only best supportive care and a pool of healthy subjects. We also analyzed the effect of AZA and VPA, alone or in combination, on HL60 cell line, which shows a hyper-methylation of PI-PLCβ1 and is affected by AZA treatment. We performed MTT experiments, cell cycle analyses and quantified the expression of PI-PLCβ1, cyclin D3 and p-Akt before and after the treatment. *Results.* We report that p-Akt, PI-PLCβ1 and cyclin D3 are affected by AZA and VPA, alone or in combination. In particular, the combination of the two treatments not only increases the demethylation effect of AZA on PI-PLC-beta1, but also induces cell cycle progression in the G1 phase. This is demonstrated by an increase in both PI-PLCβ1 and cyclin D3 expression, as well as MTT and cell cycle analyses. As for p-Akt, it is slightly down-regulated by AZA and by the combination of AZA with VPA. Conclusions. Our findings indicate that VPA enhances the effect of AZA on inositide signalling pathways. *In vitro* experiments on HL60 cells showed that these treatments increase PI-PLCβ1 and cyclin D3, thus inducing cells towards the G1 phase of the cell cycle. Taken together, our results demonstrate for the first time that, at a molecular level, AZA and VPA can modulate key molecules of the inositide signalling involved in cell cycle progression, thus hinting at a role for these pathways in the MDS pathogenesis.

C012

AZACITIDINE LOW-DOSE SCHEDULE IN SYMPTOMATIC LOW-RISK (IPSS: 0-1) MYELODISPLASTIC PATIENTS. CLINICAL AND BIOLOGICAL EFFECTS

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Background. Nucleoside 5-Azacitidine (5-Aza) in high risk MDS patients (pts) at a dose of 75 mg^2/d subcutaneously for 7 days, every 28 days, induces high hematologic response rates and prolongation of survival. *Aim.* The role of 5-Aza in low-risk MDS pts is not well defined but its use in the earlier phases of disease could be more effective and useful to control the expansion of MDS clone and disease progression. In our phase II, prospective, multicentric trial a low-dose schedule of 5-Aza (75 mg^2 daily for 5 consecutive days every 28 days) was given to low-risk MDS patients in order to evaluate its efficacy and tolerability. Furthermore pharmacogenomic studies (GEP, SNP), cytokine network and PI-PLC-beta1 expression, before and at the end of 4^{th} and 8^{th} course of 5-Aza treatment, were planned to identify new biological markers to predict the response. *Methods.* From September 2008 to February 2010, 34 patients were enrolled into the study. According to WHO criteria 15 patients had RA, 5 patients RARS, 7 patients RCMD and 7 patients RAEB-1. Age at diagnosis ranged between 56 and 84 years. All patients failed previously EPO therapy and were in chronic red blood cell (RBC) supportive care. The response treatment criteria was according to IWG 2006. *Results.* At present time 31 out of 34 pts are evaluable: 15/31 pts (48%) completed the treatment plan (8 courses), 8/31 pts (26%) performed the first 4 courses, 4/31 (13%) made 1 to 3 courses and 4/31 (13%) died during the treatment period. Out of 15 pts who completed the 8 courses of therapy 10 (67%) obtained an HI, 5/15 (33%) maintained a stable disease. Out of 10 pts who obtained HI, 4 pts (40%) achieved a CR. Generally the drug was very well tolerated. The most commonly reported hematologic toxicities were neutropenia (55%) and thrombocytopenia (19%). 2/4 pts died early after the 1th cycle for septic shock and gastrointestinal hemorrage respectively whereas 2/4 pts died in a condition of stable disease after the 4th cycle for pneumonia and respiratory distress. Preliminary data on the lipid signalling pathways suggested a direct correlation between PI-PLC- 1 gene expression and 5-Aza responsiveness. *Conclusion.* Interim analysis of our study based on the small number of cases who completed the treatment program, shows that 67% of pts obtain an HI and 40% obtain a CR. These response rates are higher than those observed in the setting of high risk MDS pts and have been obtained by using low doses of 5-Aza. 4 patients died during the treatment and even if the causes were reported as no related to the therapy it has been considered that caution has to be

reserved in given 5-Aza in these pts who are elderly and frail. Preliminary data of PI-PLC-β1 gene expression suggest that probably there are biologic markers which can help us to know a priori who are the patients who have more chances to respond. Furthermore pharmacogenomic studies will help us to better understand which patients may benefit from chronic maintenance therapy. *Acknowledgments.* This work was supported by cofin 2007.

C013

PROGNOSTIC ROLE OF GLOBAL DNA METHYLATION AND HISTONE ACETYLATION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Introduction. Epigenetic aberrations are now well recognized as very frequent and also as early events in the process of malignant transformation. These molecular mechanisms regulate gene expression without changing the DNA sequence and include alterations in the methylation status of DNA, covalent modification of histone tails, chromatin remodeling, and microRNAs. Somewhat contradictory, in patients with myelodysplastic syndrome (MDS), the induction of hypomethylation using DNA methyltransferase inhibitors leads to very promising clinical response rates and haematological improvement. We investigated global DNA methylation and histone H3 lysine 9 acetylation by immunohistochemistry in bone marrow trephine biopsy specimens in a cohort of 120 MDS patients comprising all subgroups. Results were compared to an age-matched control group of healthy subjects and to a group of AML patients. *Methods.* Immunohistochemistry was performed on paraffin-embedded sections, using anti-5-methylcytosine/5mc and anti-Acetyl-Histone H3 (Lys9)/AcH3K9 antibodies. Scoring of immunohistochemistry was evaluated with a four-point scale for both the number of positive tumor cells and their intensity of immunoreactivity. *Results.* Our results showed that in MDS the 5 mc immunostaining score was intermediate between normal controls and AML cases, whereas the AcH3K9 immunostaining score was lower than normal control. The 5 mc score correlated significantly with the risk score according to the International Prognostic Scoring System, the blast count and the karyotype. Our results suggest that global hypermethylation and histone hypoacetylation correlate with MDS aggressiveness. *Conclusions.* These results may provide a molecular explanation for the success in treating MDS patients with hypomethylation-inducing agents and why patients with a poor karyotype respond best. Future studies have to analyse whether the determination of global methylation and histone acetylation levels may serve as a new predictive marker for therapy response.

C014

GENE EXPRESSION PROFILE CHANGES IN BONE MARROW OF MDS PATIENTS WITH DEL5Q DURING LENALIDOMIDE TREATMENT

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Introduction. The commonly deleted region in patients with 5q syndrome contains key genes involved in the pathogenesis of the disease, including SPARC and RPS14. Lenalidomide has proven efficacy in MDS patients with del(5q), resulting in transfusion-independence, suppression of the 5q clone, and improvement in bone marrow features. Haploinsufficiency of RPS14 has been demonstrated and predicts response to lenalidomide, while over-expression salvages erythroid differentiation *in vitro*. *Aim.* To investigate changes in gene expression patterns during lenalidomide treatment in MDS patients with del5q and low or Int-1 risk IPSS. *Study Design.* In a multicenter Italian Phase II prospective single-arm trial, 23 patients received a starting dose of lenalidomide 10 mg p.o once daily on a continuous daily schedule for a maximum of 12 months. Dosing was based upon clinical and laboratory findings. Bone marrow cytogenetics and gene expression profiling were performed on study entry and every 12 weeks up to end of study (week 52). Gene expression assays of 51 candidate genes were carried out with TaqMan® Low Density Array Fluidic card. Target gene expression was normalized with expression of similar gene derived from a bone marrow pool of normal healthy subjects. Baseline values for 23 patients (mean age 73±10 years) were available. Mean Hb was 8.6±0.9 g/dL and 20 patients were transfusion-dependent. Seven patients had additional cytogenetic abnormalities. *Results.* At baseline, RPS14 level was under-expressed in 19 out of 23 patients evaluated. After 12 weeks 13 patients were re-evaluated and RPS14 increased significantly from 0.07 (IQ Range 0.03-0.13) to 76.1 (0.73-304.0, P=0.002). SPARC expression was under-expressed in 15/23 patients and variations during treatment were not significant. Baseline FAS gene was under-expressed in all patients and increased above reference normal values (P=0.006) after 12 weeks in 7/14 cases. IL7R gene was over-expressed in all patients at baseline (median 3263.3, IQ range 1998.3-5027.1) and was significantly reduced after 12 weeks (median 0.17, IQ range 0.05-2.20, P<0.0001). WT1 gene was over-expressed in almost all patients (median 3246, IQ range 1590-7785) and, though it reduced during treatment, still remained highly expressed at 12 weeks (P=0.148). The expression of the other genes did not vary significantly during lenalidomide treatment. *Conclusion.* Our preliminary data evaluating short-term effects of lenalidomide in low and Int-1 IPSS risk MDS with del5q demonstrated that RPS14 was down-expressed in most patients at baseline. A significant increase in its expression was observed *in vivo* during therapy in patients with erythroid response. Reduction of IL7R suggested a probable drug anti-apoptotic effect with reversible action on RPS14 haploinsufficiency. Further results during this trial may develop new insights in long-term effects of lenalidomide on bone marrow changes. The authors are engaged in this direction.

C015

AZACITIDINE FOR THE TREATMENT OF HIGH-AND-LOW-RISK MYELODYSPLASTIC SYNDROMES. HEMATOLOGIC AND MOLECULAR RESPONSE TO 2 ALTERNATIVE DOSING SCHEDULES

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Introduction. Azacitidine (AZA) is effective treatment for myelodysplastic syndromes (MDS) at a dosing schedule of 75 mg/m^2/d subcutaneously for 7 days every 4 weeks (AZA 7) (Silverman, 2002; Fenaux, 2009). Recently 3 different AZA dosing regimens (AZA 5-2-2, AZA 5-2-5 and AZA 5) which avoid week-end dosing, have shown to induce therapeutic responses consistent with the currently approved schedule (Lyons, 2009). In particular, two of them, AZA 5 (75 mg/m^2/d subcutaneously for 5 days) and AZA 5-2-2 (75 mg/m^2/d subcutaneously for 5 days, followed by 2 days no treatment, then 75 mg/m^2/d for 2 days), save 4 vials/month (28% of the actual cost). However, the Lyons' community-based study mainly involved lower-risk MDS patients (pts). These data prompted us to investigate the therapeutic effect of the more convenient and less expensive AZA 5 and AZA 5-2-5 regimens in MDS pts with a more aggressive disease. *Methods.* From December 2007, in our Institution, 22 MDS pts (15 males), with a median age of 70 (52-85) yrs, were treated with AZA, following 2 different treatment regimens. Group 1 (12 pts), with low-risk but more aggressive disease (IPSS low-or-int-1), refractory to erythropoietin (EPO) or with a poor EPO-response profile and/or with severe neutropenia or thrombocytopenia, were treated with the AZA 5 regimen. Group 2 (10 pts.), with high-or-int-2 risk MDS, received the AZA 5-2-5 regimen. Moreover, as our group (Follo, 2009) previously demonstrated that the lipid signalling pathways, in particular phosphoinositide-phospholipase C (PI-PLC) β1, may represent a target for AZA, we quantified the degree of PI-PLCbeta1 methylation and gene expression before and during AZA administration in pts of group 1 and 2, and in a control group of pts previously treated with the cur-

rently approved AZA 7 regimen. *Results.* Group 1: 10 pts (83%) completed at least 6 AZA cycles, and were considered evaluable for response. 5 pts (50%) showed a favourable response, as defined by Cheson (2006): Complete Remission (CR): 2 pts, Hematologic Improvement (HI): 3 pts. 2 pts died because of sepsis, after 1st and 5th cycle. 4 responder pts discontinued AZA after 8 courses, and maintained the response for a median of 9 (3-11) months. Group 2: 8 pts (80%) evaluable for response (≥6 cycles); 6 responder pts (75%); CR: 1 pt, HI: 5 pts; 4 pts died (2 because of AML). PI-PLCβ1 methylation and gene expression appeared to be related to the therapeutic response, but not to the dose schedule. *Conclusions.* Our data confirm, in a population of higher risk MDS pts, the effectiveness, in terms of hematologic response, of these 2 alternative and more convenient AZA dosing schedules. No significant differences were found, as regards the demethylating effect on PI-PLCbeta1, between these 2 alternative regimens and the control group (conventional AZA 7 dosing schedule). These data need to be confirmed by larger studies, and the effect on survival also needs to be assessed.

C016

FISH TO REVEAL TET2 ABNORMALITIES IN MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA

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Haplo-insufficiency of the TET2 gene occurs in distinct onco-hematological disorders (MDS, myeloproliferative disorders and t-AML) through different cytogenetic and molecular mechanisms. In MDS/AML this unifying tumor-initiating event has an incidence of 24% if patients are investigated with DNA sequencing and of 5% if they are investigated with FISH. Based on these findings, the present study was aimed at establishing the incidence of band 4q24 deletions/structural defects in a series of 35 MDS/AML patients examined between January 2003 and December 2009 and to test whether TET2 deletions were correlated with any peculiar gene mutation and clinical findings. Our patients were eighteen females and seventeen males, whose median age was 62 years (range 29-85). According to WHO classification twenty-one patients were diagnosed as MDS [8 refractory anemia, one 5q- syndrome, 7 RA with excess of blasts type 1 (RAEB-1) and 5 RAEB type 2 (RAEB-2)] and 14 patients as AML (3 M0, 3 M2, 5 M4 and 3 M5). A clinical progression occurred in two RA, in two RAEB-1 and in three RAEB-2 patients. Conventional cytogenetic analyses revealed a normal chromosome pattern in twenty-nine patients and a structural defect of chromosome 4 in five (two patients with a single defect involving chromosome 4 [a t(1;4)(p32;q35) and a t(3;4)(p13;q31)] and three with a complex karyotype [≥3 abnormalities). In order to evaluate TET2 deletions, FISH with the BAC probes RP11-144B4 (mapped at 14q22.3), RP11-810D13, RP11-571L19, RP11-414I7 (all mapped at 4q23), RP11-356L5 and RP11-16G16 (both covering the TET2 gene at band 4q24), RP11-642P17, RP11-788K3, RP11-752J12 (all mapped at 4q24) and RP11-66J16 (mapped at 4q25) was applied. All these probes were obtained from BACPAC Resources Center at C.H.O.R.I. (Oakland, USA), labelled and applied as previously reported. The cut-off values for interphase FISH (i-FISH) were obtained from the analysis of 300 nuclei from ten normal samples and were fixed at 10%. A TET 2 deletion was found in three patients. The patient with the t(1;4) translocation showed the loss of the 356L5, 16G16, 788K3 and 642P17 BAC probes and the maintenance of the 752J12 and 66J6 BAC probes in 88% of the nuclei and in all the mitotic figures examined. So, this patient presented a loss of the TET2 gene and of the 788K3 and 642P17 probes even if the breakpoint of the chromosomal translocation was localized at band 4q25. Other two patients, one without and one with a structural defect of chromosome 4, presented a cryptic deletion of the 356L5, 16G16 and 788K3 probes. In conclusion, i) FISH is a good method for identifying cryptic TET2 deletions, as the chromosomal area containing this gene is often deleted independently of the chromosomal breakpoints; ii) TET2 deletions are rare events which in our series occurred at an incidence of 8.5%; iii) TET2 deletion is not always associated with chromosome 4 rearrangements on conventional cytogenetics.

Hematopoietic Stem Cells

C017

THE IMMUNOREGULATORY ENZYME INDOLEAMINE 2,3-DIOXYGENASE (IDO1) IS EXPRESSED BY NATURAL KILLER (NK) CELLS DURING CYTOKINE-MEDIATED ACTIVATION

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Introduction. Human NK cells are large, granular cells derived from lymphocyte lineage. They are critical mediators of the innate immunity as they rapidly respond to pathogens infected and tumour cells through cytotoxic and cytokines-producing responses. Indoleamine 2,3-dioxygenase (IDO1) is an enzyme catalyzing the degradation of the essential amino acid L-tryptophan into kynurenines. Several cell types, including dendritic cells, have been shown to express IDO1, which acts as a potent immunosuppressive agent. Recently, in addiction to IDO1, the new variant IDO2 was described but its immunosuppressive role is still under investigations. The aim of the present work is to study the expression and the role of IDO1 and IDO2 in NK cells. *Methods.* $CD3^-CD56^+$NK cells were immunomagnetically purified from healthy donors. IFN-γ production was measured with ELISA assay. IDO1 and DAP12 transcript levels were normalized on ABL. IDO2 expression was measured by semi-quantitative PCR. *Results.* peripheral blood NK cells were treated with IL-2 for 16h, 40h, 88h and 160h. At the end of each IL-2 stimulation, supernatants were collected and IFN-γ production was measured by colorimetric assay. IDO1 expression in NK cells was evaluated by quantitative real time PCR and Western blotting. Our data show that, during IL-2-mediated activation, IDO1 is up-regulated at mRNA and protein levels. This effect is maximum after an overnight incubation and it decreases at later time points. IDO1 expression is clearly correlated with IFN-γ production by NK cells whereas IFN-γ receptor expression is not affected. Culturing NK cells with a combination of IL-2 and anti-IFN-γ antibody demonstrated that IDO1 expression is regulated by IFN-γ. Interestingly, IDO1 mRNA upregulation is associated with downregulation of DAP12, a gene whose expression has been showed to be inversely correlated to IDO1 in dendritic cells. IDO2 expression seems to be not affected by IL-2 stimulation. Treatment with other inflammatory cytokines demonstrated that IDO1 is modulated by different stimuli. Overnight incubation with IL-12, IL-15 and a combination of IL-2 and IL-6 showed strong IDO1 induction. *Conclusion.* our results demonstrate that NK cells upregulate IDO1 expression during cytokine-mediated activation and that IDO1 expression is regulated, at least in part, by IFN-γ. Similarly to DCs, NK cells express IDO gene in association with DAP12, thus suggesting a common regulatory pathway among different cell subsets.

C018

CYTOKINE INDUCED KILLER CELLS FOR CELL THERAPY OF ACUTE MYELOID LEUKEMIA: IMPROVEMENT OF IMMUNE ACTIVITY BY EXPRESSION OF CD33-SPECIFIC CHIMERIC RECEPTORS (CAR)

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Introduction. Cytokine induced killer (CIK) cells are *ex vivo* expanded effector cells with potent antitumoral activity. We demonstrated that CIK cells' infusion in AML patients relapsing after allogeneic transplant is well tolerated, but limited clinical responses were observed. *Aims.* To improve anti-leukemic CIK cell functions. *Methods.* CIK cells were genetically modified with SFG-retroviral vectors coding for anti-CD33-zeta or anti-CD28-OX40-zeta CAR and their ability to kill leukemic targets was analysed either by 4h-51Cr-release assays and long-term co-culture on stromal cells. CD33-specific proliferation was assessed by 3H-thymidine incorporation and cytokine release by flow cytometry. *Results.* Anti-CD33-zeta or anti-CD28-OX40-zeta CAR expressing CIK cells (average CAR expression, 65%) acquired potent cytotoxicity against several AML targets: after 4-hour incubation we observed, at effector:target (E:T) ratio of 5:1, a mean lysis of the HL-60 cell line of 79% and 75%, for anti-

CD33-zeta and anti-CD33-CD28-OX40-zeta cells respectively (P<0.005 *vs.* untransduced CIK cells). Analogous lytic efficiency was registered against the KG-1 cell line, known to be resistant to Gentuzumab-Ozogamicin, and primary AML blasts, with cytotoxicity >50%, contrarily from non transduced CIK cells, that showed <10% lysis against both targets (P<0.005).The strong anti-leukemic activity of manipulated CIK cells was confirmed in long-term killing experiments, where leukemic cells were co-coltured with CIK cells for 6 days on human stromal layer without exogenous IL-2 at an E:T ratio of 1:200.In these CIK cells eliminated almost all leukemic assays, anti-CD33-CD28-OX40-zeta cells, with 16% mean residual primary AML cells compared to 91% (P<0.005) of untransduced CIK cells. Anti-CD33-zeta CIK cells showed analogous but lower efficiency, with 31% mean residual primary AML (P<0.05). Moreover, a prominent CD33-specific proliferative activity was observed, with a mean proliferation index of 2.2 after HL-60-mediated stimulation and 2.4 after primary AML cells-mediated stimulation in anti-CD33-zeta transduced cells compared to 0.9 (P<0.005) and 1.4 (P<0.05) for un-transduced CIK cells. Expression of the anti-CD33-CD28-OX40-zeta CAR on CIK cells resulted in a higher expansion rate, with a mean proliferation index of 4.4 (P<0.005) after HL-60-mediated stimulation and 3.7 (P<0.005) after primary AML cells-mediated stimulation. In addition, when stimulated with irradiated HL-60, anti-CD33-zeta and anti-CD33-CD28OX40-zeta CAR-transduced CIK cells secreted 11-fold and 10-fold higher amount of IFN-gamma (P<0.005 for anti-CD33-zeta and anti-CD33-CD28-OX40-zeta), 120-fold and 180-fold more TNF-alfa (P<0.005 for anti-CD33-zeta and anti-CD33-CD28-OX40-zeta), 250-fold and 600-fold more TNF-β (P<0.005 for anti-CD33-zeta and for anti-CD33-CD28-OX40-zeta), 1400-fold and 3800-fold more IL-2 (P<0.05 for anti-CD33-zeta and for anti-CD33-CD28-OX40-zeta) compared to unmanipulated cells. Importantly, anti-CD33-CAR expressing CIK cells showed a transitory toxicity against normal hematopoietic $CD34^+$progenitors, with a reduced, but consistent number of clonogenic progenitors recovered in colony forming-assays after up to 48-hour co-culture with transduced CIK cells. Conclusions:our results indicate that anti-CD33 CARs strongly enhances anti-leukemic CIK functions, suggesting that CAR-expressing CIK cells might represent a promising tool for AML immunotherapy.

C019

GPR171, A PUTATIVE P2Y-LIKE RECEPTOR, COUNTERACTS MONOCYTE LINEAGE SPECIFICATION WHEN OVER-EXPRESSED IN MURINE HEMATOPOIETIC PROGENITORS

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Introduction. Despite intense investigation, the molecular mechanisms underlying the multi-step process that leads from committed progenitors to terminally differentiated cells are still poorly understood. Due to the capability of responding to a variety of chemically different compounds, G protein-coupled receptors (GPRs) have been shown to play a pivotal role in regulating the interaction of hematopoietic cells with the surrounding microenvironment. A novel GPR gene, Gpr171, encodes an orphan receptor putatively related to the family of purinergic receptors (P2YRs) for extracellular nucleotides, a group of mediators previously shown to be involved in the regulation of HSCs. Gpr171 has been indicated as a putative, novel member of the family of P2Y receptors, but no information is currently available on the ligand responsible for its activation. Likewise, its functional role in biological systems remains completely unknown. *Methods.* By sequence analysis, we reconstructed Gpr171 phylogenesis in mice. We explored the functional role of Gpr171 in the hematopoietic system by transducing $Sca\text{-}1^+$ hematopoietic progenitor cells (HPCs) with MSCV bicistronic retroviral vectors (thus driving the enforced expression of Gpr171 along with GFP). Transduced HPCs were analyzed both *in vitro* and *in vivo*. *Results.* By phylogenetic analysis, we confirmed that Gpr171 is evolutionally related to members of a P2Y gene-cluster localized on mouse chromosome 3. As a first step towards unveiling a role for Gpr171, we investigated its expression profile and function in the murine hematopoietic system. As opposed to other P2YRs, Gpr171 emerged to be significantly repressed in cells undergoing myeloid differentiation. We therefore hypothesized that Gpr171 might be involved in repressing monocyte lineage-specification. Consistent with our assumption, we observed a decreased expression of myeloid markers upon induction of Gpr171 in hematopoietic progenitors (HPCs), as well as a dramatic reduction in the capability of generating myeloid colonies *in vitro*. Furthermore, mice transplanted with HPCs over-expressing Gpr171 display a significant reduction in the percentage of Mac-1+Gr-1- monocytes. *Conclusions.* Our findings establish, for the first time, Gpr171 biological function and unveil its unique role in repressing monocyte differentiation. Gpr171 enforced expression induced a significant decrease in the proliferation rate of myeloid cells, as well as a reduction in the expression of monocyte-specific markers, both *in vitro* and *in vivo*. Our findings contribute to the delineation of a peculiar evolutionary history for Gpr171 as compared to other members of the P2Y family, suggesting a rather unique role in counteracting myeloid differentiation.

C020

NEW INSIGHTS INTO THE MIRNA SIGNATURE OF CORD BLOOD-CD34 POSITIVE CELLS

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Introduction. The therapeutic use of multipotent stem cells depends on their differentiation potential. The differences are the result of key changes in their epigenetic profiles mediated by microRNAs (miRNAs). miRNAs are known to interfere with the expression of mRNAs-encoding factors that control developmental timing, stem cell maintenance, and other physiological processes. A decrease in the differentiation potential of adult stem cells (ASC) is mediated by the silencing of pluripotency genes downregulated through the expression of certain miRNA, as indicated by Aranda's studies. *Aim.* To investigate the epigenetic regulation of stemness property at the microRNA levels in well characterized populations of multipotent adult stem cells arise from different human cell sources. *Materials and methods.* We utilized five human cord blood (CB) samples and five human bone marrow (BM) samples from healthy donors. All CB and BM samples were subjected to immune-selection system in order to obtain purified $CD34^+$ stem progenitor populations (SC), (MACS MicroBeads- Miltenyi Biotec). MiRNAs were isolated in each purified SC sample using mirVan™ miRNA Isolation Kit-Ambion. By combining the 5 single miRNAs of each SC sample group (CB, BM), miRNA-pools were created (300 ng/μL of product for each SC pool). TaqMan miRNA Array Analysis was performed to determine expression of 760 mature human miRNAs listed in the Sanger Registry (7900HT Sequence Detection System Applied Biosystems). CB-SC pool miRNAs was our studying target, whereas BM-SC pool miRNAs was considered as calibrator. Each CB-miRNA was normalized respect to corresponding BM-miRNA. It was assumed that BM expression value of each miRNA was 1 unit.

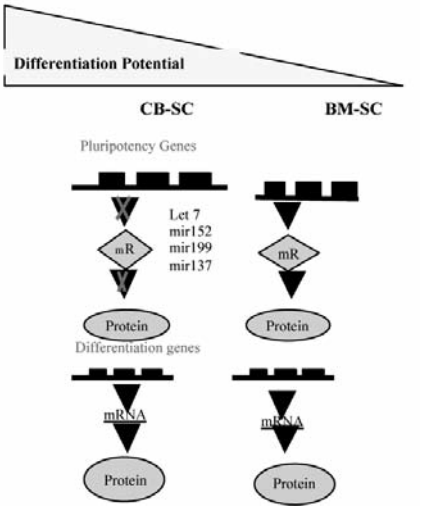

Figure. Model of epigenetic regulation of pluripotency genes in CB-SC and BM-SC.

Results. In CB-SC were identified 3 miRNAs blocks with different expression levels: 1st block with down-expressed miRNAs (value <1), 2nd block with miRNAs that showed comparable amount between CB-SC and BM-SC (value =1), 3rd block with up-expressed miRNAs (value >1), The great majority of miRNAs have poorly defined roles in cell fate decisions, but in CB-SC we observed the over-expression of mir-150 and mir-223 wich are known to be involved in the block of early B cell development and erythroid differentiation, respectively. miR-362 regolator of cell differentiation control was over-expressed, too. It's remarkable the up-espression of let 7family, mir 152, mir 137 and mir 199 in CB-SC. All together these mirRNAs are evoked in pluripotency program restriction in ASC. *Conclusion.* We observed a specific miRNA signature in CB-CD34+ cells, suggesting a different regulation versus BM-CD34+ cells, figure A. Our preliminary data help us to understand that in CB-SC exists the same pattern of up-expressed miRNAs that was identified in other adult stem cells and we may suppose that BM-SC epigenetic regulatory plan is different. These data provide a starting point for further studies aimed at choice the right cell source for regenerative medicine therapy.

C021

RELEVANCE OF THE MEVALONATE BIOSYNTHETIC PATHWAY IN THE REGULATION OF BONE MARROW MESENCHYMAL STROMAL CELL-MEDIATED EFFECTS ON B CELL SURVIVAL

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Introduction. Bone marrow mesenchymal stromal cells (BMSC) can exert immunosuppressive effects on T lymphocytes; in turn, they can promote survival and proliferation of B cells. Thus, they have become a possible target for therapeutic intervention. Thalidomide or lenalidomide are currently used in the treatment of multiple myeloma, due to their effect on bone marrow stroma. Recently, it has been proposed to use lenalidomide together with statins: these drugs are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-CoenzymeA (HMG-CoA) reductase involved in cholesterol biosynthesis. *Methods.* We have analyzed whether inhibition of the HMG-CoA reductase affects BMSC function. to this aim, BMSC were pre-treated with the competitive inhibitor fluvastatin; then cytoskeleton assembly, RhoA activation, adhesion molecule expression were analyzed. Moreover, the effects of statin-treated BMSC on B lymphocyte survival were studied. *Results.* BMSC could elicit in B lymphocytes a sustained calcium flux and enhance the transcription of anti-apoptotic proteins, as Bcl-xL and Bcl-2, leading to increased B cell survival. Pro-apoptotic effect of dexamethasone on B lymphocytes was partially counteracted by BMSC. Exposure of BMSC to the HMG-CoA-reductase competitive inhibitor fluvastatin led to alterations in the assembly of actin microfilaments due to inactivation of RhoA small guanosin triphosphate (GTP) binding protein. Preincubation of BMSC with fluvastatin down-regulated the expression of ICAM1, beta1 integrin and CD105 molecules, reduced B cell binding to BMSC and the subsequent calcium mobilization in B cells. Also, fluvastatin significantly reduced the BMSC-mediated rescue of B cells in the presence of dexamethasone, although it did not function in the absence of corticosteroids. The effects of fluvastatin were mainly due to the lack of isoprenylation of small GTP binding proteins occurring when the lack of mevalonate blocks farnesyl transferase activity and isoprenylation. Indeed, the farnesyl transferase inhibitor manumycin A strongly reduced adhesion molecule expression on BMSC and cell-to cell-interaction. These findings were further supported by the observation that mevalonic acid, the metabolic product of HMG-CoA reductase, could revert morphologic, phenotypic and functional effects of fluvastatin but not those of manumicin A. Conclusions. Altogether these results suggest that the mevalonate biosynthetic pathway can regulate BMSC functions and contribute to the control of B cell survival and potentiate the effects exerted on B cells by dexamethasone. This would further reinforce the idea that fluvastatin may be used for the treatment of diseases where stromal cells present in the microenvironment play a key role in promoting neoplastic growth.

C022

IMMUNE RECONSTITUTION AND THYMIC FUNCTION AFTER REDUCED INTENSITY ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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Introduction. The thymus is fundamental for the generation of T-cell diversity following allografting even though its function declines with age. Non-myeloablative conditionings have extended the eligible age for allografting to 65-70 yrs for patients with hematological cancers. The thymic generation of the TCR diversity occurs through the recombination of gene segments coding for the TCR alpha and beta chains generating by-products defined as signal joint TCR excision circles (sjTRECs). sjTRECs are extrachromosomal DNA fragments, most frequently found in naive T cell, that do not replicate with subsequent cell divisions. *Methods.* sjTRECs evaluation by quantitative PCR and the kinetics of naive and memory T cells by flow-cytometry were used to asses the thymic function. Moreover, TCR repertoire analysis of V-beta families was evaluated, in a subset of patients, by spectratyping up to 1 year post-transplant. Finally, TRECs values were compared with 67 paediatric patients (median age 9, r 1-19 years). Subpopulations studied included peripheral mononuclear cells (PMC), sorted CD4− and CD8− T cells. *Results.* Overall, 55 patients, median age 51 (r 34-64) years, conditioned with low dose TBI (200 cGy), with/without fludarabine, followed by G-CSF mobilised donor peripheral blood stem cell infusion from HLA identical siblings or unrelated donors, were evaluated at different time points: baseline, at 28, 56, 84, 100, 180 days, and at 1, 2, 3, 4, 5 years post-transplant. Naive CD4+CD62L+CD45RA+bright T cells and memory CD4+CD62L+CD45R0+bright T cells showed a gradual increase up to 3 years post-transplant with median values of 882/μL and of 532/ul respectively. Median values of sjTREC copies/100ng DNA in PMC increased 5 fold at 1 yr, 20 fold at 3 yrs and 75 fold at 5 yrs from 0.7 (pre-transplant), whereas median sjTREC copies/100ng DNA from sorted CD4+ cells (purity>95%) increased 7 fold at 1 yr and 15 fold at 5 yrs from 5 at 3 months post-transplant. A significant correlation was demonstrated between TREC values and CD4+CD62L+CD45RA+bright T cells (P<0.001). Importantly, a 60 y/o patient thymectomised 10 years before transplant showed no thymic output and a very poor recovery of the TCR repertoire. However, in the subset of studied patients, though slow, TCR repertoire of V-β families required at least 1 year to clearly become polyclonal. sjTRECs levels in both sorted CD4− and CD8− T cells at 1 year were significantly higher in patients without chronic GVHD (P=0.0135 and 0.007). At 2 years post-transplant, sjTRECs levels were significantly higher in paediatric patients versus adults (median values 240 *vs.* 56.9, P=0.001). *Conclusions.* Detectable thymic function persisted post-transplant in all patients except one who had previously been thymectomised. T cell reconstitution was somewhat slow especially during the first year post-transplant. The thymus may be an important target of chronic GVHD. Age played an important role in thymic output.

C023

CHROMOSOMAL ABNORMALITIES AND IMMUNOPHENOTYPE IN ELDERLY MULTIPLE MYELOMA PATIENTS AT DIAGNOSIS ENTERED IN A PROSPECTIVE RANDOMIZED TRIAL OF VELCADE-MELPHALAN-PREDNISONE AND THALIDOMIDE *VS.* VELCADE-MELPHALAN-PREDNISONE

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Introduction. The relevance of chromosomal abnormalities in the clinical course and response to therapy has been demonstrated in Multiple Myeloma (MM). Moreover, clinical and prognostic significance of plasma cell immunophenotype have convincingly been shown. The aim of the present study was to evaluate chromosomal abnormalities and

immunophenotype in a large series of elderly uniformly treated MM patients to identify prognostic characteristics correlated with response to therapy. *Methods.* Between May, 2005 and January, 2009, 511 patients aged ≥ 65 years were randomized to receive VMPT-VT (N=254) or VMP (N=257). In 336 patients FISH analysis was performed on bone marrow plasma cells (BMPC) purified using anti-CD138-coated magnetic beads. Nuclei from fixed PC were prepared for interphase FISH using standard methods. DNA probes were used to detect 13q14, 12p13, 1p36 and 17p13.1 deletions; t(4;14)(p16;q32), t(14;16)(q32;q23), t(11;14)(q13;q32); 11q23 (MLL) and 1qter gains. Ploidy status was analyzed by chromosome 9,11,15 enumeration. Moreover, the immunological phenotype of BMPC was assessed in 399 patients using quadruple combinations of MoAbs for the detection of CD38, CD138, CD56, CD45, CD40, CD19, CD20, CD52, CD117, and cytoplasmic kappa/lambda ratio. *Results.* Del13 was identified in 53.9% of patients. A significant correlation was observed between del13 and higher levels of LDH (P=0.009) and β2-microglobulin (P=0.03), lower levels of Hb (P=0.002) and female gender (P=0.02). Moreover, BMPC with del13 were more frequently CD45 and CD19 negative (P=0.003 and P=0.006, respectively) and correlated with delp53 and t(4;14)(P=0.005 and P=0.0002, respectively). Delp53 was found in 16.4% of all patients and no significant correlation was found according to clinical and phenotypic parameters. t(4;14) was detected in 18.9% of all patients and a significant correlation was found with t(14;16) (P=0.00003) and lower levels of CD117 (P=0.00001). t(14;16) was present in 9.8% of 173 patients and did correlate with lower expression of CD45 e CD19 (P=0.01 and P=0.02, respectively). t(11;14) was found in 16.2% of all patients and was associated with lower levels of C Reactive Protein (CRP) (P=0.0002) and higher expression of CD20 (P=0.000001). Deletion of 12p13 and 1p36 was identified in 15.6% and 20.6% of 109 and 50 patients, respectively. Gain of 11q23 e 1q21 was found in 57% and 42% of 159 and 50 patients, respectively. Ploidy status was analyzed in 105 patients and 59.1% showed a non-hyperdiploid status. Significantly higher expression of CD20 was identified in patients not achieving CR+VGPR, in both VMPT-VT and VMP groups. Other phenotypic and cytogenetic features significantly correlating with response to therapy are shown in Table 1. Conclusions Our results indicate that: 1- Higher expression of CD20 negatively influences the response to therapy in both arms. 2- Higher expression of CD117, higher frequency of t(11;14) and non-hyperdiploid status correlate with absence of CR+VGPR only in VMPT-VT arm. 3- Higher expression of CD45 and CD19 correlates with absence of CR+VGPR only in VMP arm. The analysis of chromosome 1,11 and 12 abnormalities on a larger series of patients can lead to a more precise prognostic stratification in MM.

Table 1.

	VMPT-VT			*VMP*		
%	*CR+VGPR*	*No CR+VGPR*	*P value*	*CR+VGPR*	*No CR+VGPR*	*P value*
CD20 mean expression	8.8	16.3	0.007	10.3	18.4	0.01
CD117 mean expression	17.4	25.2	0.04			ns
CD45 mean expression			ns	9.2	15.2	0.02
CD19 mean expression			ns	18.6	26.7	0.01
t(11;14)+ patients	8.6	27	0.001			ns
Non-hyperdiploid patients	51.3	81.8	0.02			ns

C024

INDUCTION OF PREFERENTIALLY EXPRESSED ANTIGEN OF MELANOMA (PRAME)-SPECIFIC IMMUNITY BY PRAME-OVERLAPPING PENTADECAPEPTIDES IN PATIENTS WITH HEMATOLOGIC MALIGNANCIES, INCLUDING CHRONIC MYELOGENENOUS LEUKEMIA

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Introduction. Several groups, including ours, provided evidence that the cancer testis antigen PRAME is a potential target for adoptive T-cell or vaccine therapy of many hematologic malignancies and solid tumors. PRAME-specific T cells (PRAME-CTL) can be detected in patients with hematologic malignancies and we have shown that they can be generated and expanded ex-vivo, using artificial antigen presenting cells (aAPC) (K562 cell line genetically modified to express the HLA-A*02, CD80, CD40L) (Quintarelli *et al.*, Blood 2008). So far, four PRAME-derived epitopes have been identified by a proteosome mediated digestion assay. However, this strategy may fail to identify of putative peptides generated *in vivo* rather than the proteosome major cleavage site. *Methods.* We have now adopted an alternative method that uses a peptide-library consisting of 135 synthetic pentadecapeptides, overlapping by 11aa, spanning the entire PRAME protein. We evaluated whether novel HLA-A*02 restricted $CD8^+$ T-cell responses to multiple immunogenic epitopes can be identified and used to generate polyclonal PRAME-CTL lines from patients with hematologic malignancies. $CD8^+$ T lymphocytes from 21 HLA-A*02 healthy donors and 7 patients with CML were primed with autologous dendritic cells loaded with the entire PRAME-peptide library, and then expanded by weekly re-stimulation with peptide loaded aAPC. *Results.* Using this approach we consistently generated PRAME-CTLs in 19/21 healthy donors (457±412 SFC/105 cells as assessed by IFNg Elispot assay) and all 7 CML patients (936±136 SFC/105). These PRAME-CTLs were also able to target autologous CML cells (57±6 IFNγ SFC/105 when cultured with PRAME+ CML blasts), demonstrating that the same peptides were presented physiologically. A Cr51 release assay confirmed that the PRAME-reactive T cells were cytotoxic, lysing autologous-PHA blasts loaded with the peptides derived from the PRAME-library (63±14% at a 20:1 E: T ratio), but not with irrelevant peptides. *Conclusions.* Using pentadecapeptides sub-pools, we found that the responses of our expanded PRAME-CTLs were polyclonal, since they consistently released IFNg in response to 1 to 6 pentadecapeptides pools (59% were specific for 1 or 2 pools, 25% to 3 pools, and 16% to 6 pools). In conclusion, this novel approach allowed us to identify several new immunogenic peptides that should facilitate expansion of polyclonal PRAME-CTLs for adoptive transfer or after vaccine administration to patients with PRAME+ hematological malignancy.

Erythropoiesis, Megakaryocytopoiesis and Thrombosis

C025

KINETICS OF COMPLEMENT PROTEIN 3 (C3) BINDING TO PNH (GPI-NEGATIVE) ERYTHROCYTES UNDER COMPLEMENT BLOCKADE BY ECULIZUMAB

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Introduction. C5 blockade by the monoclonal antibody eculizumab prevents intravascular hemolysis and produces a significant clinical improvement in most of PNH patients. However, in almost all PNH patients on eculizumab a proportion of GPI-negative (GPI^-) red blood cells (RBC) become bound with C3 ($C3^+$). In most cases this phenomenon has relatively little clinical relevance; however, in some patients it may limit the clinical benefit from eculizumab because $C3^+$ RBC are possible target of macrophage phagocytosis. *Methods.* It has been set up an *in vitro* model to investigate the C3 binding to RBCs of PNH patients on eculizumab: RBCs from untreated PNH patients are incubated with AB0-compatible sera from either healthy subjects or PNH patients on eculizumab, and then the complement alternative pathway is activated by either mild acidification or spontaneous complement activation. C3 binding was assessed at serial time points by flow cytometry. *Results.* No evidence of C3 binding has been observed on normal and GPI-neg RBCs in untreated PNH patients, and when RBCs from these patients (n=11) were incubated with acidified sera from healthy subjects. At variance, when RBCs from untreated PNH patients were incubated with acidified sera from patients on eculizumab, a population of $C3^+$ GPI^- RBCs has been observed in every case. Just as in PNH patients on eculizumab, this population coexists with a population of GPI^- RBCs without C3 and with a population of normal RBCs. The size of $C3^+$ GPI-neg RBCs population increased with time from 6±2% at 1 hour to 64±15% at 24 hours. The addition of 1.25 mM $MgCl_2$ resulted in a further increase of the proportion of $C3^+$ GPI^- RBCs, in some cases up to nearly 100%. Serum acidification *in vitro* produces discrete hemolysis of GPI-neg RBCs (20-70%), whereas intravascular hemolysis is not observed *in vivo* in PNH patients on eculizumab. Thus, in order to reproduce more physiological conditions, the spontaneous complement activation has been investigated: under these conditions, there was only a slight hemolysis of GPI-neg RBCs in sera from patients on eculizumab (0-10% at 5 days); however, even under these very mild conditions of complement activation, C3 binding on GPI-neg RBCs was still substantial ranging from 20±18% at 3 days to 29±23% at 5 days. *Conclusions.* The phenomenon of C3 binding on GPI^- RBCs of PNH patients on eculizumab has been fully reproduced *in vitro*. In fact, *in vitro* C5 blockade by eculizumab results, just as *in vivo* (PNH patients on eculizumab), in the generation of a discrete population of $C3^+$ GPI^- RBCs that always coexist with a population of GPI^- RBCs without C3 binding. The kinetics of C3 binding, that has been possible to study *in vitro*, strongly suggests that the two populations of GPI^- RBCs (with and without C3 binding), do not result from intrinsically different properties of RBCs, but rather from how long they have been exposed to activated complement.

C026

ISOLATED ERYTHROCYTOSIS: STUDY OF 67 PATIENTS AND IDENTIFICATION OF THREE NOVEL MUTATIONS IN THE PROLYL HYDROXYLASE DOMAIN PROTEIN 2 GENE

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Introduction. Patients with persistent erythrocytosis, secondary to any apparent cause of comorbidity, not fitting a polycythemia vera diagnosis according to WHO 2008, are characterized by isolated increase of red cell mass and have low to increased erythropoietin (Epo) levels. These cases are referred to as idiopathic/isolated erythrocytosis (IE) and may have different genetic causes. The oxygen sensing pathway (OSP) modulates Epo expression. In normal cells, intracellular oxygen concentrations are sensed by prolyl hydroxylase domain (PHD)-containing proteins. PHD2 isozyme (Figure 1A) has a key role in tagging hypoxia-inducible factor (HIF)-α subunits for polyubiquitination and proteasomal degradation. Erythrocytosis associated-PHD2 mutations reduce hydroxylation of HIF-α. *Aim.* 67 consecutive patients with IE, either sporadic (55 cases) or familial (12 cases), were investigated to identify the underlying genetic lesions. *Methods*. Genomic DNA was extracted from peripheral blood samples at diagnosis. PCR of the PHD2 coding region and sequencing analyses were performed under standard conditions. The three VHL exons, HIF2A-exon 12, JAK2 (exon 12 and V617F mutations) were also analyzed. To confirm the identity of the newly identified mutations, allele-specific PCR were carried out. *Results.* Three novel PHD2 heterozygous mutations were identified (Figure 1C) - two in two different families and one sporadic - in the catalytic domain of the protein. N203K substitution occurred in a male aged 80 years with familiar erythrocytosis. The patient resulted positive for JAK2(547insL+ I540F547dup8) mutation, and his Epo level was low. One of his niece and her respective son were affected by mild erythrocytosis but they did not carry PHD2 mutation and they were both JAK2 wild-types. The same N203K PHD2 substitution was detected on DNA extracted from four patient's Epo-independent endogenous erythroid colonies. N203 flanks the M202 residue in the helice 1 (Figure 1B), previously found to be mutated in erythrocytosis, and introduces a positive charged amino acid with a long side chain in place of a polar asparagine. K291I occurred in a 29-year-old male - with low Epo level - that referred a familial history of erythrocytosis observed in his father, uncle and first cousin, too. K291I lies at the beginning of the strand 4 of the hydroxylase domain (Figure 1B) and, differently from the other PHD2 mutations, this novel one involves a codon that was not conserved in other species, nor in the human isoforms PHD1 and PHD3. The last PHD2 mutation, K423E, lies three residues from the end of the enzyme. It was found in a case of sporadic erythrocytosis, a male aged 60 years. Mutation analyses of the catalytic domains of the PHDs suggested that removal of the C-terminus of the enzyme promotes uncoupled turnover of substrate. The germ line origin of this genetic lesion was assessed by its identification on DNA obtained from the proband's epithelial cells. *Conclusions.* The study of IE can yield additional insight into the human OSP that regulate Epo. Identification of the disease-causing genes will enable better classification of familial and acquired IE as, to date, in many cases the molecular basis of this disorder remain unknown. The coexistence of PHD2 and JAK2 mutations in one of the studied patient and the contrasting serum 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 functional consequences on the substrate binding affinity and the differential effect of these new mutations on HIF-α hydroxylation.

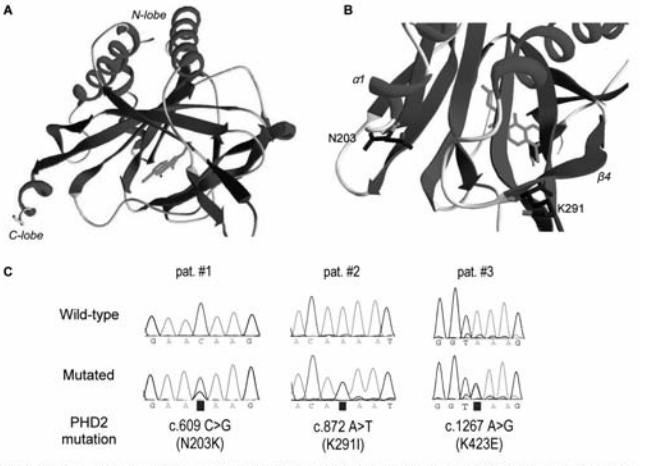

Figure 1. (**A**) Three dimensional ribbon representation of the catalytic domain of the human PHD2. The structure was generated using Swiss-PDBViewer v3.7 software from Protein Data Bank coordinates of the X-ray structure, 2G1M. Compound A (a 2-oxoglutarate competitive inhibitor) is shown near the Fe(II) sphere. (**B**) Location of the mutated residues is highlighted. The C-terminal K423 is not visible as it falls outside the available PHD2 X-ray maps (residues from 188 to 403). (**C**) 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.

C027

ENOXAPARIN, ASPIRIN, OR WARFARIN FOR THROMBOPROPHILAXIS IN NEWLY DIAGNOSED MYELOMA PATIENTS RECEIVING THALIDOMIDE: A RANDOMIZED CONTROLLED TRIAL

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Background. Thromboembolism (TE) is a common adverse events in newly diagnosed myeloma (MM) patients who receive thalidomide-based regimens. Anticoagulant prophylaxis is recommended, but controversies exist on the best thromboprophylactic regimen to adopt. *Aims.* To evaluated the safety and the efficacy of low-dose aspirin (ASA) or low-fixed dose warfarin (WAR) or low-molecular weight heparin (LMWH) as anticoagulant prophylaxis. Primary end-points were incidence of TE, acute cardiovascular events and sudden death. *Methods.* 991 newly diagnosed MM patients were assessed for eligibility. Younger patients were randomized to receive 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) as induction before autologous transplant; elderly patients were randomized to receive VMPT-VT (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 for 9 courses followed by continuous therapy with bortezomib 1.3 mg/m^2 every 15 days and thalidomide 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 for 9 courses without any further therapy). 667 patients receiving thalidomide regimens were enrolled in the sub-study, 659 received at least one dose of the study treatment and were included in the efficacy and safety analyses (220 in the ASA [aspirin 100 mg/d], 220 in the WAR [warfarin 1.25 mg/d] and 219 in the LMWH group [enoxaparin 40 mg/d]). The prophylaxis was administered during the induction therapy in younger patients and for the first 6 cycles in elderly patients (treatment period). Patients treated with VMP did not receive any prophylaxis. *Results.* During the treatment period, any grade 3-4 TE had occurred in 11 patients (5%) in the ASA group, 14 (6%) in the WAR group and 5 (2%) in the LMWH group. The absolute differences in the ASA group and in the WAR group as compared with the LMWH group were 2.71 (95% CI -0.88-6.73, P=0.13) and 4.08 (95% CI 0.30-8.39, P=0.04), respectively. The reduction in the incidence of any grade 3-4 TE in the ASA group and in the LMWH group was maintained during the follow-up period. Multivariate analysis confirmed these results (HR 1.76, P=0.24 for the ASA group, HR 2.69, P=0.03 for the WAR group in comparison with the LMWH group) and detected no significant interaction between treatment groups and risk factors. There were no significant differences among all groups in the incidence of cardiovascular events or sudden deaths during the treatment or follow-up period. The incidence of major bleedings (only gastrointestinal tract bleeding recorded) was 0.5% in the ASA group and 0% in the WAR group and in the LMWH group. The incidence of minor bleeding (gastrointestinal, urinary tract, skin, eye and ear) was 2% in ASA, 0.5% WAR and 1% in LMWH group. *Conclusion.* In MM patients with standard risk of thrombosis who received thalidomide regimens, prophylactic treatment with LMWH safely reduced the risk of thrombosis, ASA could be considered a valid cost-effective alternative.

C028

MOLECULAR CHARACTERIZATION OF AN ITALIAN COHORT OF 10 PATIENTS WITH TYPE 3 VON WILLEBRAND DISEASE: EXPRESSION STUDIES OF 3 MISSENSE MUTATIONS

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Introduction. Type 3 von Willebrand disease (VWD3) is a severe autosomal recessive inherited bleeding disorder caused by a virtually complete absence of von Willebrand Factor (VWF). Usually patients are homozygous or compound heterozygous for null alleles due to nonsense mutations, small insertions/deletions, splice site defects and, more rarely, large gene deletions. Nevertheless, several missense mutations have been reported. To better understand the molecular defects of VWD3, we have genetically analyzed 10 patients previously diagnosed with VWD3. *Methods.* Molecular defects of VWF were evaluated by DNA direct sequencing, High Resolution Melting (HRM) analysis and duplex PCR. Moreover, we performed *in vitro* expression studies of the missense mutations identified, to confirm their link with the disease. Mutant and wild-type (WT) expression vectors were used for transient transfection, alone and together (hybrids), in COS-7 cells. Conditioned media and cell lysates were collected and recombinant VWFs (rVWFs) were quantified by VWF:Ag and evaluated for multimeric structure. *Results.* Twenty-six exons were analyzed by direct sequencing whereas, the remaining twenty-five were evaluated by both HRM and sequencing analysis. Direct sequencing (Table) revealed ten mutations (shown in italics); HRM analysis detected two defects (shown in bold) and one large deletion was identified by duplex PCR. Nine out of thirteen identified mutations were novel (*), which are 5% of VWD3 previously reported mutations. Four patients were found to carry mutations in the homozygous state, but only two of them were known to be born from a consanguineous marriage. The large deletion involving exons 1-3, recently reported as the most frequent in the Hungarian population, was found in two patients. Expression studies of missense mutations (C2184S, C2212R and C2325S) showed a strongly reduced secretion of mutant proteins, with only dimers being visualized using multimer analysis. Hybrids rVWFs showed mildly reduced secretion and a full set of multimers. Higher VWF:Ag levels were detected in cell lysates of mutants and hybrids rVWFs in comparison to the WT.*Conclusions.* HRM analysis, used for the first time in the molecular diagnosis of VWD3, shows a reduced ability in detecting mutations on VWF. The presence of many polymorphic sites in the VWF coding region has, so far, limited the use of this technique to twenty-five exons of the gene only. The three detected missense mutations were confirmed to be disease related by expression studies. All of them involve a cysteine residue, important in the correct folding/processing/secretion of the neo-synthesized VWF. Further analysis should be performed on the two partially characterized patients to evaluate intronic mutations or heterozygous large deletions, whereas, mRNA analysis should be carried out to confirm the candidate splice site mutations identified.

Table.

Pz	Mutation	Mutation effect	Exon intron involved
1	**2157delA**/ *7729+7C>T*	**D720TfsX21**/*Splice site*	16/IVS45
2	*6651G>C*/?*	C2184S*/?	37/?
3	*4576C>T**/**6973T>A***	*Q1526X**/**C2325S***	28/40
4	*6634T>C*/6634T>C**	*C2212R*/C2212R**	38/38
5	del ex 1-3/*3940delG**	Large deletion/*V1314SfsX33**	1,2,3/28
6	*8155+1G>T*/8155+1G>T**	*Splice site*/Splice site**	IVS50/IVS50
7	*4645G>T*/7344C>A**	*E1549X*/C2448X**	28/43
8	*2269delCT/2269delCT*	*L757VfsX22/L757VfsX22*	17/17
9	*658-2 A>G*/658-2 A>G**	*Splice site*/Splice site**	IVS6/IVS6
10	del ex 1-3/?	Large deletion/?	1,2,3/?

C029

THROMBOPROPHYLAXIS FOR NEWLY DIAGNOSED MYELOMA PATIENTS TREATED WITH LENALIDOMIDE-BASED REGIMENS: AN INTERIM ANALYSIS OF A PROSPECTIVE, RANDOMIZED STUDY OF ENOXAPARIN VS ASPIRIN

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Introduction. Preliminary studies with MM patients receiving the association of lenalidomide (R) and dexamethasone have shown an improvement in the incidence of thrombosis and a calculeted odd ratio of about 3.5. A prospective, multicenter phase III trial has been conducted to treat newly diagnosed patients by using lenalidomide+low-dose dexamethasone (Rd) as induction; patients were then randomized to receive consolidation with lenalidomide+melphalan+prednisone (MPR) or high dose melphalan (MEL200). The objective of this sub-study was to evaluate the safety and the efficacy of low-molecular weight heparin (LMWH) or low-dose aspirin (ASA) as anticoagulant prophylaxis during Rd induction and MPR consolidation. End-points were incidence of venous thromboembolism (VTE), acute cardiovascular events, sudden death, major and minor bleeding. *Methods.* in the randomized trial RV-MM-PI-209, 402 patients with newly diagnosed MM were enrolled. The treatment consisted of induction: four 28 day cycles of lenalidomide (25 mg days 1-21) and low-dose dexamethasone (40 mg days 1,8,15,22) (Rd); consolidation: patients were randomized to receive six 28-day cycles of melphalan (0.18 mg/kg days 1-4), prednisone (2 mg/kg days 1-4) and lenalidomide (10 mg days 1-21) (MPR, N=202) or tandem melphalan 200 mg/m^2 with stem-cell support (MEL200, N=200). Eligible patients were randomly assigned to receive LMWH (Enoxaparin 40 mg/d, N=166) or ASA (Aspirin 100 mg/d, N=176) during the induction and consolidation therapy in the MPR group; because of indication for anticoagulant/antiplatelet therapy or high-risk of bleeding, 60 patients were excluded from this sub-study.

Table 1. Incidence of thrombotic events, acute cardiovascular events, sudden death and bleeding events.

Events	*LMWH (N =152)*	*ASA (N =165)*
Grade 3-4 thrombotic events, (%)	1	2
Acute cardiovascular events, (%)	0	0
Sudden deaths, (%)	0	0
Major and minor bleeding, (%)	1	0
Total events, (%)	2	2

Results. in both groups, patient characteristics and distribution of major risk factors were similar. 370 patients during Rd induction and 151 during consolidaiton were evaluable at the time of the present analysis. The overall incidence of any grade 3-4 thrombotic events during induction was 1% in the LMWH group, 2.4% in the ASA group (p not significant). VTE, mostly of the lower limbs, were equally distributed in the two groups (1%; p not significant), while pulmonary embolism was observed only in the ASA group (2%; p not significant). In patients who received LMWH or ASA, the median time to onset of thrombotic events was 2.1 and 1 months, respectively. No acute cardiovascular events and in the LMWH group were observed, and only 1% of minor bleeding was detected. In the MPR group, no thrombotic events were seen during the consolidation phase and only one central venous catheter thrombosis was observed in the MEL200 group. *Conclusions.* in all groups, the overall incidence of thrombotic events was less than 5%, and this further supports the safety of low dose dexamethasone in association with Lenalidomide. For patients treated with Lenalidomide, LMWH and ASA are likely to be effective thromboprophylactic regimens.

C030

B AND T CELL INTERACTIONS IN GRAFT VERSUS HOST DISEASE

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Introduction. Recent literature is investigating the role of B cells in the setting of graft versus host disease (GVHD), although no exhaustive mechanistic explanation has been provided. We described a new interaction between autologous CD B cells and activated CD8 cytotoxic T lymphocytes (CTLs) in preliminary experiments on normal donors (Deola *et al*. 2008). *In vitro* antigen-stimulated CTLs serially engage autologous B lymphocytes independently from antigen presentation. CTL and B cells form stabilized couplets. The effect of coupling enhances CTL survival and proliferation, and stimulates cytokine release by B cells, including a select pattern of T-cell attracting chemokines: Mig, IP-10 and I-TAC which interact with the same receptor CXCR3, and are involved in inflammation and GVHD pathobiology. Studies on leukocyte migration and GVHD describe CXCR3 as pivotal for migration of T cells to the parenchymal GVHD target organs.

Table 1.

Disease	*Source of bmt*	*Mo. after BMT*	*CD20$^+$*	*Couplets*	*Histol OGY (grading)*	*Involved organ*
T-NHL	MUD PBSC	13,3	++	+	AGVHD (II)	Skin
AML	MUD PBSC	2,8	+	+	AGVHD (II)	GI
AML	MUD BM	3,7	NEG	NEG	AGVHD (III)	Skin
ALL	MRD BM	6,2	++	+	AGVHD (III)	Skin
CLL	MRD PBSC	2,7	NEG	NEG	AGVHD (III)	Skin
ALL	MRD BM	2,8	+	+	AGVHD (III)	Skin
AML	MRD BM	6,9	+++	NEG	NO GVHD (*)	GI
AML	MRD BM	16,8	NEG	NEG	NO GVHD (*)	Skin
MM	MRD PBSC	2,8	+++	NEG	NO GVHD (*)	GI
AML	MRD PBSC	15,0	NEG	NEG	NO GVHD (**)	None
AML	MRD PBSC	4,9	NEG	NEG	NO GVHD (**)	None
AML	MRD PBSC	4,1	NEG	NEG	NO GVHD (**)	None

No 0 Cells CD20/Slice; +, >1 <=3 Cells CD20/Slice; ++, >3 <=10 Cells CD20/Slice; +++, >10 Cells CD20/Slice reactive pattern; (*) Inflammation; (**) Control biopsies.

Methods. We analyzed the presence of B and T cells in 6 cases of acute GVHD (grade 2 or more), including 5 skin, and 1 gastrointestinal biopsies, obtained at 3-13 months after BMT (Table 1). Six biopsies without features of GVHD were also included. Biopsy specimens were double-immunostained with monoclonal antibodies for CD8 and CD20. A simple quantitative scoring (no, up to 3 cells, from 4 to 10 cells, more than 10 cells) was used to evaluate the results of the immunohistochemical stainings. *Results.* Four out of 6 GVHD$^+$ biopsies showed a limited number (up to 10) of B cells, at least focally coupled with CD8 T cells. Two further GVHD$^+$ were negative for CD20. Four out of 6 GVHD$^-$ were CD20$^-$. Two further GVHD$^-$ samples showed an evident CD20$^+$ and CD8$^+$ infiltration with an aspecific inflammatory pattern, and no evidence of couplets was present. Noteworthy, both patients were affected by inflammation involving the analyzed organ. None out of 3 control biopsies, taken from transplanted patients without signs of GVHD

at 4-15 months after BMT resulted positive for B cells. *Discussion.* We suggest that the interaction between CTL and B cells could play a role in GVHD and inflammation target organs by locally amplifying the inflammatory signal. B cells contribution could act by attracting additional immune cells, and supporting CTL cells survival and expansion. Further experiments are undergoing to confirm these preliminary results on a larger sample size, to distinguish the source of B cells and the cytokine patterns released in GVHD and inflammation settings.

C031

SCF TREATMENT DOWNREGULATES FPN1 AND GDF15 MRNA EXPRESSION IN HUMAN β-THALASSEMIC ERYTHROID CELLS *IN VITRO*

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Introduction. Progressive iron overload is the most salient and ultimately fatal complication of β-thalassemia. However, little is known about the relationship among ineffective erythropoiesis, the role of iron-regulatory genes, and tissue iron distribution in β-thalassemia. Recent observations has suggested that the cytokine growth differentiation factor-15(GDF15), secreted by erythroid precursors and highly espressed in serum from thalassemia patients, might contribute to iron overload in β-thalassemia by inhibiting hepcidin expression. A potential for therapeutic intervention in β-thalassemia by means of manipulating iron metabolism may hold great promise. We previously demonstrated that, in β-thalassemia, the Stem Cell Factor(SCF) induced an *in vitro* expansion of effective erythropoiesis and a reactivation of -globin synthesis up to fetal levels, paving the way to its potential use in the therapeutic treatment of this disease. Hence, we started *in vitro* studies in order to evaluate a potential SCF effect on FPN1 and GDF15 expression in human normal and β-thalassemic $CD34^+$ hematopoietic progenitors grown in erythroid unilineage culture. *Methods.* We examined two groups of β-thalassemia intermedia(βTI) and major(βTM) patients, classified at clinical and laboratory level in terms of genotype, haematologic values, splenectomy and dependence on red blood cell transfusions. The $CD34^+$ hematopoietic progenitor cells, isolated from peripheral blood of thalassemic patients, were grown in unilineage erythroid cultures supplemented or not with SCF and analyzed for FPN1 and GDF15 genes expression at different days of differentiation by quantitative real-time PCR. FPN1 and GDF15 protein levels were analyzed by Western blot and ELISA, respectively. *Results.* FPN1 and GDF15 mRNA levels on human normal PB $CD34^+$ erythroid cell cultures supplemented or not with SCF were not modulated by SCF treatment. In contrast, on thalassemic $CD34^+$ erythroid cultures we observed a strong up-regulation of FPN1 and GDF15 mRNA levels both in β-TI and β-TM patients, particularly in the final stages of erythroid maturation. The most surprising results were observed after treatment with SCF: both in β-TI and β-TM we observed a down-modulation of FPN1 and GDF15 mRNA levels reaching values comparable to normal controls especially in β-TM. Western blot analysis of FPN1 protein levels confirmed these observations. Also the concentrations of GDF15 in the culture medium or human sera confirmed these results. *Conclusions.* Our results suggest a role of FPN1 and GDF15 in β-thalassemia iron overload. More important, SCF inducing effective erythropoiesis, reactivation of fetal hemoglobin and down-regulation of FPN1 and GDF15 expression levels, may be considered as a potential therapeutic agent for this disease. In conclusion we hypothesized that the SCF treatment induced a link between -globin reactivation and iron genes expression downmodulating GDF15 overexpression.

C032

IMMUNE THROMBOCYTOPENIA: A NEW FUNCTIONAL CHARACTERIZATION BASED ON ALTERED CROSS-TALK BETWEEN DENDRITIC CELLS AND REGULATORY T CELLS

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Introduction. $CD4^+CD25^+$ regulatory T cells (Tregs) are critical in maintaining self-tolerance and preventing organ-specific autoimmune diseases. However, the role of Tregs in the pathogenesis of Immune Thrombocytopenia (ITP) has not yet been clarified in depth. In the present study we further investigated the bidirectional interactions between dendritic cells (DCs) and Tregs. Specifically, we studied whether, in ITP patients: 1) the frequency and the suppressive function of Tregs is impaired; 2) DCs maturation is modulated by Tregs from ITP patients as compared with healthy donors; 3) the mechanism of Tregs generation is altered. Methods. Forty adult ITP patients, newly diagnosed or with persistent or chronic ITP, off therapy by at least two months, were studied. Circulating and *in vitro* generated Tregs were identified by flow cytometry. Allogeneic Mixed Leukocyte Reaction (MLR) was performed to test the suppressive activity of Tregs. To analyze the *in vitro* ability of $CD4^+CD25^+$ T cells to inhibit DCs maturation, normal immature CD14-derived DCs were cultured alone and with allogeneic $CD4^+CD25^+$ T cells from healthy subjects or ITP patients in the presence of Lipopolysaccaryde. CD80 and CD86 expression on DCs was then tested at flow cytometry. To evaluate the *in vitro* conversion of non- Tregs into Tregs, $CD4^+CD25^-$ T cells were cultured alone and with autologous mature CD14-derived DCs from ITP patients and healthy subjects. The percentages of $CD4^+CD25^+FoxP3^+$ Tregs were quantitated at flow cytometry. mRNA IDO expression of immature and mature CD14-derived DCs was evaluated by Real-time RT-PCR. Results. We found that in ITP Tregs show lower ability to suppress T cell proliferation and to inhibit DCs maturation because they do not affect the expression of the costimulatory molecules CD80 and CD86. This is due to altered cytokine microenvinronment and to the reduced number of circulating Tregs. We found that the absolute number of Tregs was significantly decreased in ITP patients in comparison to healthy subjects ($CD4^+CD25^{high}Foxp3^+$ T cells (5.5±4.3 *vs.* 11.6±6.9 cells/μL; p below 0.01) and $CD4^+CD25^{high}CD127^{low-}$ T cells (50.9±27.3 *vs.* 80.5±37.7 cells/μL; P<0.02)). In addition, we document that the low number of circulating Tregs is in part due to the reduced ability of mature DCs to convert non-Treg cells ($CD4^+CD25^-$) into Tregs ($CD4^+CD25^+FoxP3^+$ Tregs). This finding was associated with reduced expression of the immunomodulatory enzyme Indoleamine 2,3-dioxygenase (IDO) in mature DCs. Conclusions. In conclusion, taken together our data demonstrate that in ITP the cross-talk between Tregs and DCs is impaired and plays a pathogenetic role. As a consequence, we found the generation of more immunogenic DCs and defective Tregs. The understanding of novel regulatory mechanisms in ITP is essential for the development of therapeutic strategies.

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Multiple Myeloma

C033

MET MRNA VALUE PREDICTS OUTCOME IN MULTIPLE MYELOMA PATIENTS

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Background. Multiple myeloma (MM) plasma cells growth and survival is sustained by several stimulating factors produced by bone marrow (BM) microenvironment. Hepatocyte Growth Factor (HGF) is released by surrounding cells and directly stimulates plasma cells via its ligand, the TK-receptor MET. In MM cell lines, the inhibition of this pathway causes growth arrest and cell death. Few information are available on the role of MET in MM patients. *Aim.* to investigate the role of MET mRNA expression as predictor of response and as prognostic marker in MM. *Patients and methods.* eighty four samples of $CD138^+$ cells obtained from newly diagnosed MM patients have been evaluated for MET mRNA expression. HGF serum levels have been investigated at diagnosis on the same patients too. Fifty one patients received the PAD-MEL100-LP-L regimen (Palumbo A, JCO 2010) and 33 received 9 VMP courses (Palumbo A, 2009 ASH Meeting, abs 128). MET mRNA expression has been evaluated using a quantitative Real-Time PCR with a relative quantification based on DDCt approach. JUM2 cell line was utilized as calibrator and Gus as housekeeping gene. ROC analysis have been employed to reach the cut-off value of MET mRNA on samples of patients treated with PAD-MEL100-LP-L schedule (learning series) and then validated on samples of patients treated with VMP (validation series). ELISA assay has been employed to determine HGF serum value. *Results.* Eighty four patients (39 female/45 male) with a median age of 69 yr (range 46-86) have been evaluated. No differences in baseline albumin, β2-microglobulin, BM plasma cell infiltration and cytogenetic abnormalities have been observed between patients with high and low MET mRNA levels. Considering PAD-MEL100-LP-L patients, MET mRNA expression was significantly higher in those achieving partial response (PR) or less, compared to patients reaching at least very good partial response (VGPR). In patients enrolled in the PAD-MEL100-LP-L trial this difference was observed after PAD-induction (median MET value 94.03 range 9.7-586.1 *vs.* median 37.92 range 1.1-170.0, P=0.001) and was maintained after autologous bone marrow transplantation (ABMT) (median 148.6 range 27.1-586.1 *vs.* median 65.8 range 11-466.3, P=0.007). On the whole population of 84 patients, after a median follow-up of 27.7 months, the 2-year PFS in the low and in the high MET mRNA groups was 92% and 62% respectively (P=0.0001). The 2-year OS was 96.6% and 83,8% respectively (P=0.0460). The prognostic value of MET mRNA expression resulted stronger than those of β2 microglobulin or cytogenetic abnormalities. HGF serum values resulted similar in both groups and did not predict PFS and OS. *Conclusions.* 1) high MET mRNA expression identified a group of patients with suboptimal response and inferior PFS and OS in both ABMT- and non ABMT-based regimen; 2) high MET mRNA expression seems to be a hallmark of more aggressive disease thus MET can be a target for anti myeloma therapy.

C034

SEROTONIN INDUCES OSTEOLYTIC LESIONS IN PATIENTS WITH MULTIPLE MYELOMA

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Background. The biologic mechanisms involved in the pathogenesis of multiple myeloma (MM)-induced osteolytic bone disease are poorly understood. Physiological interactions between the serotoninergic and skeletal systems have been implicated by clinical observations. Brainstem-derived serotonin positively regulates bone mass following binding to 5-HT2C receptors on ventromedial hypothalamic neurons. This is opposed by platelet-derived serotonin that induces bone lysis and osteoclast activation. Circulating monoamine serotonin [5-hydroxytryptamine (5- HT)] is principally stored in platelet-dense granules. It has been shown that aggregated immunoglobulins derived from all the IgG subclasses, isolated from healthy controls or myeloma patients, induced platelet granule release in the absence of antigen or particulate matter, in a dose dependent manner. Immunoglobulins have been shown to induce platelet release when participating in immune reactions as antigen-antibody complexes. While MM patients with evidence of osteolytic lesions have been shown to have elevated concentrations of serum tryptophan and serotonin the consequences of this elevation have not been previously studied in the bone itself. *Aims.* Test the hypothesis that increased circulating-serotonin levels may alter the osteoblast/osteoclast differentiation rate in the bone marrow microenvironment to promote MM osteolytic lesions. Correlate the serotonin associated bone remodeling signal pathway proteins in human MM bone marrow with the presence of osteolytic disease. *Methods.* We retrospectively measured bone remodeling signal pathway perturbations in 15 bone marrow core biopsies from patients diagnosed with MM, at different clinical stages, and correlated this with the presence of osteolytic bone disease. Our multiplexed protein kinase signal pathway mapping technology, reverse phase protein microarrays (RPMA), provides quantitative information regarding post-translational modifications (e.g. phosphorylation, cleavage, acetylation) and/or total cell signaling kinase levels. We measured circulating serotonin levels by ELISA in a pilot set of MM patients (n=20) to confirm observations obtained by RPMA. *Results.* Bone marrow core biopsies exhibited significant elevation of Serotonin, RANK, MMP-11, TNFα, TNF-R1, and Ezrin Tyr353 by RPMA in patients with osteolytic lesions compared to patients without evidence of bone disease. Cytokines IL-1β, IL-6, and IL-10 were also significantly elevated in the bone marrow cores of patients with bone disease. Free circulating serotonin in MM sera was elevated compared to healthy controls. *Conclusions.* These data suggest that the 5-HT system plays an important role in serotonin mediated signaling cascades related to the pathogenesis of MM-induced bone disease. This insight could provide strategies for reducing osteolysis with agents that regulate serotonin, either alone or in combination with other molecular targeted inhibitors.

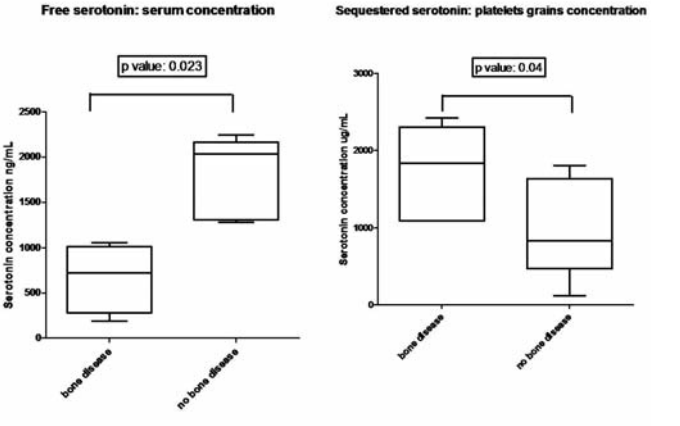

Figure.

C035

FIRST LINE THERAPY WITH LENALIDOMIDE AND DEXAMETHASONE IN PRIMARY PLASMA CELL LEUKEMIA

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Introduction. Primary Plasma Cell Leukemia (PPCL) is an aggressive, rare variant of multiple myeloma, which accounts approximately for 2% to 4% of all myeloma diagnoses. The prognosis of PPCL patients is usually poor, with less than half of patients responding to conventional chemotherapy and a median survival of 7 months. Even by using autologous or allogeneic transplant procedures, survival generally does not exceed three years. Bortezomib has recently provided some promising results in this setting, but, given all the above, new treatments for PPCL are greatly awaited. Lenalidomide is a new immunomodulating agent with proven efficacy in multiple myeloma, especially when associated with dexamethasone or other drugs. There are, indeed, some sporadic case reports of PPCL patients treated with lenalidomide as salvage therapy, but no data are currently available on the use of this drug as first line treatment in this disease. *Methods.* On March, 2009, we started an open label, multicenter, exploratory, single arm, two-stage study (CROB0108/1 -- RV-PCL-PI-350, EudraCT No. 2008-003246-28), aiming to evaluate safety and antitumor activity of the lenalidomide/low dose dexamethasone combination (Rd), as initial therapy in patients with PPCL. The primary endpoint was early response rate according to International Uniform Criteria. The secondary endpoints were TTP, PFS, OS, percentage of eligible PPCL patients able to collect peripheral blood stem cells and to undergo autologous or allogeneic stem cells transplantation after Rd, and safety. According to this study protocol, all eligible, newly diagnosed adult patients with PPCL receive Lenalidomide at a dose of 25 mg daily for 21 days every 28 days. Oral dexamethasone is administered at a dose of 40 mg daily on days 1, 8, 15, and 22 for each 28-day cycle. After 4 cycles, patients who achieve at least PR and not eligible for autologous or allogeneic stem cell transplantation, continue with Rd until clinically appropriate (disease progression, unacceptable toxicity, patient's decision to leave the protocol). In these patients, a maintenance dose of lenalidomide alone equal to 10 mg/die days 1-21 every month is considered after at least 8 full-dose Rd cycles. Patients responding after 4 Rd cycles and eligible for transplant procedures, proceed according to single Centre transplant policy. Patients not responding after 4 cycles or progressing under Rd treatment are considered off-study. Appropriate contraception methods and anti-thrombotic prophylaxis are planned. *Results.* So far, fourteen out of sixteen enrolled patients (6 male, 8 female, mean age 65 years, range 45-81) are evaluable for response. At baseline, circulating plasma cells ranged from 655 to 34.000×10^9/L. Seven patients had a moderate degree of renal failure (serum creatinine levels comprised between 1.5-2.8 mg/dL). Nine patients had increased LDH levels, five had extramedullary disease. Eleven patients had Hb levels below 10 g/dl, three patients had less than 50×10^9/L platelets. Among 11 patients so far analyzed for cytogenetic abnormalities, nine had del13 (two in combination with del17 and t(14;16), two with t(14;16), one with del17 and t(11;14), one with del17, one with t(4;14). One patient evidenced del17 alone. After a median of 4 Rd cycles (range 1-8), 1 CR, 2 nCR, 4 VGPR, and 4 PR were achieved (overall response rate 78.5%), with disappearance or near complete clearance of circulating plasma cells in all cases. Three of the responding patients underwent peripheral blood stem cell collection followed by autologous transplantation after Rd therapy. Three patients did not respond: one of them died during the first cycle of treatment because of progressive disease. The other 2 patients developed extramedullary disease under Rd therapy, despite the complete absence of circulating plasma cells. One of responders died in PR, due to causes unrelated to PPCL or treatment. The most relevant grade 3-4 hematological toxicities were neutropenia (four patients), anemia (three patients) and thrombocytopenia (three patients). Two patients experienced pneumonia, two relevant fatigue, one an intestinal perforation due to fecalith, one a combination of dermatological and neurological side effects. After a median follow-up of 9 months, 12 patients are alive (85.7%), 9 of whom with responsive disease. *Conclusions.* These findings suggest that Rd may be a promising initial therapy for PPCL patients, which can rapidly control the disease, allowing following single patient-adapted therapeutic strategies. An update of this study, planned to be concluded after inclusion of 22 patients, will be presented at the Meeting.

C036

THE HOMEOBOX GENE HOXB7 REGULATES THE PRODUCTION OF PRO-ANGIOGENIC MOLECULES BY MYELOMA CELLS AND IT IS A TARGET IN MYELOMA-INDUCED ANGIOGENESIS

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Bone marrow (BM) angiogenesis is increased in multiple myeloma (MM) patients in relationship with disease progression and supports MM cell growth. MM-induced angiogenesis is mainly due to an overproduction of pro-angiogenic molecules by MM cells and the BM microenvironment. However, the molecular mechanisms at the basis of the angiogenic process are currently under investigation. The deregulation of the homeobox genes has been previously associated to tumor progression and neo-angiogenesis. Particularly, overexpression of the homeobox HOXB7 is involved in tumor-associated angiogenic switch in solid tumors. Interestingly, we have recently shown that HOXB7 is one of the overexpressed genes in BM micorenvironment cells in MM patients as compared to healthy subjects. In this study we have investigated the expression of HOXB7 by MM cells and its potential role in MM-induced angiogenesis *in vitro* and *in vivo*. Here we investigated the potential role of HOXB7 in the pro-angiogenic properties of MM cells. HOXB7 was expressed in 10 out of 22 MM patients analyzed at the diagnosis related to bone marrow angiogenesis but not in normal plasma cells (nPC) and overexpressed in about 40% of myeloma cell lines compared to nPC. Enforced HOXB7 expression in MM cells by a lentiviral vector significantly modified their transcriptional and angiogenic profile, checked by combined microarray and angiogenesis PCR analyses, up-regulating VEGFA, FGF2, MMP2, WNT5a and PDGFA and down-regulating TSP2. The pro- and anti-angiogenic HOXB7-related gene signature was also validated in a large independent dataset of MM patients. Accordingly, MM-induced vessel formation was significantly increased by HOXB7 overexpression both *in vitro* angiogenic and chorioallantoic membrane assays as well as the HOXB7 silencing by siRNA inhibited the production of angiogenic factors and the pro-angiogenic properties of MM cells. Finally, in SCID-NOD mice we confirmed that HOXB7 overexpression by MM cells stimulated tumor growth, increased MM-associated angiogenesis and the expression of pro-angiogenic genes by microarray analysis. Our data demonstrate the critical role of HOXB7 in the production of pro-angiogenic molecules by MM cells and in the regulation of MM-induced angiogenic switch suggesting that HOXB7 could be a potential therapeutic target in MM.

C037

THE MEK INHIBITOR PD0325901 POTENTIATES THE ACTIVITY OF STATINS IN MULTIPLE MYELOMA CELLS

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Introduction. The MEK/ERK pathway is overexpressed in hematologic malignancies, where it promotes proliferation and survival of the neoplastic cells. We have previously demonstrated that the selective MEK inhibitor PD0325901 (PD) exerts a potent growth-inhibitory action in pre-clinical multiple myeloma (MM) models with effects mostly related to the cell cycle progression arrest rather than on apoptosis induction. Statins function in the mevalonate pathway as small-molecule inhibitors of HMG-CoA reductase, which lowers cholesterol. These agents are effective in cancer prevention because of pleiotropic effects primarily related to their anti-inflammatory, immunomodulating and anti-angiogenetic activities. It was also reported that statins induce apoptosis by regulating several signalling pathways, including the MEK/ERK module. Here, we analyzed the impact of the simultaneous inhibition of these two pathways on cell proliferation and apoptosis on MM cell lines and on primary CD138$^+$ malignant plasma cells. *Results.* First, we exposed different MM cell lines to increasing concentrations of PD (1-100 nM) and Mevinolin (Mev) (1-100 M), alone and in combination with a constant ratio of 1:1000. While Mev as single compound dose-dependently inhibited cell growth, as assessed by the MTT assay, its combination with PD synergistically enhanced this effect with combination indexes (CI), as measured by isobologram analysis, of 0.13 and 0.15 for KMS18 and KMS27 cells, respectively. Moreover, while individual exposure of the KMS27 cell line to 10 nM of PD or 10 M of Mev had minimal cytotoxic effects, exposure of cells to both molecules resulted in a striking increase in mitochondrial dysfunction with loss of mitochondrial membrane potential and apoptosis induction. The last was demonstrated by an increase of the subG1-pick, after 72 hours of liquid culture, from 14.86±6.4% (control) to 18.4±1.1% with 10 nM of PD, 25.5±0.1% with 10 M of Mev and to 79.2±15.2% when the two small-molecules were used in combination. Similar findings were noted in other MM cell lines (e.g., KMS18, OPM-2). Conversely, in the PD resistant MM cell line ARH-77, Mev was still able to induce apoptosis, but its effects were not significantly potentiated by MEK inhibition. The effects of the PD/Mev combination were then examined on purified CD138$^+$ primary cells cultured *in vitro* with 10 nM of PD or 10 M of Mev or with their combination. Preliminary results indicate that the PD/Mev co-exposure enhanced the lethal effects of the single compounds toward CD138$^+$ cells with a net apoptosis induction, at 72 hours, of 51.5±40%, compared to those obtained with individual treatments (18.7±2.8% with 10 nM of PD and 34.16±25.9% with 10 M of Mev). *Conclusions.* Together, these findings indicate that MEK inhibitors and statins cooperate to trigger mitochondrial dysfunction and apoptosis in human MM cells and suggest that this approach may warrant further evaluation as an antimyeloma strategy.

C038

AURORA KINASES TARGETING ENHANCES THE PROAPOPTOTIC ACTIVITY OF TRAIL/APO2L IN MULTIPLE MYELOMA CELLS SENSITIVE AND RESISTANT TO BORTEZOMIB

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Introduction. Aurora kinase inhibitors have been shown to induce apoptosis in human myeloma cells lines (HMCLs) and primary myeloma cells. In addition, previous studies have demonstrated the antimyeloma activity of Apo2L/TRAIL as a single agent or in combination with certain chemotherapeutic agents. The aim of this study was to investigate whether the combined treatment with pan-Aurora kinase inhibitor MK-0457 and Apo2L/TRAIL has cytotoxic effects on MM cells. *Methods.* Reagents: MK-0457 (Vertex/Merck), killerTRAIL and caspase inhibitors (Alexis biochemicals), siRNAs (Dharmacon); western blotting, annexin-V and propidium flow cytometric apoptosis assays. *Results.* We found that the combined treatment MK-0457/Apo2L/TRAIL resulted in the synergistic (Chou-Talalay method) induction of apoptosis in RPMI 8226 (highly sensitive to Apo2L/TRAIL: median lethal dose (LD50) at 48 hours was 4.9 ng/mL), and the bortezomib-resistant 8226/R5 HMCLs (barely sensitive to Apo2L/TRAIL: LD50 at 48 hours was 90.9 ng/mL). Consistent with these results we demonstrated that the functional knock-out of Aurora-A or -B gene expression by siRNAs increased (P less.001 Dunnett test) the TRAIL-induced apoptosis in both HMCLs. We demonstrated the involvement of primarily caspase-8 and -3 in MK-0457/Apo2L/TRAIL-induced apoptosis in RPMI 8226 and 8226/R5: the inhibition of caspase-8 reduced (P<001 Dunnett test) the MK-0457/Apo2L/TRAIL-induced apoptosis in both cell lines. The pancaspase inhibitor Z-VAD-FMK protected MM cells from MK-0457/Apo2L/TRAIL-induced apoptosis, confirming that caspase activity was indispensable in MK-0457 /Apo2L/TRAIL-induced apoptosis. Interestingly, the combined treatment MK-0457/Apo2L/TRAIL showed ex vivo antimyeloma activity in 2 of 3 patients studied with relapsed MM refractory to conventional chemotherapy. Since antiapoptotic Mcl-1 and proapoptotic Bim play a pivotal role in controlling MM cell survival and apoptosis and Bim can interfere with the activation of both intrinsic and extrinsic apoptotic pathways in MM cells, we analyzed their expression in MK-0457/Apo2L/TRAIL treated cells. We found that monotreatment with neither MK-0457 nor Apo2L/TRAIL (or their combination) was able to substantially modulate the expression of Mcl-1 or Bim in RPMI 8226; in contrast in HMCL 8226/R5, that showed low sensitivity to Apo2L/TRAIL, the treatment with Apo2L/TRAIL increased the intracellular amount of the antiapoptotic protein Mcl-1, and MK-0457 reverted Apo2L/TRAIL-induced upregulation of Mcl-1. Consistent with these results, we demonstrated that abrogation of Mcl-1 significantly enhanced TRAIL citotoxicity in Bortezomib resistant RPMI 8226/R5 cell line. *Conclusions.* Our data indicate that targeting Aurora kinase potentiates the apoptotic effect of Apo2L/TRAIL in MM cells with differential sensitivity to Apo2L/TRAIL through the activation of the extrinsic pathway. More importantly, MK-0457/Apo2L/TRAIL can induce apoptosis in MM cells displaying resistance to bortezomib.

C039

MULTIPLE MYELOMA IMMUNOGLOBULIN HEAVY CHAIN (IGH) SEQUENCES SHOW NO INTRA-DISEASE CLUSTERING BUT ARE OCCASIONALLY RELATED TO REPERTOIRES FROM NORMAL B-CELLS AND OTHER LYMPHOID TUMORS

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Introduction. The characterization of stereotyped immunoglobulin receptors has improved our knowledge on the antigen-driven patho-

genesis of several lymphoid tumors, including chronic lymphocitic leukemia (CLL), mantle-cell (MCL) and marginal-zone lymphoma (MZL). Multiple myeloma (MM) is a mature neoplasm no longer expressing surface immunoglobulin heavy chain (IGH); however antigen stimulation might have played a role during earlier disease phases. As yet, IGH genes have not been extensively investigated in MM, mostly because of lack of large sequence databases. In this order we created a database of MM sequences including our institutional records as well as sequences from the literature. We characterized first the MM repertoire, performing intra-MM clustering analysis; then we compared the MM series to a large public database of IGH sequences from neoplastic and non-neoplastic B-cells. Methods: 131 MM IGH genes were amplified and sequenced as described (Voena *et al*., Leukemia 1997). 214 MM IGH sequences were derived from published databases (NCBI-EMBL-IMGT/LIGM-DB) for a total of 345 MM sequences. 28590 IGH sequences from other (benignant and malignant) B-cells were retrieved in the same way. All the sequences were analyzed using the IMGT tools (Lefranc *et al*., Nucleic Acid Res. 2005) to identify IGHV-D-J gene usage, to assess the somatic hypermutation (SHM) rate and to identify HCDR3. HCDR3 aminoacidic sequences were aligned using the ClustalX 2.0 software (Larkin *et al*., Bioinformatics, 2007). Stereotyped IGH receptors were defined according to Messmer *et al*. (J Exp Med 2004) and Stamatopoulos *et al*. (Blood 2007). *Results*. IGHV-D-J usage and HCDR3 lenght in MM was more in keeping with the normal B-cell repertoire compared to other lymphoid tumors, with only modest over-representation of IGHV3-9, IGHV3-21, IGHV5-51 genes and under-representation of the IGHV3-23 and IGHV4-34; 98% of MM sequences showed a SHM rate >2%. Intra-MM search for HCDR3 similarity never met minimal requirements for stereotyped receptors. Compared to the public database, only a minority of MM sequences (2.9%) clustered with those from lymphoid tumors and normal B-cells (Table 1): two MM sequences could be assigned to previously identified CLL subsets (n.37 and n.71, Murray *et al*., Blood 2008). In addition, three mixed MM/CLL and one MM/MZL provisional clusters were identified. Finally three provisional clusters were found between MM and IGH sequences from normal lymphocytes. Conclusions: 1) MM IGH repertoire follows a nearly physiological distribution; 2) MM specific HCDR3 clusters do not occur to a frequency detectable with currently available databases; 3) 98% of MM sequences are not related to other "highly-clustered" lymphoid disorders; 4) Occasional clustering of MM with IGH from normal and neoplastic B-cells was noticed. In conclusion, our analysis does not support a critical role for antigen selection in the majority of MM patients.

Table 1. Mixed clusters of IGH sequences between multiple myeloma (MM) and normal or neoplastic B-lymphocyties.

Source of IGH sequence	*Number of IGH sequences analyzed*	*Number of MM clustering with other sequences*	*% of present MM database clustering with other sequences*	*MM: probability clustering with other sequences*
Non neoplastic B-lymphocytes	24085	3 (3*)	0.9%	1.2×10^{-4}
Chronic lymphocitic leukemia	3185	5 (3*)	1.5%	16×10^{-4}
Marginal-zone lymphoma	195	1 (1*)	0.3%	51×10^{-4}
Mantie-cell lymphoma	104	0	0%	0

**Newly identified provisional cluster; IGH, immunoglobulin heavy chain.*

C040

ANALYSIS OF CIRCULATING MICRORNAS EXPRESSION PROFILE IN PATIENTS AFFECTED BY MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATIES OF UNCERTAIN SIGNIFICANCE

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Introduction. The role of small noncoding transcripts, such as microRNAs (miRNAs), in Multiple Myeloma (MM) pathogenesis has been recently highlighted by several studies. However little is known about the role of circulating miRNAs in this malignancy. MicroRNAs circulate in a stable cell-free form in the blood-stream and they can serve as ideal biomarkers for cancer. We analysed miRNAs expression profile in the plasma of newly-diagnosed MM patients in order to detect a distinctive signature of circulating miRNAs that could be used for disease monitoring. *Methods.* After defining the method for the isolation of miRNAs from blood plasma by adjusting the mirVana™ miRNA Isolation Kit (Ambion Inc), miRNAs have been isolated from peripheral blood (PB) plasma, bone marrow (BM) plasma and $CD138^+$ plasma cells of MM patients and healthy donors. Each sample has been analysed by low density array technology (Applied Biosystems, TaqMan® Human microRNA cards). This method allows the study of the expression of 365 human miRNAs by quantitative RT-PCR. Plasma samples have been collected from MM patients and healthy subjects as well. The data have been normalized respect to miR-16, which was observed to be a stably expressed reference gene. The calibrator sample was calculated using the average of the data of normal subjects plasma samples. Relative quantification of miRNA expression was calculated using the $2\text{-}\Delta\Delta^{Ct}$ method. Differentially expressed miRNAs have been identified using the "Significant Analysis of Microarrays" (SAM) algorithm, the t-test and the nonparametric Wilcoxon rank sum test. *Results.* Having obtained an informed consent, we collected plasma samples of 15 healthy donors, 5 Monoclonal gammopathy of undetermined significance (MGUS) patients and 15 newly diagnosed myeloma patients. The miRNA expression profile observed in the PB plasma faithfully traces that of the bone marrow plasma and that of $CD138^+$ isolated plasmacells. Furthermore, by comparing the miRNA expression profiles, we identified a group of 5 miRNAs that are significantly upregulated in the plasma of newly diagnosed MM patients. Interestingly, among these, miR-124 is also overexpressed in MGUS patients and miR-455-5p targets CAPRIN1 adhesion molecule and GAS8 growth arrest specific protein. A group of 23 miRNAs was found to be down-regulated in MM patients. MicroRNA-101 (P=0.00015) is a tumor suppressor, miR-127 (P=0.03) is involved in apoptosis and cell proliferation and miR-199a (P=0.00001) targets the MET and the ERK dependent apoptotic pathway. *Conclusions.* Circulating miRNAs can be detected and analysed by quantitative RT-PCR in peripheral blood plasma samples. Further studies will be aimed at the identification of the role of the significantly different miRNAs found in the plasma of Myeloma and MGUS patients. Nonetheless, these preliminary results indicate that specific miRNAs present in the peripheral blood could be used for disease monitoring in myeloma patients.

Lymphomas

C041

BENDAMUSTINE AND CYTOSINE ARABINOSIDE EXHIBIT A HIGH SYNERGISTIC APOPTOTIC EFFECT ON MANTLE CELL LYMPHOMA CELL LINES

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Introduction. Bendamustine is a bifunctional compound that has been recently reported to be extremely active and with low toxicity profile when combined with Rituximab in the treatment of patients with mantle cell non-Hodgkin's lymphomas (MCL). Cytosine arabinoside (Ara-C) has been incorporated in most regimens designed for treating young patients with MCL because of its recognized efficacy in this disease. Since Bendamustine and Ara-C exhibit individual and unique mechanisms of action in MCL, a synergistic or additive effect might be expected when they are used in combination. Our aim is to investigate whether the addition of Ara-C would enhance the apoptotic power of Bendamustine in two MCL cell line models (JEKO-1 and GRANTA-519). *Methods.* JEKO-1 and GRANTA-519 cells were incubated with Bendamustine or Ara-C at concentrations ranging between 0 and 1000 μg/mL in RPMI medium supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin. Drug dosages were chosen according to IC25, IC50 and IC75 previously established for both cell lines. The two drugs were incubated simultaneously (24h and 48h, respectively) or consecutively (drug A or B for 2h, then washed and incubated for further 21h without any drug, then supplemented with drug B or A for 24h). Apoptosis was measured with the fluorescent DNA-binding agent 7-AAD (7 amino-actinomycin D) by flow cytometry. To evaluate the combined effect of the two drugs we used CalcuSyn software. This software calculates an index, called combination index (CI), that is a quantitative measure of the degree of drug interaction in terms of additive effect (CI=1), synergism (CI<1) or antagonism (CI>1). The experiments were repeated a minimum of 4 times with 2 biological repeats.

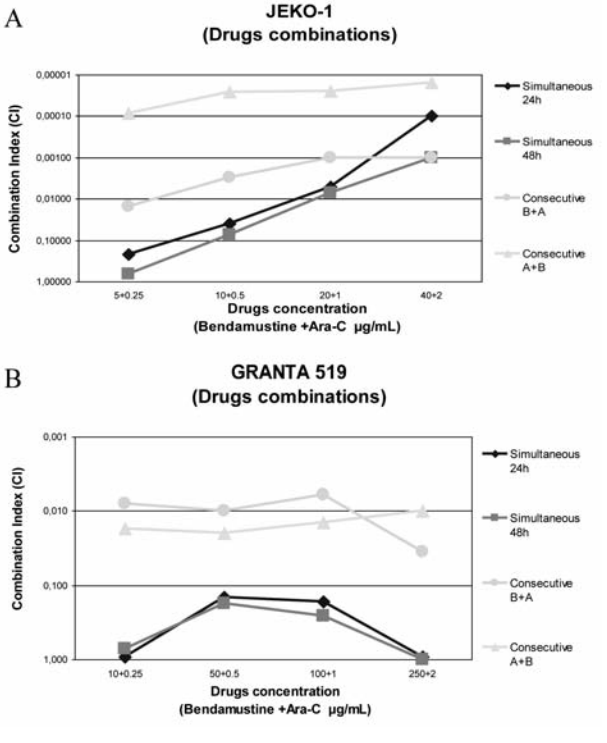

Figure 1. Combination index (CI) values obtained after 24h and 48h (simultaneous) or after consecutive incubations of the two drugs (B+A: Bendamustine then Ara-C; A+B: Ara-C then Bendamustine, see text) calculated for JEKO-1 (A) and GRANTA-519 cells (B).

Results. For JEKO-1 cells, simultaneous incubation of Bendamustine and Ara-C for 24h revealed higher cytotoxic activity compared to the two drugs alone with an improvement of 30% of apoptosis at highest drug combinations. This indicates that the drugs in the two simultaneous conditions were synergistic. Noteworthy, consecutive incubations were significantly very synergistic in inducing apoptosis even at lowest drug combinations, with a CI over 3log lower than the additive effect defined as CI=1 (Figure 1A). For GRANTA-519 cells the mixture at 24 and 48h of incubation were only additive, where consecutive incubations were synergistic reaching about 85% of apoptosis (Figure 1B). For both cell lines, the cytotoxicity of Bendamustine alone was dose dependent. The lowest CI (very synergistic effect) were obtained with the consecutive incubations of Bendamustine and Ara-C (not significantly different changing the drug order). *Conclusions.* Our data confirm the strong apoptotic effect of Bendamustine on JEKO-1 and GRANTA-519 cells and reveal a considerable synergistic effect of the two drugs when used in combination, particularly in consecutive schedules, irrespective of the incubation order. These findings give a rationale for the use of these two drugs in combination for the treatment of patients with MCL. We are now running a Phase II study with a regimen combining Rituximab, Bendamustine and Ara-C (R -BAC) in the treatment of MCL patients.

C042

ANALYSIS OF T-CELL RECEPTOR REARRANGEMENTS IN PEDIATRIC PATIENTS WITH CUTANEOUS LYMPHOMA

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Cutaneous lymphomas are rare in pediatric patients and are mostly denoted by T-cells types. Representing 1% to 3% of all childhood non-Hodgkin's lymphomas, they are frequently characterized by good prognosis, even though a few cases are lethal. Because cutaneous lymphomas share features of some benign skin disorders, differential diagnosis is often challenging only on the basis of clinical manifestations and immuno-histopathology. With the aim of investigating the role of T-cell receptor rearrangements in their molecular characterization, we collected DNA samples from skin biopsies of 33 patients less than 18 years of age: 7 diagnosed with mycosis fungoides (MF), 8 with parapsoriasis, 12 with primary cutaneous CD30$^+$ lymphoproliferative disorder (10 lymphomatoid papulosis, LYP and 2 anaplastic large T-cell lymphoma, ALCL), 2 with pityriasis lichenoides, 2 with subcutaneous panniculitis-like CD8$^+$ α:β T-cell lymphoma (SPTL) and 2 with CD4$^+$ small/medium-sized pleomorphic T-cell lymphoma (PTL). Genomic DNA samples at diagnosis were screened by PCR amplification using the BIOMED-1 and BIOMED-2 primer sets for complete and incomplete TCR delta, gamma and beta rearrangements. When possible, junctional regions of monoclonal PCR products were directly sequenced, confirming the presence of a T-cell clone. All cases of MF presented clonal rearrangements, while a T-cell clone in the skin was unveiled only in 2/8 patients with parapsoriasis. In the CD30$^+$ lymphomas group, monoclonal populations were observed in 9/10 LYP and 1/2 ALCL. In our group of pediatric patients, 4 presented rare entities as SPTL and PTL. In all cases it was possible to demonstrate the presence of a T-cell clone in the skin. Pityriasis lichenoides, a benign skin disorder included as control, showed a polyclonal pattern. The presence of a T-cell clone was confirmed by sequencing in 17 patients showing involvement of different V and J segments. In one case of lethal MF, we observed the presence of the same clone in skin biopsies and peripheral blood. Overall, we found clonal rearrangements in 23 patients, while considering only the most used TCR γ target the number decreases to 21. These data suggest the higher sensitivity of the more complete screening (delta, gamma and beta). To the best of our knowledge, this is the first exhaustive molecular study of TCR rearrangements in pediatric patients with cutaneous T cell lymphomas. Due to the highly variable presentation of these malignancies

and to the relevance of prognostic issues in children, we conclude that clonality assessment can be very important to improve diagnosis, particularly in early-stage of the disease.

C043

GENOMIC PROFILING OF B CELL LYMPHOMA RL CELL LINE TREATED WITH ENZASTAURIN

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Follicular lymphoma (FL) is an indolent lymphoma typically containing the chromosomal translocation t(14;18), which leads to over expression of the anti-apoptotic intracellular protein, Bcl-2. FLs are usually sensitive to several chemotherapies; however, patient relapses occur and response duration becomes progressively shorter. The majority of patients eventually die from the disease. Thus, there is a need for new, less toxic, and more active treatments. To provide new insights into the molecular mechanisms of the anti-tumour action of enzastaurin in B cell lymphoma, we investigated its effects on the gene expression profiles (GEP) of the RL cell line by oligonucleotide microarrays analysis. Using Affymetrix Gene 1.0 ST arrays, we analyzed profile of RL cell line treated with enzastaurin for 48h at low concentration, corresponding to IC25. Material and methods. RL cell line, carrying the t(14;18), was purchased from DSMZ. Citotoxicity values were calculated from curves based on enzastaurin concentration ranging from 1 to 10 μM using both MTT assay and cell viability assessment by Trypan Blue exclusion. Cell apoptosis was assessed by flow cytometer after staining with Annexin V-FITC/ propidium iodide and by western blot studies on caspases activation (caspase 9, caspase 8 and PARP). RNA was isolated from three independent replicas of RL cells, either treated or untreated. The labelled cRNAs were hybridized to Gene 1.0 ST Arrays (Affymetrix Inc., Santa Clara, CA, USA). The chips were scanned with an Affymetrix GeneChip1 Scanner 3000 and subsequent images analyzed using GCOS 1.4 Western blot analysis was performed to validate expression data for selected genes, utilising antibodies against MYC, STAT and CyclinI. *Results and conclusions.* A functional analysis of deregulated genes following enzastaurin treatment revealed a significant number of gene involved in signal transduction, in immune and inflammatory responses, in transcription regulation, in cellular adhesion and apoptosis processes. Notably, genes that are thought to play an important role in the pathogenesis of tumour, such as the transcription factors HBP1, MYC and BCL-6 are negatively modulated by enzastaurin. Among genes upregulated it is interestingly the presence of STAT, cyclinI and of several genes belonging to IFN family. Western blot analysis of expression of STAT, cyclinI and Myc confirms gene expression results. These observation provide new insights into the mechanisms involved in the induction of apoptosis by enzastaurin in B cell lymphoma cell lines and identify possible pathways which could significantly contributes to the induction of apoptosis process.

C044

ANEMIA AT DIAGNOSIS AND DURING THERAPY IN HODGKIN LYMPHOMA: THE ROLE OF BASELINE LEVELS OF INTERLEUKIN-6 AND HEPCIDIN

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Introduction. Approximately 40% of patients with Hodgkin lymphoma (HL) present with anemia at diagnosis, which is typically a mild normochromic, normocytic anemia of chronic disease seen in a wide variety of inflammatory states. We have recently shown that IL-6 is the principal mediator of this anemia: IL-6 stimulates the overproduction of hepcidin as an acute phase reactant, and elevated levels of hepcidin in HL correlated with iron-restriction, and contributed to anemia at diagnosis (Hohaus *et al*, JCO 2010, Apr 20. [Epub ahead of print]). Treatment of HL, in particular with intensive treatment regimens as BEACOPP, is associated with a further decrease of hemoglobin levels, eventually requiring red blood cell (RBC) transfusions and therapy with erythropoisis-stimulating agents (ESA). Our objective now was to study the potential predictive role of baseline cytokine and hepcidin levels in HL for changes of hemoglobin levels during chemotherapy. *Methods.* We studied 65 patients with HL. Plasma samples at diagnosis were analyzed for levels of the cytokines IL-6, IL-10, and the chemokine TARC using ELISA techniques (R&D Dignostics), while hepcidin levels were determined using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (TOF MS), as described previously (Swinkels *et al*, PLOS ONE 2008; 3:e2706). Standard treatment of patients was ABVD (n=38), while young patients with advanced stage disease were treated with BEACOPP (dose-escalated)(n=24). *Results.* Anemia defined as hemoglobin level <12 g/dL was present in 31 patients, and did not differ according to treatment regimen. Hemoglobin levels were lower in female patients, patients with age >45 years, in the presence of B-symptoms, stage IV disease and with a higher IPS score (>2). As expected, changes of haemoglobin levels during treatment strongly depended on the type of chemotherapy: Patients treated with BEACOPP regimen had a steeper decrease of haemoglobin levels in comparison to patients treated with ABVD (-1.07 g/dL per month versus -0.19 g/dL), were more likely to receive RBC transfusions (42% *vs.* 11%) and were more often treated with ESAs (81% *vs.* 7%)(all P<0.01). IL-6 levels at diagnosis not only correlated with haemoglobin levels, but also predicted for development of anemia - with haemoglobin levels under 10 g/dL necessitating therapy with ESAs and/or RBC transfusions - during therapy of initially non-anemic patients (P=0.04). Hepcidin levels inversely correlated with haemoglobin values at diagnosis in anemic patients (r=-0.45, P=0.01), but did not predict for development of anemia during therapy. *Conclusion.* Baseline IL6 levels correlated with haemoglobin levels at diagnosis and also predicted for development of therapy-induced anemia.

C045

GENOMIC PROFILE OF NON-NODAL MANTLE CELL LYMPHOMA IS CHARACTERIZED BY A DOWN-MODULATION OF GENES INVOLVED IN TUMOR INVASION

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Introduction. MCL is an aggressive neoplasm with a median survival of 3 years. Cases with leukemic MCL and splenomegaly without adenopathies (non-nodal MCL) seem to have a more indolent course, associated with a higher rate of mutated IGHV genes, a less frequent expression of CD38 and SOX11-negativity. To gain further insights into the biologic features underlying this presentation, we investigated the gene expression profile (GEP) of a cohort of leukemic MCL cases. *Methods.* GEP of 29 peripheral blood samples with more than 75% of clonal B lymphocytes from untreated patients affected by NHL (i.e. 16 MCL, 11 SMZL, 2 FL) and 33 CLL samples were evaluated by HGU133 Plus 2.0 arrays (Affymetrix). Diagnosis of MCL was confirmed in all cases by t(11;14) and/or cyclin D1 detection. GEP data were examined by the dChip software. Functional annotation and enrichment analyses were performed by DAVID and FATIGO software. IGHV sequencing followed ERIC recommendations. *Results.* Unsupervised clustering segregated samples into 2 clusters: one included all CLL and the other all leukemic lymphoma samples. Within the latter, one branch grouped the majority of MCL (14/16), the other 8/11 SMZL and the 2 FL. To focus on the transcriptional profile of MCL, we performed two separate t-tests (MCL *vs.* CLL and MCL *vs.* SMZL). Functional annotation analysis of the overlapping genes revealed that the MCL signature was enriched for the following gene categories: mithocondrion, oxidoreductase activity, response to stress and to DNA damage and TP53-pathway. Then, we considered the clinical and biological presentation of MCL cases: 9 had palpable and/or deep lymphadenopathies (nodal group), 7 had no nodes (non-nodal group). IGHV mutational status proved unmutated in all nodal and mutated in all non-nodal MCL. CD38 was positive in 8/9 nodal and 1/5 non-nodal evaluable cases. When a t-test comparing nodal and non-nodal groups was performed, 389 differentially expressed genes were selected; the genes down-modulated in non-nodal MCL cases deserved particular attention, as they were classified as follows: cell projection (P=9.31E-03), actin cytoskeleton organization (P=1.24E-02) and ubiqui-

tin-mediated proteolysis (P=1.51E-02). A conspicuous number of down-modulated genes was related to TP53-pathway (P=4E-02) and to response to DNA damage stimulus. Interestingly, FATIGO highlighted a prevalence of genes involved in cell adhesion (IGAE, CELSR1, PCDH9, P=4.5E-01); consistently, genes involved in tumor invasion/progression (PGF, ST14, ETS1, OCIAD1, EZR) were under-expressed. We could confirm a significant down-modulation of SOX11 (P=0.04). *Conclusions.* MCL cases are characterized by a more active metabolism and response to cell injuries than other lymphoproliferative disorders; non-nodal MCLs seem to display a transcriptional profile lacking tumor invasion properties, that might justify the absence of nodal involvement.

C046

NEW INSIGHTS INTO THE MOLECULAR PATHOGENESIS OF CLASSICAL HODGKIN LYMPHOMA AS REVEALED BY GENE EXPRESSION PROFILING OF MICRODISSECTED HODGKIN/REED-STERNBERG CELLS

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Introduction. Owing to the rarity of hodgkin/reed-sternberg (HRS) cells in the lymph node, gene expression profiles of classical hodgkin lymphoma (CHL) have been so far derived either from whole tissue sections (mainly reflecting the rich inflammatory background) or from cHL cell lines. The latter, however, do not fully reflect in-situ HRS cells, as they were generated from anatomical sites of end-stage chemorefractory patients (such as bone marrow and pleural effusions) that are not typically affected by cHL and that do not recapitulate the cross-talk between HRS cells and their lymph node microenvironment. *Methods.* ~1000-2000 tumor cells were laser-microdissected from hematoxylin/eosin--stained frozen sections of lymph nodes taken at disease onset from patients with cHL or with different B-cell non-Hodgkin lymphomas, including primary mediastinal B-cell lymphoma (PMBL) and nodular lymphocyte-predominant Hodgkin lymphoma (nLPHL). Following two rounds of linear amplification, mRNA was hybridized to Affymetrix HG-U133 Plus 2.0 chips. Expression profiles were similarly generated from sorted HL cell lines and various normal mature B-cell populations. Microarray data mining was performed using different bioinformatic tools, including GeneSpring 7.3.1 and Gene Set Enrichment Analysis. *Results.* Primary and cultured HRS cells share typical cHL signatures like high NF-κB activity and loss of the B-cell transcriptional program, but appear to differ in their interaction with the microenvironment and proliferation attitude: primary HRS cell profiles are enriched in chemokine/chemokine receptor activity, extracellular matrix remodeling and cell adhesion, whereas cHL lines upregulate a number of proliferation-related genes. Unsupervised hierarchical clustering and supervised principal component analyses showed that primary HRS cells constitute a transcriptionally distinct lymphoma entity, overall closer to nLPHL than to PMBL but with differential behavior of the cHL histological subtypes. In particular, whereas HRS cells of the lymphocyte-rich and mixed-cellularity subtypes are relatively close to nLPHL cells, HRS cells of the nodular sclerosis and lymphocyte-depletion subtypes display greater resemblance to PMBL cells. Primary HRS cells also appear to downregulate a large number of genes involved in cell cycle checkpoints and in the maintenance of genomic integrity, while upregulating gene and gene signatures involved in various oncogenic signaling pathways and in cell phenotype reprogramming. When compared to normal B cells, primary HRS cells do not show a consistent relatedness with bulk germinal center (GC) B cells or with plasma cells but, interestingly, display a more pronounced similarity to $CD30^+$ GC B cells and $CD30^+$ extrafollicular B cells. *Conclusions.* Gene expression profiling of primary HRS cells offered new insights into the molecular pathogenesis of cHL, its relatedness to other lymphomas and to normal B cells, and its intriguing phenotype.

C047

IN AGGRESSIVE NHL PATIENTS THE CASES RESULTED AS POSITIVE AT CEREBROSPINAL FLUID FLOW CYTOMETRY ANALYSIS SEEMS TO HAVE AN HIGHER RISK OF SNC RELAPSE IN COMPARISON TO PATIENTS FCM NEGATIVE

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Introduction. In NHL at high risk for leptomeningeal disease intrathecal prophylaxis likely reduces the incidence of CNS relapse but may increased the toxicity of systemic chemotherapy. The identification of patients subgroups that may benefit of prophylaxis is therefore important. Recently, flow cytometry (FCM) assessment of cerebrospinal fluid (CSF) has been known to increase the proportion of positive cases with leptomeningeal disease in comparison to conventional cytologic examination (CC) but its prognostic value is still unknown. *Methods.* The primary aim of this prospective, multicenter trial was to compare CC *vs.* FCM in a large cohort of NHL patients at high risk for LD. The secondary aim was to assessed the impact of FCM^+ on PFS and OS. Patients were enrolled if they were diffuse large B-cell lymphoma (DLBCL) with IPI 2-3, elevated LDH along with at least two extranodal sites or with bone marrow, testis, palate or paravertebral involvement; Burkitt lymphoma (BL); blastoid variant of mantle-cell lymphoma (B-MCL); B-cell precursor lymphoblastic lymphoma (B-LL); HIV^+ patients. All patients were required to have no evidence or signs of neurological disease. All patients received intrathecal standard prophylactic therapy with 12 mg of methothrexate +/- cytarabin except for BL that were given prophylaxis with 50 mg of liposomial aracytin. The incidence of positive test for occult LD with FCM and CC^- was compared using the McNemar test for paired data and 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, 145 patients were enrolled by 12 centres. Clinical characteristics were: 96 males, median age 55 years (IQR:43-63); 111 patients (76%) with DLBCL, 22 pts (15%) with BL, 7 pts (5%) with B-MCL and 5 pts (3%) with B-LL. Twenty-seven patients (19%) were HIV^+. FCM was able to detect a clonal population in 17 out of 145 patients (12%) whereas CC detected abnormal cells only among 7 pts (5%)(P=0.0002). Therefore, 10 patients (7%) were discordant: FCM^+/CC^-. From date of diagnosis, overall median follow up of survivors was 26 months. We observed 39 (30%) systemic progressions, 6 (5%) CNS progressions and 32 (25%) deaths. Among the 17 pts FCM positive, we observed 10 (59%) systemic progression, 3 (18%) CNS progression (in 2 cases the disease of CNS was isolated whereas 1 pts presented a CNS progression among the systemic progression) and 8 (47%) deaths (6 PD, 2 missing). PFS at 1 year was 71.2% (95%CI:62.1-78.5) in the whole group of patients. The progression risk was significantly higher in patients FCM^+/CC^+ compared with patients FCM^-/CC^-(P=0.003). An higher but not significant risk of progression was found in FCM^+/CC^- with respect to patients FCM^-/CC^-. At 24 months the cumulative incidence of CNS progression by FMC test result, accounting for competing events, was statistically higher in FCM^+ respect to FCM^- patients (18% *vs.* 4%)(P=0.010). *Conclusion.* FCM assessment of CSF is more sensitive than CC for detection of LD, but it's clinical relevance is still to be clearly defined. Our preliminary data suggest that patients FCM^+/CC^+ have an higher risk of progression compared with those FCM^-/CC^-, whereas discordant cases seem to have an intermediate prognosis. Moreover, pts FCM^+ seems have an higher risk of SNC relapse in comparison to patients FCM^-.

C048

GENETIC AND EPIGENETIC INACTIVATION OF NEGATIVE REGULATORS OF THE CITOKINE AND B-CELL RECEPTOR SIGNALING PATHWAYS IN IMMUNODEFICIENCY-RELATED LYMPHOMAS

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Background. Immunodeficiency-related non Hodgkin lymphomas (ID-NHL) occur in the context of immune deregulation and chronic B-cell activation. Most of the stimuli targeting B-cells derive from signals that are mediated by the B-cell receptor and by cytokine receptors. SHP1, SOCS1 and SOCS3 are negative regulators that act downstream of these receptor complexes mainly through the inhibition of the JAK2/STAT pathway. Several observations point to induction of STATs as an important mechanism of lymphomagenesis. *Aim.* To test the involvement of SHP1, SOCS1 and SOCS3 inactivation by methylation or somatic mutation in ID-NHL. *Methods.* Tumor samples from 76 HIV-related non-Hodgkin lymphoma (HIV-NHL) and 21 post-transplant lymphoproliferative disorders (PTLD) were analyzed for SHP1, SOCS1 and SOCS3 methylation by methylation specific PCR and by direct sequencing for SOCS1 and SOCS3 mutation status. The tumor panel included 43 HIV-diffuse large B cell lymphoma (HIV-DLBCL), 23 HIV-Burkitt lymphoma (HIV-BL), 10 HIV-primary effusion lymphoma (HIV-PEL), 16 post-transplant DLBCL (PT-DLBCL) and 5 polymorphic PTLD. *Results.* Among HIV-NHL, SHP1 methylation occurred in 44/76 (58%) cases, including 100% HIV-PEL, 27/43 (63%) HIV-DLBCL and 7/23 (30%) HIV-BL. When considering immunohistochemical classification, SHP1 methylation occurred in 9/12 (75%) germinal center B cell (GCB) DLBCL and in 10/15 (67%) non-GC DLBCL. All HIV-BL with SHP1 methylation showed plasmacytoid differentiation. Western blot analysis demonstrated that methylation of the SHP1 gene was associated with downregulation of SHP1 protein and upregulation of p-STAT3. SOCS1 methylation occurred in 5/76 (6.57%) HIV-NHL, including 3/10 HIV-PEL and 2/43 (4.65%) HIV-DLBCL. SOCS3 methylation occurred in 4/76 (5.26%) HIV-NHL, including 2/23 (8.69%) HIV-BL and 1/43 (2.42%) HIV-DLBCL. Among PTLD, SHP1 methylation was detected in 12/21 (57%) cases, including 8/16 (50%) PT-DLBCL and 4/5 (80%) polymorphic PTLD. SOCS1 methylation was detected in 3/21 (12%) PT-DLBCL, whereas SOCS3 methylation was consistently negative. No association was observed between EBV infection of the tumor clone and methylation status. SOCS1 mutations were found in 8/43 (19%) HIV-DLBCL, 2/16 (12%) PT-DLBCL and 4/5 (80%) polymorphic PTLD. A total of 25 mutational events were observed. Mutations where mainly represented by single base-pair substitutions (n=23), with a prevalence of transversions (N=17) over transitions (N=8). In 10 cases mutations introduced aminoacid substitutions with likely functional consequences. *Conclusions.* The implications of our data are threefold. First, SHP1 inactivation by aberrant methylation is observed in the majority of ID-NHL, although with a variable incidence in the different clinico-patological entities of the disease. Second, similar to observations in NHL of the immunocompetent host, SOCS1 is mainly inactivated by somatic mutations, presumably introduced by the aberrant somatic hypermutation process. Third, SOCS3 inactivation is rarely implicated in the pathogenesis of ID-NHL. This notion is supported by the phenotype of SOCS3-deficient mice, that do not develop a lymphoproliferative disease.

Chronic Myeloproliferative Disorders

C049

V617F MUTATION INDUCES NUCLEAR LOCALIZATION OF JAK2 IN CD34$^+$ CELLS BUT NOT GRANULOCYTIC, MEGAKARYOCYTIC OR ERYTHROID CELLS OF PATIENTS WITH PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE DISORDERS

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Introduction. Recently, Dawson *et al*. identified a previously unrecognized nuclear role for JAK2 in the phosphorylation of the tyrosine 41 of the histone H3 with the exclusion of HP1a from chromatin and resulting in a disregulation of several JAK2-regulated genes such as IMO2 in haematopoietic cell lines and in one case on peripheral CD34$^+$ cells from a JAK2V617F mutated primary myelofibrosis (PMF) patient. Activation of JAK2 by chromosomal translocations or point mutations is a frequent event in haematological malignancies particularly in Philadelphia negative myeloproliferative disorders (MPDs). *Methods.* To investigate and confirm a possible nuclear localization of JAK2 in presence of V617F mutation, we stably transfected K562 with pMSCV-Puro-JAK2V617F construct and compare with K562 expressing pMSCV-Puro-wild type-(WT)-JAK2 and performed immunofluorescence and western blot analysis. To confirm the *in vitro* results we searched the possible nuclear localization of JAK2 in total BM of 10 patients affected by all types of JAK2V617F positive MPDs [PMF n=3, polycitemia vera (PV) n=3, essential thrombocythemia (ET) n=4] and 5 patients with WT MPDs (PMF n=2 , ET n=3). To define which cells show nuclear JAK2, we selected by fluorescence activated cell sorting (FACS) 4 cell populations: CD34$^+$, CD15$^+$, CD41$^+$ and CD71$^+$ cells from total BM of 3 JAK2-mutated-MPDs (1 ET, 1 PV, 1 early PMF). *Results.* Confocal immunofluorescent images on nuclear and cytoplasmic fractions confirmed nuclear JAK2 in K562 although with the strongest nuclear signal in JAK2V617F expressing cells. This latter was also seen by western blot analysis which showed nuclear and cytoplasmic JAK2 only in JAK2V617F expressing K562 comparing with untransfected and WT cells. No differences in JAK2 nuclear signal was observed by the addiction of the nuclear export inhibitor leptomycin B suggesting that export is not involved in nuclear JAK2 shuttling. We found a strong nuclear signal within the nuclei of 3-5% of mononucleated cells in 10 of 10 JAK2 mutated patients but not in un-mutated cases. We found nuclear JAK2 in CD34$^+$ cells but not in other cell populations of the 3 studied patients. Western blot performed on nuclear and cytoplasmic fractions of the JAK2V617F-CD34$^+$ cells confirmed the result. No nuclear JAK2 was detected in differentiated erythroid, granulocytic or megakaryocytic colonies obtained from all the studied patients (n=15). *Conclusions.* Our data corroborate the recent findings, obtained in hematopoietic cell lines, of a role of JAK2 in direct nuclear signaling. Furthermore we report, for the first time, a nuclear JAK2 in total BM and in sorted CD34$^+$ cells of patients affected by all subtypes of JAK2 mutated MPDs and not in patients with WT diseases. We described also the absence of nuclear JAK2 in sorted mature cells and in differentiated colonies derived from the same patients. Possible chromatin modification due to JAK2 nuclear localization has to be better assessed in patients, where further studies are needed to understand the effects of mutated JAK2 in the nuclei, its target proteins and consequences on gene expression. This intriguing insight reveals a new scenario in the pathogenesis of malignant haematopoiesis and in myeloproliferative phenotype.

C050

OVEREXPRESSION OF MICRORNA-16-2 CONTRIBUTES TO THE ABNORMAL ERYTHROPOIESIS IN POLYCYTHEMIA VERA

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Introduction. Recent reports show that microRNA-16 expression increases during *in vitro* erythropoiesis, and preliminary results from our labora-

tory show raised levels of miR-16 in CD34+ cells from PV patients. We hypothesized that miR-16 overexpression contributed to the abnormal erythropoiesis observed in Polycythemia Vera. *Methods.* miR-16 levels have been measured by RTQ-PCR in CD34+ cells purified from BM of PV, ET, and healthy donors and the PB of PMF patients. MiR-16 expression was also tested in T-lymphocytes from PV patients and in granulocytes purified from patients with reactive erythrocytosis. SMC4 expression has been measured by RTQ-PCR. Direct sequencing and RTQ-PCR approach together with SNP Array 6.0 (Affymetrix) have been used to exclude genetic abnormalities. Transfection of cells has been performed using amaxa nucleofector. The JAK2 inhibitor AZD1480 has been used to in JAK2V617F mutated cell lines. Phosphotiorate-modified and cholesterole conjugated antisense oligonucleotides (antagomiRs) have been used to silence miR-16 expression *in vivo* in C57BL6 mice. *Results.* We observed a significantly higher expression of miR-16 in PV CD34+ cells compared to controls, but not in ET and PMF cells. This alteration is restricted to myeloid lineage, since T lymphocyte had normal miR-16 levels in PV. No acquired mutation or copy number alteration involving miR-16 genes have been found. MiR-16 overexpression is mechanistically related to the enhanced JAK/STAT activity in PV cells, as demonstrated by the significant decrease of miRNA levels in JAK2 mutated cell lines treated with AZD1480. Three different evidences demonstrated that miR-16 levels in PV cells are mainly originated by pre-miR-16-2 overexpression. First, pre-miR-16-1/pre-miR16-2 ratio is lower in PV cells comparing to controls. Second, miR-16 levels decreased significantly in PV cells transfected with anti-pre-miR-16-2 siRNA, but not in cells treated with anti-pre-miR-16-1. In addition, miR-16 expression levels directly correlates with SMC4 expression, to whom pre-miR-16-2 is intronic. We then investigated the role of miR-16 both in normal and pathological erythropoiesis *in vitro* and *in vivo* in mice treated with Epo. MiR-16 expression is significantly higher during *in vitro* erythroid differentiation of PV patients' cells compared to controls. MiR-16 overexpression in CD34+ cells induces enhanced expansion of erythroid progenitors, as we observed in liquid cultures and clonogenic assays. In contrast, miR-16 silencing in PV patient's progenitors cells reduced the formation of Epo-dependent and –independent erythroid colonies, but did not affect CFU-GM number. We finally demonstrated *in vivo* that specific antagomiRs induced mir-16 silencing and caused blunting of erythroid response to Epo treatment. *Conclusions:* Our data establish a role for mir-16 in PV pathogenesis and prospectively identify novel target for therapy.

C051

SEVERE TELOMERIC LOSS OCCURS IN PH-NEGATIVE HEMATOPOIESIS EMERGING AFTER SUCCESSFUL TREATMENT OF CHRONIC MYELOID LEUKEMIA AND ASSOCIATES WITH ACQUIRED CYTOGENETIC LESIONS AND LOW HEMOGLOBIN LEVELS

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Introduction. Telomere attrition represents a useful marker of proliferative and oxidative stress and monitor the genetic integrity of the hematopoietic compartment. This approach has been used to assess the genetic integrity of Ph-negative hematopoietic cells (HCs) repopulating the bone marrow after successful chronic myeloid leukemia (CML) treatment. *Patients and methods.* We investigated 81 CML patients with persistent (>12 months) complete cytogenetic remission (CCyR). Median age was 62 (23-88), M/F ratio was 1.5. Median time from diagnosis and CCyR were 54 months (25-217) and 37 months (12-191) respectively. 15 patients had acquired cytogenetic abnormalities (CA) (del7: 4 patients, +8: 5 patients, del5q: 2 patients, del or +Y: 2 patients, other CA 2 patients). Telomere length (TL) analysis was performed by Southern Blotting on polymorphonucleates (PMN) and on monocyte-depleted PBMC (MD-PBMC) to monitor the myeloid and lymphoid compartments. As control group we analyzed 76 age matched healthy donors. Prospective follow-up monitoring of TL was performed on 64 patients (median time 11 months, range 6-20). *Results.* PMN (but not MD-PBMC) from CML patients showed a major erosion of their telomeric DNA (median loss 1294 bp P<0.001). A multivariate general linear model (GLM) was applied on the whole population (CML patients and controls): age, as expected, and interestingly previous history of CML were predictors of TL attrition (both P<0.001), while sex had no significant impact (P=0.6). Focusing only on CML population we found no associationbetween TL and sex, Sokal score,or treatment schedule. We found a correlation between TL attrition and the lack of complete molecular remission (CMolR). Moreover surprisingly we found an increased TL shorthening in patients with acquired CA compared to CML patients without CA (P<0.030, Figure 1A). When a multivariate GLM was applied to the CML series only the presence of CA, the age, and the lack of CMolR were predictors of TL damage (P=0.013, 0.003 and 0.001 respectively). When TL shortening was correlated to hematopoietic function we found no association with WBC or platelets level. However an association between telomere shortening and G1 or higher anemia (Hb<10 gr/dL, P=0.003) was evidenced (Figure 1B). TL was substantially stable overtime in the majority of patients. In none of the patients TL recovery overtime was observed. However in 16 (25%) patients a non-physiological telomeric loss was noticed (Figure 1C). *Conclusions.* i) Ph-negative HCs display severe telomeric loss, compared to healthy controls ii) telomere erosion is more pronounced in patients with CA and without CMolR; iii) an association between short telomeres and anemia was observed; iv) telomere loss is persistent and potentially worsening in a subset of cases. Our results suggest that Ph-negative hematopoiesis in CML patients has undergone significant genetic damage and warrants careful clinical monitoring.

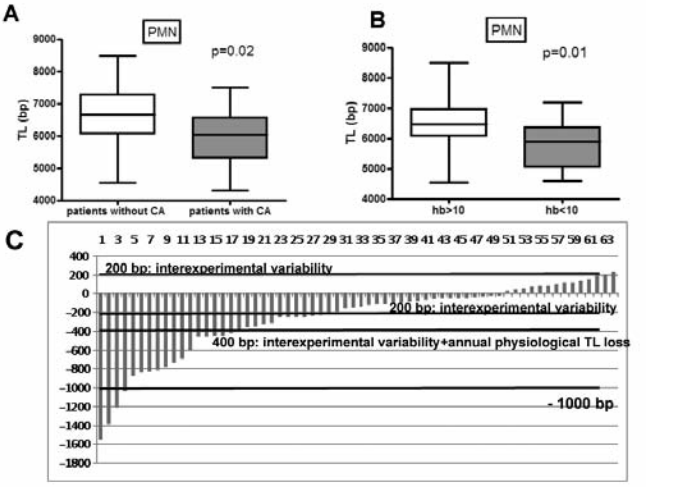

Figure 1.

C052

JAK2V617F MUTATION PERSISTS IN BLASTS AND MATURE CELLS OF TRANSFORMED-JAK2V617F-POSITIVE-MYELOPROLIFERATIVE NEOPLASIA: A EUROPEAN LEUKEMIA NET (ENL) STUDY

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Introduction. Transformation to acute myeloid leukemia (AML) is a known complication of MPN but the role of JAK2V617F mutation is still undefined. In 2006, Campbell described a possible model for the development of a JAK2WT-AML in a patient with JAK2V617F-MPN and more recently, Theocharides *et al*. reported that in up to 53% of the

patients who developed secondary AML from a JAK2-mutated MPN the mutation was no longer detectable; however the results obtained with DNA extracted from cells scraped or laser-capture-microdissected from bone marrow (BM) or peripheral blood (PB) smears, were confirmed in fresh samples only in few cases. *Methods.* In this study, we collected, by cell sorting, blast cells and mature myeloid cells (granulocytes, GRA) from whole BM aspirates of 40 newly diagnosed patients with AML secondary to MPN (18 derived from PMF; 13 from PV and 9 from ET) and analyzed the JAK2 status before and after leukemic transformation in selected cell compartments. To evaluate the modification in the JAK2 status before and during leukemic transformation we performed ASO-PCR and (QRT)-PCR assay on total BM of the MPN phase and sorted cell populations from AML phase. *Results.* At the time of MPN diagnosis, JAK2V617F was detectable in 28 of 40 patients (70%) (10 of 18 PMF; 12 of 13 PV and 6 of 9 ET). No cytogenetic abnormalities or MPL and JAK2-exone 12 mutations were detected at this stage. Median time to AML progression (TTP) was 5.09 years (yrs) (range 0.38-27.81). A significant difference (P=0.02) in TTP was found grouping patients according to JAK2 status during the MPN phase [JAK2WT-MPN n=12, TTP median 15.10 yrs (0.38-16.32); JAK2 mutated-MPN n=28, TTP median 4.07 yrs (0.67-27.81)]. Eight patients showed additional abnormalities involving chromosomes 1, 5, 7, 8, 9, 12, 14, 17 and 20 while no other AML-associated mutations (FLT3, NPM, CEBPA, RUNX1) were detectable at this stage. In our cohort of patients we found that JAK2V617F mutation was still present at the blast transformation in both compartments: $CD34^+$ cells (blasts) and $CD15^+$ cells (GRA) in 24 of 28 JAK2 mutated MPN (86%). Four of 28 patients (14%) developed JAK2V617F negative AML starting from a mutated PV with a mean TTP of 5.14 yrs. Interestingly, the WT status was confirmed in blast cells but also in GRA. Surprisingly we found 2 cases of JAK2V617F mutated AML transforming from a WT-PMF. Also in this case the JAK2V617F positivity in the AML phase occurred in both GRA and blast compartments. No differences (P=0.3) in the allele burden were found comparing MNCs from chronic phase with MNCs of leukemic transformations or comparing GRA with blasts in AML phase. *Conclusions.* In conclusion, these results contrast with the previous study in which the JAK2 mutation was lost in 53% of blasts during leukemia transformation. In our work, the loss of JAK2V617F mutation during AML progression is a rare event (14%). Additional studies in larger patient series and multivariate analysis are needed before a prognostic role of JAK2V617F mutation regarding time to leukemia transformation can be definitely assessed.

C053

CHARACTERIZATION OF AN INTRONIC SNP VARIANT IN JAK2 (RS56241661) AS PART OF THE 46/1 HAPLOTYPE

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Introduction. Recent studies demonstrated the existence of a JAK2 haplotype, named 46/1 or "GGCC", that predisposes to the development of myeloproliferative neoplasms (MPNs). In this study we have characterized an intronic 5bp deletion SNP, rs56241661 [-/TCTTA], located in JAK2 gene, to assess possible associations with an increased risk of MPNs and whether it is part of the 46/1 haplotype. *Methods.* SNP genotyping of rs56241661 locus was performed by High resolution Melting Analysis. Genotyping of 46/1 tagging SNP rs12343867 was performed by Taq-Man Allelic Discrimination Assay. *Results.* We analyzed 234 MPN patients (59 PV, 76 ET, 44 PMF, 55 PPV/PET-MF), 58 subjects with reactive erythrocytosis and 39 healthy controls. We found a significantly different incidence of rs56241661 in MPN cohort ([-] allele freq. 43%; [TCTTA/TCTTA] 31%; [-/TCTTA] 49%; [-/-] 20%) compared to reactive erythrocytosis ([-] allele freq 22%; [TCTTA/TCTTA] 60%; [-/TCTTA] 36%; [-/-] 4%) and donors ([-] allele freq 26%; [TCTTA/TCTTA] 61%; [-/TCTTA] 26%; [-/-] 13%). rs56241661 [-] allele frequency was significantly higher in PV (52%) and PPV-MF patients (n=29; 66%) than in healthy donors. PPV-MF pts displayed the highest incidence of rs56241661 [-/-] homozygosis (45%). Patients with ET harboring JAK2 V617F mutation (48 cases), showed a higher incidence of [-] allele than JAK2 wt ET patients (28 cases) (34% *vs.* 25%, [TCTTA/TCTTA] 42% *vs.* 61%; [-/TCTTA] 48% *vs.* 28%; [-/-] 10% *vs.* 11%). Also JAK2 V617F PMF patients (n=26) demonstrated an increased frequency of [-] allele compared with JAK2 wt PMF (n=18) (48% *vs.* 33%; [TCTTA/TCTTA] 31% *vs.* 39%; [-/TCTTA] 38% *vs.* 56%; [-/-] 31% *vs.* 5%). To evaluate whether the presence of rs56241661 affected splicing we analyzed RNA by PCR amplification with different primer sets but fail to find evidence of exon skipping. We tested the co-expression of rs56241661 and the C allele of rs12343867, that is in complete LD with 46/1 haplotype, on a subset of 183 MPN patients (52 PV, 71 ET, 36 PMF, 24 PPV/PET-MF), 36 healthy controls and 15 reactive erythrocytosis. Data demonstrated a strong association of the two polymorphisms. 95% of PV patients harboring at least one rs56241661 [-] allele has at least one rs12343867 C allele. Similarly, 96% of donors and 100% of erythrocytosis that did not display the 5bp deletion were rs12343867 [T/T] (ie, not 46/1 haplotype). Overall 93% of all subjects analyzed presented concordance of rs56241661 and rs12343867 SNP. *Conclusions.* Genotyping for rs56241661 in MPN patients showed a higher incidence in comparison with healthy controls. Analysis of MPN patients grouped by JAK2 mutational status showed a higher frequency of rs56241661 in V617F subjects providing evidence that this variant predisposes to the development of JAK2 positive Myeloproliferative Neoplasms. The strong association with the 46/1 tagging SNP rs12343867 suggests that also rs56241661 is part of 46/1 haplotype.

C054

TELOMERE LENGTH IS REDUCED IN PH - NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS ACCORDING TO JAK2 V617F MUTATION ALLELE BURDEN BUT IT IS MAINTAINED IN SECONDARY ERYTHROCYTOSIS

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Introduction. Telomeres erosion is an important marker of previous cell proliferation and ultimately of cell ageing. Reduction of telomere length (TL) has been described in many hematological malignancies. In particular, some recent studies have reported a marked TL reduction in patients with Ph-negative Chronic Myeloproliferative Neoplasms (Ph-neg-CMNs) (Ferraris AM *et al.*, Br J Haematol 2005; Bernard L *et al.*, Leukemia 2009). This supports the possible influence of TL in the development of CMNs. Moreover, TL might be a putative indicator of the rate of neoplastic proliferation. We here report the analysis of TL in a series of patients with Polycythemia Vera (PV), Essential Thrombocytemia (ET) and Myelofibrosis (MF). Aims of our study were: i) to verify the rate of TL reduction in Ph-neg-CMNs; ii) to compare TL in ET, PV and MF; iii. to investigate the role of TL as a marker of neoplastic proliferation, evaluating differences in TL compared to both healthy subjects and subjects with Secondary Erythrocytosis (SE). *Methods.* Peripheral blood (PB) samples were obtained from 261 Ph-neg-CMNs patients (median age 69 yrs, range 10-91): 85 had PV, 112 had ET and 64 MF. Among the 77 evaluable PV patients, a JAK2V617F mutation was found in 71 (92%) and an exon 12 mutation was identified in 3 patients. A JAK2 V617F mutation was detected in 50 (54%)ET patients. Samples were obtained either at diagnosis or during follow-up. As control, PB samples from 60 healthy age-matched subjects (median age 64 yrs, range 57-99) and from 10 SE patients (58 yrs, 28-72) were analyzed. TL was assessed by Southern blot analysis, according to standard procedures (TeloTAGGG Telomere Length Assay Kit, Roche Diagnostic, Mannheim, Germany). JAK2V617F mutation analysis was performed by ASO-PCR and digestion with BSAXI (Guerini *et al.*, Leukemia 2008) *Results.* PV, ET and MF patients showed individual progressive TL shortening correlated with age as observed in the healthy population. However, CMNs patients had TL significantly shortened (5,720 bp, range: 3,090-9,770) compared to healthy age-matched individuals (median: 6,670 bp, range: 3,780-9,940) (P<0.001). PV and MF showed the most pronounced TL loss among CMNs, with TL values respectively of 5,390 bp (range: 3090-9570) and 5,028 bp (range: 3,290-8,940). ET patients had a median TL of 6,300 bp, (range: 4,170-9,770). PV and MF but not ET had significantly shorter TL compared to healthy subjects (P<0.0001)(Figure 1). At univariate analysis, short TL correlated significantly with JAK2 V617F mutation allele burden >50% (P=0.0003). SE patients had a median TL of 7390 bp (range: 5950-8990) with no difference from healthy subjects (P=NS). Conclusions: i. Ph-negative CMNs are confirmed to present with TL reduction compared to the age-matched healthy population; ii. PV and MF show higher degree of TL loss while ET seems to have almost normal TL; iii.

high JAK2 V617F mutation burden is strongly correlated to TL loss; iv. SE patients and healthy subjects do not differ in TL. These datas indicate a possible role of TL as a possible tool to differentiate neoplastic from secondary myeloproliferation. Further studies are needed to confirm these findings in a larger population.

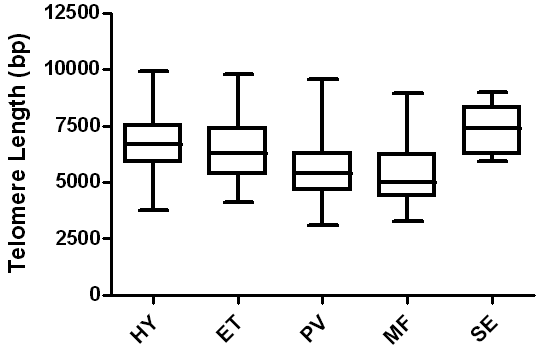

Figure 1. Median TL in healthy subjects (HY), Ph-ve CMNs (ET, PV, MF) and secondary erythrocytosis (SE)

C055

PHD2 GERM-LINE MUTATION IN A FAMILY WITH MYELOPROLIFERATIVE DISEASE

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Introduction. Philadelphia-negative chronic myeloproliferative disorders (MPDs) are a large group of clonal hematologic neoplasms that include polycythemia vera (PV), essential thrombocythemia and primary myelofibrosis. The JAK2(V617F) mutation is present in almost all patients with PV, a disease also characterized by the presence of an absolute erythrocytosis with low or normal level of serum erythropoietin (Epo). Anyway, several evidences suggest that multiple genetic lesions are involved in the pleiotropy of MPDs, including the existence of a pre-JAK2 mutated clone, even if multiple (still unknown) oncogenic events seem involved in the pathogenesis of MPDs. Recent insights of the genetic causes of erythrocytosis came from the functional and biochemical investigation of the oxygen-sensing pathway. Heterozygous mutations in prolyl hydroxylase domain protein 2 (PHD2) have been associated with erythrocytosis, either familial or sporadic. *Aim.* To investigate the implication of the oxygen-sensing pathway key genes in the pathogenesis of MPDs, we studied twenty-five patients with familial MPD from 12 different families. *Methods.* The PHD2, von Hippel-Lindau tumor suppressor (VHL) and the exon 12 of the 2 -subunit of the hypoxia-inducible factor (HIF2A) genes were analyzed for their role as key proteins of the oxygen-sensing pathway. PCR of the PHD2 (exon 1-5) and VHL (exon 1-3) coding regions and sequencing analyses were performed. To confirm the identity of the newly identified mutation, allele-specific PCR were carried out (Figure 1B). We could prove the germ line nature of the new PHD2 mutation by comparison with non-hematopoietic tissue, using genomic DNA from oral epithelial cells of the index case. JAK2 and MPL mutational status were already assessed. *Results.* A G>C missense heterozygous mutation at coding nucleotide position c.471, resulting in a Q157H replacement in the amino acid sequence, was found in two subjects (Figure 1A, B). The index case was a PV patient with normal serum Epo level, increased hematocrit (55.2%) and high platelet 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. The son of the proband was also affected by mild erythrocytosis (Figure 1 C), resulting positive for the new heterozygous Q157H variation while free of JAK2^{V617F}. The patients with the new PHD2 mutation did not carried VHL, HIF2A-exon 12, MPL or JAK2-exon 12 mutations. *Conclusions.* A novel PHD2 mutation outside the catalytic domain was detected in two members of a family with MPD. Q157H substitution is the most N-terminal mutation reported to date in the PHD2 gene and is the first published germ line PHD2 mutation associated with PV, as other partial-loss PHD2 mutations are all erythrocytosis-associated.

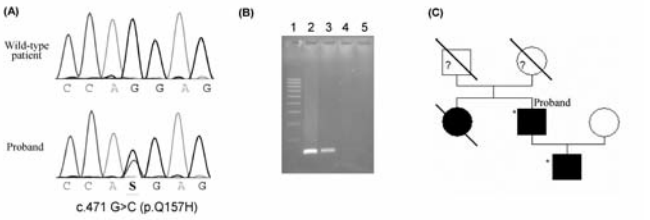

Fig 1. Molecular analyses results. (A) Sequencing electropherograms of mutated allele of the proband against the wild-type control. Nucleotide position, nucleotide change and corresponding amino acid change are indicated below. (B) AS-PCR. Lane 1: molecular weight marker 100 bp; Lane 2: proband's DNA amplified with mutation specific primer; Lane 3: DNA of the son of the proband amplified with mutation specific primer; Lane 4: control DNA (wild-type) amplified with mutation specific primer; Lane 5: reaction control (no template). (C) Pedigree of the family with MPD. Squares represent males, circles females, affected individuals are indicated in black and slashes indicate deceased members. An asterisk indicates the genetically tested individuals.

The co-presence of PHD2 and JAK2^{V617F} genetic lesions supports the hypotesis that JAK2 mutation is a secondary, not a disease-initiating, genetic event and that the JAK2^{V617F} allele might occur as a secondary oncogenic event on the background of an inherited genetic predisposition.

C056

INHERITED THROMBOPHILIA AND THROMBOTIC EVENTS IN BCR-ABL NEGATIVE MYELOPROLIPHERATIVE NEOPLASMS: A RETROSPECTIVE STUDY IN 148 PATIENTS

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Background. Subjects with BCR-ABL negative Myeloproliferative Neoplasms (MPN), Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) have an increased risk of venous (VTE) and arterial (ATE) thromboembolism. *Aims.* to investigate the possible association between inherited thrombophilia (FV/G1691A and FII/G20210A) as well as fasting homocysteine (HCY) and ATE/VTE in MPN. *Patients and methods.* A cohort of 148 patients with MPN followed-up at the Division of Hematology, L. Sacco University Hospital were enrolled in the study. They were diagnosed according to WHO criteria: PV=44 (median age 67, SE 2.3); ET=87 (median age 59, SE 1.97); and PMF=17 (median age 68, SE 4.2). ATE/VTE were managed according to CHEST recommendations. Mutation analysis of JAK2 V617F/G1849T, FV/G1691A, and FII/G20210A, and fasting plasma homocysteine (HCY) were tested. Statistics were performed by SPSS-11.5. *Results.* The JAK2V617F, FV/G1691A, and FII/G20210A mutations were identified respectively in: PV=36 (81,8%), 1 (2.3%), 2 (4.5%); ET=54 (62.1%), 0 (0%), 5 (5.7%); PMF=10 (58.8%), 3 (17.6%), 1 (5.9%) PMF. ATE/VTE events were found respectively in: PV= 8/9 in 14 (31.8%); ET= 16/14 in 25 (28.7%); PMF= 4/8 in 12 (70.5%). ATE (28 events/28 patients) were localized at the following sites: 5 TIA, 10 AMI, 7 stroke, 3 peripheral, 1 bowel ischemia and 1 placental infarction. VTE (31 events/ 23 patients) were identified by CUS or CT, occurred at the time of MPD diagnosis (23 events) or during follow up (8 events) and were localized at the following sites: 4 portal, 2 splenic, 9 DVT, 2 retinal, 1 cerebral sinus, 1 jugular, 9 thrombophebitis. Linear regression analyses of MPN showed significant (P=0.031) results in PV only taking into account FV/G1691A (P=0.006), FII/G20210A (P=0.006), HCY (P=0.008). *Conclusions.* Based on these observations, we can confirm that inherited thrombophilia (FV/G1691A and FII /G20210A) and HCY levels enhance the risk of ATE/VTE of MPN patients.

Immunotherapy and Hematopoietic Stem Cell Transplantation

C057

CD133+ PLURIPOTENT STEM CELLS FOR THE TREATMENT OF CHRONIC LIVER FAILURE

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Introduction. The potential role of bone marrow (BM)-derived stem cells (SCs) in patients with end-stage liver disease has been addressed by our group in four studies. Main objectives were: 1) to assess stem/progenitor cell mobilization in 24 patients receiving orthotopic liver transplantation (OLT); 2) to evaluate whether G-CSF can be safely administered to patients with liver cirrhosis in order to expand and mobilize BM-derived SCs; 3) to investigate the effects of transplantation of human G-CSF-mobilized CD34+ and CD133+ SCs in mice with chronic liver injury and fibrosis; 4) to evaluate the feasibility and the safety of the purification and intrahepatic reinfusion of increasing numbers of autologous BM-derived G-CSF-mobilized CD133+ SCs in patients with end-stage liver disease. *Methods.* 1) Flow cytometry analysis, clonogenic assays and RT-PCR have been performed after OLT; 2) 18 patients with advanced liver disease were consecutively treated with increasing doses of G-CSF starting from 2 μg/kg/daily; 3)C57BL/6N mice received CCl4 by inhalation for thirteen weeks and were treated with Cyclosporin-A. Transplantation was performed by injection (tail vein) of 10^6 CD34+ or CD133+ SCs of three cirrhotic patients. After four weeks from transplantation all mice were sacrificed; 4)G-CSF at 7.5 microg/Kg/b.i.d. is administered subcutaneosly (sc) from day 1 until the completion of peripheral blood stem cells (PBSC) collection. At least 4 weeks after SC mobilization, collection and cryopreservation, highly purified autologous G-CSF-mobilized CD133+ cells are re-infused through the hepatic artery by transfemoral or transbranchial arteriography. CD133+ cells are administered to patients starting from 5×10^4/Kg patient's body weight and increased every 3 patients. The maximum infused cell dose will be 1×10^6/kg. G-CSF at 5 microg/Kg/day is administered sc for 3 days after the reinfusion of SCs for their expansion and to induce a selective proliferative advantage of reinfused cells *in vivo*. *Results and Discussion.* 1) We demonstrated that both early subsets of the hematopoietic SC compartment (CD34+/CD90+ cells) and more mature committed progenitors (CFU-C) were mobilized into PB after OLT. We also demonstrated the release from the BM of liver-committed HSCs co-expressing epithelial markers after OLT; 2) We show that the administration of G-CSF to patients with liver cirrhosis is safe and feasible and allows the mobilization and collection of BM-derived SCs at the dose of 15 microg/kg/day. 3) We demonstrated that mice transplanted with either CD133+ or CD34+ human cells appear to have less fibrotic septa than mice without SC transplantation, suggesting the potential therapeutic role of human SCs on the recovery of liver fibrosis. 4) Up to date, three patients have been enrolled in the clinical study and the preliminary results will be presented.

C058

PURINERGIC STIMULATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS MODULATE THEIR FUNCTION AND DIFFERENTIATION POTENTIAL

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Introduction. Human bone marrow derived Mesenchymal Stem Cells (hMSCs) are adult multipotent cells. hMSCs differentiate *in vitro* and *in vivo* into several tissue lineages originating from the three germinal layers making them attractive candidates for bioengineering and cellular therapy. Thus, it seems of great relevance to search putative messengers and signalling able to modulate their proliferation and differentiation. Nucleotides triphosphates are extracellular messengers binding to specific receptors (P2Rs) that modulate cell functions depending on the cell type. Controversial information is available on P2 expression and activity in hMSCs. *Methods and Results.* Here we found that hMSCs expressed several P2R subtypes. hMSCs were very resistant to the cytotoxic effects of high concentrations of ATP, as demonstrated by the lack of morphological and mitochondrial changes or release of intracellular markers of cell death. Gene expression profiling revealed that ATP treatment down-regulated cell proliferation and up-regulated cell migration genes in hMSCs. Functional studies confirmed the inhibitory activity of ATP on proliferation and clonogenic ability of hMSCs. Furthermore, ATP potentiated the chemotactic response of hMSCs to the chemokine CXCL12, and increased their spontaneous migration. *In vivo*, xenotransplant experiments showed that the homing capacity of hMSCs to murine bone marrow was increased by ATP pre-treatment. Moreover, ATP increased pro-inflammatory cytokines production (IL-2, IFN-γ, IL-12p70), while decreased secretion of the anti-inflammatory cytokine IL-10. This finding was associated with the reduced ability of ATP-treated hMSC of inhibiting T-cell proliferation. Microarrays data suggested that several genes implicated in hMSC differentiation can be modulated by ATP treatment. To further investigate this issue, hMSCs cells were cultured under adipogenic or osteogenic conditions and were transiently exposed to ATP before starting differentiation or continuously exposed to ATP for the first 3 days of differentiation induction. We demonstrated that adipogenesis-related accumulation of lipids, analyzed by Oil red O staining, was more evident in ATP treated cultures with both protocols. Furthermore, quantitative real time PCR (qRT-PCR) assay showed that mRNA expression of PPARg, a transcription factor early up-regulated during adipogenesis, was significantly increased in hMSCs differentiated cells treated with ATP. In osteogenic condition, analysis of mineralized area through Alizarin Red staining, indicated that ATP pre-incubation decreased, while ATP continuous treatment enhanced the extent of mineralization compared to untreated control. The expression of RUNX2, a key transcription factor in osteogenesis, analyzed by qRT-PCR in differentiated cells confirmed data obtained in Alizarin-based assay. *Conclusions.* These data demonstrated that purinergic signalling modulates biological functions and differentiation potential of hMSCs.

C059

ALLOGENEIC HUMAN SERUM ALLOWS AN EXTENSIVE *IN VITRO* EXPANSION OF MESENCHYMAL STEM CELL FROM CHORIONIC VILLI WITHOUT MALIGNANT TRANSFORMATION

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Introduction. Mesenchymal stem cells (MSCs) represent an interesting cell type for research and cell therapy and they have been derived from many different organs and tissues. Recently, fetal tissues have emerged as a promising alternative stem cells source to use in cell therapy and tissue engineer. We reported on the growth, characterization, immunomod-

ulatory capacity, cytokine expression profile and replicative stability of MSCs from first-trimester chorionic villi (CV) expanded in allogeneic human serum. *Methods.* We used cells taken from back up cultures of CV samples set for prenatal diagnosis. We evaluated 10 CV samples (gestation age 11-13 weeks) expanded in human serum, human platelet lysate and animal serum, until the MSCs reached senescence. We studied the immunophenotype, the differentiation capacity into mesodermal lineages, the gene expression profile, the replicative stability of CV cells after extensive *in vitro* expansion by telomere length and telomerase activity, the expression of c-myc and p53, tumor-associated genes, at different passage and the capacity of these cells to grow in an anchorage-independent manner by soft agar assay. We investigated the immunoregolatory activity of CV MSCs in mixed lymphocyte reactions for $CD4^+$, $CD8^+$ and $CD56^+$ cells. Spontaneous chromosomal alterations were excluded by cytogenetic analysis. *Results.* CV cells grow rapidly in culture in human serum, 6 log after 75 days (6 passages). Platelet lysate allowed an expansion like human serum, only in one-third of the samples. Typically, CV MSCs not expressed hematopoietic stem cell markers on their surface but a specific pattern of molecules, such as CD90, CD105, CD29, CD73 and CD44. CV MSCs showed an undifferentiated stem cell gene expression profile and the capacity to differentiate into mesodermal lineages. CV cells inhibited the proliferation of stimulated T lymphocytes, suppressing the growth of both $CD4^+$ and $CD8^+$ T subpopulations. The inhibitory activity of CV MSCs was not mediated by cell contact but was dependent on the release of soluble factors. Instead, CD56+ stimulated NK cells seemed to be significantly susceptible to the MSCs contact-dependent suppression. Despite their high proliferation capacity, CV MSCs showed no anchorage-independent growth and no telomerase activity, no hTERT and c-myc transcriptions. A constant expression level of p53 and a normal karyotype were preserved throughout long-term expansion, suggesting the safety of fetal MSCs. *Conclusions.* Our results indicate that CV MSCs had a high expansion potential in allogeneic human serum with immunoregolatory capacity on T lymphocyte and NK cells. We ascertained the safety of these cells for clinical use, excluding their potential susceptibility to malignant transformation after long term culture. CV cells appeared to be advantageous for cell therapy in terms of accessibility. These findings suggested that CV MSCs could be a good resource for cell therapy and regenerative medicine.

C060

ADOPTIVE IMMUNOTHERAPY AFTER HAPLOIDENTICAL STEM CELL TRANSPLANTATION WITH T CELLS ALLODEPLETED BY PHOTODYNAMIC PURGING

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After haploidentical stem cell transplantation, immune recovery is slow due to decaying thymic function and extensive T-cell depletion of the graft which is needed to prevent Graft-versus-Host Disease (GvHD). Consequently, infectious related mortality is about 30-40%. To address this problem, we investigated the efficacy of adoptive immunotherapy after photodynamic purging of alloreactive T cells (ATIR, Kiadis Pharma, Amsterdam, The Netherlands) in preventing GvHD and improving immune reconstitution. The 4,5-dibromorhodamine methyl ester (TH9402) is a photosensitizer structurally similar to rhodamine. When donor T cells are activated with allogeneic cells, they retain TH9402 which becomes highly cytotoxic upon activation with visible light. MLR-activated donor T cells are exposed to a fluorescent-light scanning device to eliminate alloreactive T cells. We defined optimal protocol conditions which provided 3,260±450 (mean ± SD)-fold allodepletion, retention of T-regulatory cells, and preservation of pathogen- and leukaemia-specific T-cell responses (against Aspergillus, Candida, Cytomegalovirus (CMV), Adenovirus (ADV), Herpes Simplex Virus (HSV), Varicella Zoster Virus (VZV), Toxoplasma antigens; and against leukaemia antigens, such WT1). We therefore started a clinical trial. Escalating doses of photodynamically allodeleted donor T cells, i.e., 1.25×10^5/Kg, 2.5×10^5/Kg, 5×10^5/Kg, 1×10^6/Kg and 1.25×10^6/Kg, were infused into groups of haploidentical transplant recipients. Only 1 patient developed grade III aGvHD at the 1×10^6/Kg cell dose and responded to immune suppressive treatment. Immune assessment analyses revealed that infusion of cell doses equal or greater than 5×10^5/Kg are associated with significant reconstitution of T-cell counts and appearance of pathogen-specific T-cell responses. One month after infusion, $CD4^+$ and $CD8^+$ T cells were 124±54/cmm and 327±42/cmm (*versus* 11±4/cmm and 8±4/cmm respectively, in patients receiving T-cell doses below 5×10^5/Kg, P=0.0007). Aspergillus, Candida, CMV, ADV, HSV, VZV, Toxoplasma-specific $CD4^+$ and $CD8^+$ T-cell responses had recovered to frequencies within the normal ranges while they were absent in patients who received T cell doses under 5×10^5/Kg (P=0.0002). Another phase I study (Roy D.C., Blood 2009, 114;22:212a) also demonstrated safety and efficacy of photoallodepleted donor lymphocyte infusions (DLIs) in haploidentical transplant recipients. On the basis of these phase I clinical trials, an international multicenter phase II clinical trial was initiated with the aim of evaluating the impact of photoallodepleted DLIs on transplant non relapse mortality and event free survival.

C061

THYMIC RENEWAL IN ADULT LEUKEMIA PATIENTS AFTER HAPLOIDENTICAL STEM CELL TRANSPLANTATION AND INFUSION OF SUICIDE GENE-MODIFIED DONOR T CELLS

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Introduction. In haploidentical Hematopoietic Stem Cell Transplantation (HSCT), the infusion of donor lymphocytes transduced to express the Herpes Simplex Virus Thymidine kinase (HSV-Tk) suicide gene allows to control GvHD, to mediate GvL, and to rapidly provide an effective and polyclonal anti-infective T cell repertoire (Ciceri and Bonini *et al*., Lancet Oncology, 2009). Even though their engraftment is necessary to achieve these effects, HSV-Tk^+ cells represent the minority of lymphocytes circulating in treated patients. Therefore, we investigated the putative role of HSV-Tk^+ cells in promoting thymic activity and T cell development from graft progenitors. *Methods.* Thymic function was assessed in adult patient who underwent haploidentical HSCT and infusion of suicide gene-modified donor T cells for hematologic malignancies, after validating the methods in healthy pediatric and adult controls. Single joint T cell Receptor Excision Circles (sjTREC) were quantified by qPCR in peripheral blood mononuclear cells (PBMCs) and purified T cells, and the proportion of $CD31^+$ recent thymic emigrants (RTEs) in $CD4^+$ naïve T cells was measured with immunophenotype analysis in PBMCs. Thymic output was correlated with thymic volume, assessed by Computed Tomography (CT) scans. T Cell Receptor repertoire was assessed by V spectratyping. The relative contribution of HSV-Tk^+ and HSV-Tk^- donor T cells to post-transplantation anti-host alloreactivity was studied by mixed lymphocyte cultures. *Results.* At the moment of T cell immune reconstitution (defined as $CD3^+$ cells >100/L peripheral blood), the $CD4^+$ naïve T cell subset was almost entirely comprised by $CD31^+$ RTEs, and both the frequency and the absolute number of thymic emigrants were significantly higher than in age-matched patients who received unmanipulated T cell-replete grafts. Moreover, RTE frequencies before HSCT and before HSV-Tk^+ cell infusion were in line with age-related healthy controls, strongly suggesting a direct role of the infused cells in enhancing thymic renewal. Accordingly, thorax CT scans of treated patients documented an increase in thymic volume following HSV-Tk^+ cell add-backs. Finally, a consistent increase in sjTREC counts could be detected during the post-transplantation follow-up, although absolute counts remained low, in line with the documentation of extensive peripheral T cell proliferation after transplantation. *Conclusions.* These data show that after the infusion of suicide gene-modified T cells a renewal of thymic activity takes place, driving the recovery of a polyclonal and efficient peripheral T cell repertoire.

C062

EDITING HUMAN LYMPHOCYTE SPECIFICITY FOR SAFE AND EFFECTIVE ADOPTIVE IMMUNOTHERAPY OF LEUKEMIA

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T cell receptor (TCR) gene-transfer is an attractive strategy for the adoptive immunotherapy of tumors. However, the full potential of this approach is limited by a number of technical hurdles including inefficient gene transfer, unstable transgene expression, exhaustion of gene-modified cells and, most importantly, the co-expression in the same cell of the endogenous and tumor-specific TCR. The latter results not only in reduction of expression of the introduced tumor-specific TCR, but also in acquisition of autoreactive specificities due to mispairing between endogenous and exogenous TCR chains. Mispaired TCR have been shown to be autoreactive and potentially halrmful in animal models. To address these limitations, we designed a novel strategy based on zinc finger nucleases (ZFNs) and lentiviral vectors that allows for the first time editing of T cell specificity at the DNA level, by combining the disruption of the endogenous TCR beta chain gene with the transfer of a tumor-specific TCR. We first stimulated PBL with anti-CD3 and anti-CD28 antibody-conjugated beads, and cultured the cells with low doses of IL-7/IL-15, to target and preserve early differentiated T cells. To eliminate expression of the endogenous TCR, activated cells were treated with integrase defective lentiviral vectors (IDLV) carrying a set of ZFNs specific for the constant regions of the TCR beta chain. Abrogation of surface expression of the TCR/CD3 complex was observed in up to 7% of the treated cells. The majority (>80%) of CD3 negative lymphocytes expressed CD62L, CD127, CD27 and CD28, a phenotype most consistent with central memory T cells, and could be expanded in culture with IL7 and IL15 as unmodified T cells. To re-direct tumor specific T cell activity, we selected a codon-optimized, cysteine-modified TCR specific for the Wilm's Tumor Antigen 1 (WT1), which is involved in oncogenic transformation in several tumors. To promote balanced expression of the introduced alpha and beta TCR chains, we generated lentiviral vectors (LV) encoding both chains under a single PGK bidirectional promoter (PGK-WT1 LV). Sorted CD3 negative cells were efficiently transduced (45%) with PGK-WT1 LV, and expressed stable and high levels of tumor-specific TCR, indicating that CD3 negative cells are permissive to further genetic manipulation. TCR-edited cells were enriched to 90% purity by polyclonal stimulation, indicating that surface expression of the transferred TCR/CD3 complex was functional and sufficient to promote cell expansion. TCR-edited cells killed targets pulsed with lower peptide concentrations than unedited T cells, modified by conventional TCR gene transfer, and lysed fresh $WT1^+/HLA^-A2^+$ primary acute myeloid leukemia blasts. These data demonstrate that genetic re-programming of T cell specificity in early differentiated lymphocytes is feasible and functional and represents an attractive approach to improve the therapeutic outcome of cancer immunotherapy.

C063

THE HEMATOPOIETIC RECONSTITUTION AFTER TRANSPLANT IS INFLUENCED BY THE AGE OF MICROENVIRONNEMENT

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Introduction. The hematopoietic cells (HC) are known to be strongly influenced by the hematopoietic microevironnement (HM). The HM is composed by several cell populations including: mesenchymal cells (MSC), Osteoblast, Adipocytes, Endothelial cells etc. Hematopoietic cells are transplanted in a recipient which has received chemo-radiotherapy which is known to impair both HC and HM. Therefore, ideally, one should aim to transfer both HC and HM. We therefore evaluated the reconstitution after transplant of HC and HM in patients undergoing allograft for hematopoietic malignacies. *Methods.* We evaluated the frequency of CFC, CFU-F and LTC-IC after HC transplant using standard methods 1 year after transplant. Patients' population was: (i) 24 pediatric patients (12 receiving UCB and 12 receiving adult HC (age 2-14); (ii) 40 adult patients (age 18-65) after intrabone CBT; 42 adult patients after adult BM or PBSC transplant. Chimerism was evaluated at each time point to identify donor *vs.* recipient origin of HC and MSC. *Results.* All patients were fully reconstituted at 1 year from transplant (100% donor), committed component resulted within normal range (CFC frequency/10^4MNC: 21, range=10-81, NBM:56, range 15-85 NS). However, LTC-IC frequency, as well as CFU-F resulted severely impaired (all the comparison were statistically significant):

Table.

	LTC-IC/ 106MNC	*CFU-F/ 106MNC*
CB-IBT adult	2 (0-72)	1 (0-16)
CB-IV pediatric	8.2 (3-15)	10 (0-96)
BMT adult	0.2 (0-4)	4 (0-18)
NBM (control)	33 (14-93)	46 (15-85)

In adult patients HM (MSC) was 100% recipient either they were transplanted with adult cells (BM or PBSC) or CB cell. In pediatric recipints HM origin resulted both of donor and recipient origin mostly when they were transplanted using cord blood cells (75% donor). HC progenitor reconstitution was much superior in recipient of CB cell with respect to adult HC sources. However, again, the reconstitution of hematopoietic progenitor cell reservoir was superior in pediatric recipients that in adults. *Conclusion.* The results indicate that the influence of HM origin on hematopoietic progenitor reconstitution is strongly influenced by the recipient age. In adults recipients, MSC do not engraft independently from HC sources or route of administration, whereas in peaditric recipients a proportion of MSC are of donor origin. In addition. the HM microenvironnement of pediatric patients allow a better LTC-IC (stem cell surrogate) reconstitution. This suggests that the age of recipient HM is a crucial factor affecting reconstitution. Dissecting the cellular component of HM in young subject may contribute to understanding the cellular and molecular mechanisms of the "efficient niche".

C064

PERSONALIZED MODELING BASED GENE SELECTION FOR DEVELOPING AN EARLY ACUTE GVHD DIAGNOSIS SUPPORT SYSTEM

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Introduction. Based on an expression profile, it is possible to set a diagnostic test, so a sample can be taken from a patient, the data related to the sample processed, and a profile related to the sample obtained. We apply this approach here to detect acute graft-versus-host disease (aGvHD) in allogeneic (HSCT). There is not a definitive diagnostic blood test for the aGvHD and its diagnosis is merely based on clinical criteria. *Aim.* Our primary objective was to validate a novel and not invasive method to confirm the diagnosis of aGvHD in HSCT patients at onset of clinical symptoms. The main idea was to create a model for each objective sample, which was able to discover the most important information specifically for this sample. This technique is called personalized modelling and it is a novel and very useful computational intelligence method in medical decision support systems field. *Method.* This study used a Personalized Modeling based Gene Selection method (PMGS) proposed for macroarray data analysis, Figure 1. Fifty-nine HSCT patients were enrolled between March 2007 and July 2009 in transplant unit. We used 26 peripheral blood samples (PBS) from aGvHD (YES) patients that were taken at the time of diagnosis and we selected 33 PBS from patients that didn't experienced aGvHD (NO). All together YES/NO patient groups comprised a validation set. A macroarray was carried out with TaqMan® Low Density Array Fluidic (LDA-custom card).We selected 47 candidate genes involved in immune network and inflammation pathogenesis. *Results.* In the personalized model used, the selected frequency of some gene transcripts was significantly high,

which means they can be recognized highly representative of the data pattern. For example CASP1, FOXP3, ICOS, CD52 are the most important genes for sample 20 and CASP1 was often present in the best subgroups. A leave-one-out cross validation procedure was performed to investigate the robustness of the PMGS method over the training set: in 29 runs the personalized best subset was selected 29 times (100%). From a biological point of view, the results are reliable. It is noteworthy that in our study a set of genes, indicated by computational analysis, included same mediators of Th2 response such as IL10, and signal transducer and activator of transcription 6, interleukin-4 induced (STAT6). All these were strongly down-regulated in aGVHD (YES) setting, suggesting absence of control mediated by Th2 cells. FOXP3 surrogate marker of T regulator cells was the best informative indicator of alloreactive syndrome. *Conclusions.* This study demonstrated, for the first time, that the proposed integrated methodology for the personalized selection of gene diagnostic targets and their use for diagnosis of aGVHD results in a satisfactory 92% accuracy over independent test data set of HSCT population. The author are working to develop a user-friendly software package for clinical testing from medical staff. Grant: Governative Research Program of Minister of the Health and Calabria Region.

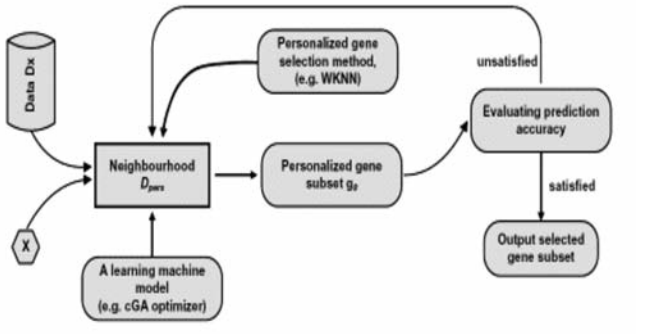

Figure 1. A diagram of personalized modeling based gene selection method (PMGS).

Chronic Myeloid Leukemia

C065

IMPROVING ON IMATINIB FOR TARGETED THERAPY OF CHRONIC MYELOID LEUKEMIA: FIRST LINE TREATMENT WITH NILOTINIB 800 MG DAILY RESULTS IN UNPRECEDENTEDLY HIGH RATE OF RAPID, "DEEP" AND STABLE MOLECULAR RESPONSES - RESULTS OF A PHASE 2 TRIAL OF THE GIMEMA CML WORKING PARTY

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Introduction. Imatinib (IM) 400 mg daily is the standard treatment for chronic myeloid leukemia (CML) in early chronic phase (ECP). Nilotinib is a second generation TKI born to be more potent and selective than IM. *Methods.* To investigate the molecular therapeutic efficacy of nilotinib 400 mg BID in previously untreated, ECP, Philadelphia-positive CML patients, the Italian GIMEMA CML Working Party is conducting an multicenter phase II study (ClinicalTrials.gov NCT00481052). The primary endpoint is the CCgR rate at 1 year. The kinetic of molecular response is being studied by Q-PCR at baseline and after 1, 2, 3, 6, 9, 12, 18 and 24 months from treatment start. *Results.* Seventy-three patients have been enrolled by eighteen GIMEMA centers between June, 2007 and February, 2008. The median age was 51 years (range 18-83), 45% low, 41% intermediate and 14% high Sokal risk. Median follow-up is currently 572 days (range 433-727 days). The CCgR rate at 1 year was 96% (71/73 patients; one patient progressed at 6 months to lymphoid blast crisis because of a T315I mutation and one patient permanently discontinued nilotinib at 9 months, while in major molecular response [MMR], because of grade 4 lipase elevation). A MMR, defined as a BCR-ABL:ABL ratio <0.1% according to the International Scale, was already achieved in 2 patients after only 1 month of nilotinib treatment; this proportion rapidly increased to 21% at 2 months, 52% at 3 months, 66% at 6 months 73% at 9 months and 85% at 12 months. By comparison, the MMR rate reported in the IRIS study for patients receiving first-line IM was 53% at 12 months and reached 80% only at 4 years. The median BCR-ABL transcripts level at 1, 2, 3, 6, 9 and 12 months was 4.704, 0.456, 0.063, 0.018, 0.018 and 0.006, respectively. A complete molecular response (CMR; defined as a BCR-ABL:ABL ratio <0.001%IS and a negative nested PCR) was already achieved in 7 patients at 12 months. The log-reduction in BCR-ABL/ABL level at 3 months was predictive of the probability of MMR at 12 months: a reduction of less than half-log, between half- and 1-log, and more than 1-log corresponded to a probability to obtain a MMR at 12 months of 42%, 62% and 95%, respectively. *Conclusions.* The molecular results achieved in our study strongly support the notion that in ECP CML patients molecular responses to nilotinib are substantially faster and "deeper" than those to IM. More rapid reduction of residual disease might help to reduce failures and to improve the late outcome of therapy.

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C066

PRE-FINAL ANALYSIS OF THE PHASE II EXPLORATIVE STUDY OF INTERMITTENT IMATINIB (IM) TREATMENT (INTERIM) IN ELDERLY PH+ CML PATIENTS WITH STABLE COMPLETE CYTOGENETIC RESPONSE (CCGR) – EUDRACT NUMBER 2007-005102-42, CLINICALTRIALS.GOV NCT 00858806

Russo D,[1] Martinelli G,[2] Malagola M,[1] Colombi C,[1] Rosti G,[2] Amabile M,[2] Fogli M,[2] Mirto S,[3] Turri D,[3] Gobbi M,[4] Pierri I,[4] Vitolo U,[5] Pregno P,[5] Morra E,[6] Pungolino E,[6] Di Raimondo F,[7] Stagno F,[7] Foà R,[8] Alimena G,[8] Breccia M,[8] Nobile F,[9] Martino B,[9] Rambaldi R,[10] Intermesoli T,[10] Saglio G,[11] Rege Cambrin G,[11] Visani G,[12] Nicolini G,[12] De Fabritiis P,[13] Abruzzese E,[13] Fanin R,[14] Tiribelli M,[14] Galieni P,[15] Bigazzi C,[15] Liso V,[16] Specchia G,[16] Angelucci E,[17] Usala E,[17] Musolino C,[17] Russo S,[18] Gaidano G,[19] Lunghi M,[19] Lauria F,[20] Bocchia M,[20] Rodeghiero F,[21] D'Emilio A,[21] Bosi A,[22] Santini V,[22] Quarta G,[23] Girasoli M,[23] Fioritoni G,[24] Di Lorenzo R,[24] Testoni N,[2] De Vivo A,[2] Soverini S,[2] Iacobucci I,[2] Baccarani M[2]

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Objectives. The phase II explorative study of InterIM in elderly Ph+ CML patients who achieved a stable CCgR after at least 2-years standard IM therapy (any dose between 300 and 800 mg/day) was started in April 2008. The accrual of patient was closed in August 2009 since more than 78 patients required by statistics were enrolled. The main objective of the study was to investigate if the CCgR achieved with standard IM therapy could be maintained with the same dose given intermittently (InterIM). After 12 month of InterIM the patients who were in continuous CCgR were advised to continue the study treatment dose but could go back to pre-study daily dose. *Matherial and methods.* Ninety-five patients were enrolled; 7 patients early went off study because of protocol violations; 88 patients were evaluable and out of them 77 (88%), 73 (83%), 65 (74%) and 50 (57%) completed 3, 6, 9 and 12 months of the treatment program, respectively. The CgR status was assessed by Interphase fluorescence *in situ* hybridization (I-FISH) on peripheral blood (≥200 cells counted) every 3 months. When I-FISH (% Ph+ cells) increased more then 1%, chromosome banding analysis (CBA) on bone marrow was performed to confirm the loss of CCgR and to check for additional cytogenetic abnormalities (ACA). *Results.* At the present time, about two-third of the patients (50 pts) completed the trial time (12 months) and none out of the 88 evaluable patients lost CCgR. From baseline to the 12 th month, 95% to 87 % of patients showed a I-FISH negative (0% Ph+ cells); 1% to 6% a I-FISH ≤1% Ph+ cells; and 4% to 11% a I-FISH >1% Ph+ cells. As concern as molecular response, 99% of the patients had a major molecular response (MMR=<0.001-0.1 BCR-ABL/ABLISX 100) at the baseline. The proportion of the patients who maintained the MMR after 3, 6, 9 and 12 months of InterIM was 95%, 92%, 92%, 87%, respectively. *Conclusions.* This interinal analysis based on the evaluation of the two-third of patients who completed the trial time 12 (months) of InterIM, strongly suggest that IM, given intermittently, may be sufficient to maintain the CCgR in all patients studied and the MMR in 85-90% of them. More detailed analysis will be done before the end of 2010 when all patients will complete the trial time and about 25-30% of them will have a follow-up long lasting 18-24 months. *Acknowledgments.* This work was supported in part by CML-Leukemia Net and Progetto Regione Lombardia.

C067

NOVEL GENE MUTATIONS REVEALED BY MASSIVELY PARALLEL SEQUENCING OF THE TRANSCRIPTOME OF A CHRONIC MYELOID LEUKEMIA PATIENT AT DIAGNOSIS AND AT THE TIME OF PROGRESSION TO BLAST CRISIS

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Introduction. Ph+ CML is generally regarded as a genetically heterogeneous disease and Bcr-Abl inhibition results in high response rates. Nevertheless, resistance may develop, especially in High Sokal risk patients, and progression to BC still represents a major concern. *Methods.* We used massively parallel sequencing on a Solexa Illumina Genome Analyzer to scan the transcriptome of a High-Sokal risk CML patient at the time of diagnosis, at the time of remission (major molecular response) and at the time of progression to lymphoid BC. Both custom scripts and published algorithms were used for read alignment against the human reference genome, for single nucleotide variant (SNV) calling, for identification of alternative splicings and for digital gene expression profiling. Sanger sequencing was used for validation and for further screening of a preliminary additional subset of 10 CP CML patients at diagnosis (4 low, 3 intermediate and 3 high Sokal risk) and 10 BC CML patients. *Results.* Comparison of the SNVs identified in the diagnosis and relapse samples with the SNVs detected in the remission sample - representing inherited sequence variants not specific for the Ph+ clone - allowed the identification of nine missense mutations at diagnosis affecting the coding sequence of AMPD3, SUCNR1, FANCD2, INCENP, BSPRY, ZWILCH, HEXDC, KIAA2018 and NUDT9 genes. Six of these mutations (FANCD2, INCENP, BSPRY, ZWILCH, HEXDC, NUDT9) were also detected in the Ph+ clone re-emerged at the time of disease progression, together with six additional missense mutations affecting the coding sequence of IDH2, DECR1, C4Orf14, MRM1, PRKD2 and TCHP genes. Mutations of isocitrate dehydrogenase 1 and 2 enzyme isoforms (IDH1, IDH2), including the same R140Q found in our study, have recently been reported in some de novo acute myeloid leukemias and in patients with leukemia evolved from a Philadelphia chromosome-negative (Ph-) chronic myeloproliferative disease, suggesting that they can be associated with leukemic progression to acute phase both in Ph- and in Ph+ chronic myeloproliferative diseases. AMPD3 (adenosine monophosphate deaminase 3) and KIAA2018 (encoding a protein with predicted DNA binding and transcriptional regulation activity) genes were found to harbour the same point mutations in 1 out of the 3 additional high Sokal risk patients analyzed. Moreover, the same MRM1 (mitochondrial rRNA methyltransferase 1) mutation detected at the time of progression was found in 3/10 additional BC patient patients analyzed. Digital gene expression profiling and alternatively spliced transcripts will also be presented. *Conclusions.* our preliminary data highlighted putative key genes whose deregulation may be recurrent in a subset of CML patients and may be linked to disease pathogenesis or progression. Further investigations in a larger series of patients are ongoing.

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C068

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

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Introduction. The t(9;22)(q34;q11), generating the Philadelphia (Ph) chromosome, is found in more than 90% of patients with chronic myeloid leukemia (CML). At diagnosis, in 5-10% of CML patients the Ph chromosome is derived from variant translocations other than the standard t(9;22). The aim of this study was to perform an accurate breakpoints identification and bioinformatic analysis of other chromosomes involved in variant t(9;22) or in concomitant chromosomal rearrangements apart from the t(9;22). *Methods.* Four hundred and fifty two consecutive CML

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

C069

DECREASED LEVEL OF SHP-1 PROVIDES AN ADDITIVE SURVIVAL ADVANTAGE TO PHILADELPHIA CHROMOSOME-POSITIVE (PH$^+$) CELLS DERIVED FROM PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML) AND ACCOUNT FOR RESISTANCE TO IMATINIB (IMA) TREATMENT

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The majority of patients with CML achieve a deep response to Ima treatment, however, a limited group of them may show or develop resistance to this therapy. While mutations of Bcr/Abl kinase domain as a cause of resistance has been described in around 50% of resistant patients, other mechanisms accounting resistance are still poorly understood. In this study, we investigated the protein-tyrosine phosphatase SHP-1, a protein with a tumour suppressor activity, as a possible determinant for resistance to Ima in an *in vitro model* constituted of the KCL22 Ph$^+$ cell lines (sensitive/KCL22-S and resistant/KCL22-R) and in patients with CML. In particular, the resistant phenotype is associated with low level of SHP-1 expression and lacks of its interaction with the proto-oncogene SHP-2, another protein-tyrosine phosphatase. Thus, consistently with epigenetic regulation of the SHP-1 expression, we found that aberrant methylation of its promoter is fundamental for the down-regulation detected in KCL22-R compared to KCL22-S cell line (0.006±0.004 *vs.* 0.8±0.2 SHP1/ABL copy numbers). Then, immunoprecipitation assay shown that in our model one of the main interactors of SHP-1 is SHP-2, that, differently from SHP-1, acts as a positive regulator of Ras/MAPK pathway. The most likely mechanism for SHP-2 activation is the posphorylation of a single tyrosine residue, Tyr-542, that has been demonstrated to be sufficient for activating MAP kinase pathway. Using western blot analysis we found that Ima exposure reduce SHP-2 Tyr-542 posphorylation only in sensitive KCL22-S but not in resistant KCL22-R cell line, where SHP-1 is expressed at lower levels. Therefore, we assumed that SHP-1 might have a negative role on SHP-2 activation modulating its Tyr-542 phosphorylation. To evaluate the functional role of SHP-1, we forced its expression in KCL22-R and assessed that this phosphatase restores Ima sensitivity, since KCL22-RSHP-1$^+$ ceases to proliferate after Ima exposure and concurrently SHP-2 is detected in the inactivated status. Consistently with this finding, SHP-2 knocking-down in KCL22-R decreases cell viability after Ima exposure and induces a significant reduction of activation status of STAT3 (60%) and ERK1/2 (70%). Both KCL22-RSHP-1$^+$ and KCL22-RSHP-2- treated with 2nd generation of TKIs (Nilotinib and Dasatinib) show a similar response of the parental sensitive KCL22-S treated with Ima, thus confirming that our model system could be even applied to study the resistance to 2nd generation TKIs. The role of SHP-1 as determinant of Ima sensitivity was further corroborated by the expression data in bone marrow (BM) samples of 60 consecutive untreated CML patients. The level of SHP-1 mRNA is significantly lower in patients classified as failure responder according to the ENL criteria, than optimal responders (p less than 0.0001). Thus, our data indicate that SPH-1 in Ph$^+$ cells may modulate their sensitivity to Ima treatment through the regulation of the activity of SHP-2.

C070

BCR-ABL TYROSINE KINASE ACTIVITY MODULATES THE PHOSPHORYLATION, LOCALIZATION AND FUNCTION OF INTERFERON REGULATORY FACTOR 5 (IRF-5) IN CHRONIC MYELOID LEUKEMIA CELLS

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Introduction. Interferon Regulatory Factor 5 (IRF-5), is a nuclear protein which modulates the immune response against viral infections and cancer. IRF-5 is a transcription factor with tumor suppressor-like properties that regulates the expression of several genes including Bak, Bax, p21 and caspase-8. Aim of this research was to study the relationship between IRF-5 and the BCR-ABL oncoprotein of Chronic Myeloid Leukemia (CML). *Methods.* We assessed IRF-5 expression by immunoblot on both primary cells derived from CML patients and three immortalized cell lines (K562, KCL22 and KYO-1). We also investigated the association between IRF-5 and BCR-ABL using immunoprecipitation assays before and after treatment with the kinase inhibitor Imatinib Mesylate (IM). IRF-5 intracellular localization was investigated through fractionation experiments while growth in soft agar or in methylcellulose media was used to determine the transforming activity of CML cells expressing different IRF-5 constructs. *Results.* We found that IRF-5 is expressed in both primary and immortalized CML cells. Co-immunoprecipitation assays demonstrated that IRF-5 associated with BCR-ABL and was a target of the oncoproteins kinase activity. In CML cells, IRF-5 was mostly confined to the cell cytoplasm. However, treatment with IM or with alpha-Interferon (IFN) relocalized IRF-5 to the nucleus suggesting that BCR-ABL modulated its nuclear-cytoplasmic shuttling. Mutagenesis of IRF-5 tyrosine 104 to phenylalanine generated a mutant that displayed reduced levels of tyrosine phosphorylation. In addition, IRF-5 Y104F mainly localized to the cell nucleus, confirming that tyrosine-phosphorylated IRF-5 is preferentially cytoplasmic. Finally, over-expression of IRF-5 Y104F significantly reduced proliferation and foci formation of both CML cell lines and CD34$^+$ cells isolated from four patients in chronic phase and one in blast crisis. *Conclusions.* Our findings demonstrate that BCR-ABL associates with IRF-5 and causes its phosphorylation on tyrosine 104, thereby preventing IRF-5 nuclear localization and transcriptional activity. Pharmacological strategies aimed at disrupting the interaction between BCR-ABL and IRF-5 may represent a novel approach to reduce the proliferation of CML cells.

C071

THE SHP1 LEVEL IS A NOVEL POWERFUL PREDICTOR OF MAJOR MOLECULAR RESPONSE (MMR) ACHIEVEMENT IN CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB

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The notion that BCR-ABL kinase is central to the pathogenesis of CML has provided the rationale for the therapeutic use of Ima, a tyrosine kinase inhibitors, that has rapidly become the standard front-line therapy for CML. However, molecular techniques indicate the persistence of variable amounts of BCR-ABL transcripts in the vast majority of patients under treatment and it is estimated that approximately 20-30% of patients will eventually develop resistance to Ima. However, although the outstanding progress in the treatment and management of CML, still today, Sokal index remains a dominant prognostic determinant of newly diagnosed CML patients. This study has explored in a large multicentric clinical trial the prognostic role of the SH2-containing protein tyrosine phosphatase SHP-1 expression assessed in CML patients at baseline. We already have shown in an *in vitro* model that this phosphatase has an important role to determine the cellular response to Ima in Ph+ cells in terms of both apoptosis and proliferative rates. To this aim we evaluated SHP-1 mRNA levels in peripheral blood mononuclear cells of 93 patients who were enrolled in the TOPS (Tyrosine Kinase Inhibitor OPtimization and Selectivity) study, a multicentre trial designed to compare outcomes of patients with newly diagnosed CML randomized 1 to 2 to receive 400mg/d (standard dose) *vs.* 800mg/d (high dose) Ima therapy. The outcome was assesses on the basis of Major Molecular Response (MMR) achievement after 12 months of treatment (primary end-point of the study). All patients were followed-up for at least 12 mo. No differences in baseline clinical features were found in patients randomized to the standard- *vs.* high-dose arms of the study. MMR rates were not significantly different between the two arms. Our results indicate that mRNA levels of SHP1, as assessed baseline by QPCR in peripheral blood of patients are significantly higher in patients who achieve MMR by 12 months (7.9±4.0 *vs.* 5.9±3.4; P=0.01). Logistic regression was used to estimate the odds rate of all clinical variables that may have influence on the outcome, including treatment arm and Sokal index, using MMR by 12 months as outcome variable in our model. Statistical analysis shown that a value of 4.1 or more in SHP1 is associated with almost 2-fold odds of achieving MMR by 12 months (OR=1.92; 95% CI=1.12, 3.29; P=0.018), and that SHP-1 is an independent variable respect to the Sokal index to MMR achievement. Indeed, those patients with high-risk Sokal associated to low SHP1 expression have 0% of probability to achieve MMR while those with low SHP1 expression and low-intermediate Sokal score have 79% probability to not be in MMR after 12 months of treatment and the probability of patients with high SHP-1 and low-intermediate Sokal score to achieve MMR is around 52% (P=0.0061 after chi square analysis of contingency table). In conclusion, these results suggest that, SHP-1 expression analysis may be used as baseline indicator of probability to obtain an early MMR to Ima treatment in CML patients.

C072

COMPUTATIONAL AND EXPERIMENTAL CHARACTERIZATION OF TWO NUCLEAR LOCALIZATION SIGNALS AND A NUCLEAR EXPORT SIGNAL THAT MODULATE BCR INTRACELLULAR LOCALIZATION

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Introduction. The Breakpoint Cluster Region (BCR) gene contributes to the pathogenesis of Chronic Myeloid Leukemia (CML) by fusing its first 12 or 13 exons to most of the coding sequence of the ABL gene. Unlike ABL, little is known about the intracellular localization of the BCR protein. Previous evidence suggested that BCR binds to heterochromatin, implying that it may be capable of nuclear import. Yet, further data assigned BCR exclusively to the cell cytoplasm. We therefore decided to investigate the mechanisms regulating the intracellular localization of BCR and to determine if and how they applied to the BCR-ABL chimaeric oncoprotein. *Methods.* Different computational analyses were carried out to identify putative Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES) in the BCR protein. The localization of both NLS and NES GFP-fusion constructs was evaluated by immunofluorescence (IF). Multiple BCR and BCR-ABL deletion mutants were then transiently expressed in HeLa cells or in the mouse pro-B Baf-3 cell line and their intracellular distribution was also determined by IF. Baf-3 cells expressing various BCR-ABL mutants were also cultured in liquid media (in the absence of IL-3) or in soft agar to investigate their different transforming ability. *Results.* We computationally identified two NLSs and a single NES in the BCR protein sequence. Both NLS sequences directed GFP in the nuclear compartment, while the NES favored the cytoplasmic localization of GFP. The latter effect was abolished after treatment with the exportin-1 inhibitor Leptomycin B (LMB). Despite these findings, wild-type BCR was an exclusively cytoplasmic protein because of a lack of nuclear import. However, removal of the N-terminus oligomerization domain (OD) and of the C-terminus Protein Kinase C conserved region 2 (DC2) and Rho-GAP domains generated a construct displaying strong nuclear staining that was further increased by LMB. Since only the OD and part of the DC2 are preserved in the BCR-ABL oncoprotein, we wanted to establish if these domains contributed to the subcellular localization of BCR-ABL. Hence, we assembled a catalytically-proficient ΔDO and ΔDC2 BCR-ABL construct that was transiently expressed in HeLa cells. This mutant failed to relocalize to the cell nucleus but did not display transforming activity in Baf-3 cells, leading instead to cell death. *Conclusions.* Our results suggest that BCR displays two putative NLS and one potential NES, which are inhibited by the OD, DC2 and Rho-GAP domains. A kinase-active BCR-ABL construct devoid of these regions retains its cytoplasmic localization but does not display transforming activity, inducing massive cell killing.

Acute Leukemias II

C073

RESIDUAL BONE MARROW CD34+ CELLS AFTER INDUCTION CHEMOTHERAPY IN PATIENTS WITH NPM1-MUTATED AML DO NOT BELONG TO THE LEUKEMIC CLONE

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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 [Falini B *et al.*, Blood 2007;109:874-885] which include negativity for CD34 antigen expression at both immunohistochemistry and gene expression profiling. At diagnosis, flow cytometric analysis shows that, in most NPM1-mutated AML, percentages of CD34+ cells are in the low range (<5-10%). We previously reported the small CD34+ cell fraction in NPMc+ AML harboured NPM1 mutant protein and gene, indicating they belong to the leukemic clone [Martelli MP *et al.*, Blood (ASH Annual Meeting Abstracts), 2008, 112: 307; and 2009, 114: 480]. Moreover, flow cytometric analysis showed CD34+ cells exhibited the phenotype CD34+/CD38-/CD123+/CD33+/CD90-, characteristic of leukemic stem cell (LSC). When transplanted into immunocompromised mice, CD34+ but not CD34- cells generated a leukemia recapitulating, both morphologically and immunohistochemically (aberrant cytoplasmic nucleophosmin, CD34-), the original patient's disease. After induction chemotherapy, we observed immunohistochemical examination of bone marrow biopsies from some patients displayed scattered CD34+ cells of uncertain nature in bone marrow. Bone marrow smears from the same patients showed immature-looking cells ranging between 6 and 9 %, questioning achievement of complete remission. To establish the nature of these cells, in 3 patients we purified by MACS cell sorting CD34+ cells from bone marrow after induction chemotherapy (purity 86.3, 86 and 67%, respectively). Unlike what was observed at diagnosis, flow cytometric analysis showed CD34+ cells contained CD38- cells which were also CD123 and CD33-, a phenotype characteristic of the normal hemopoietic stem cell (HSC). Western blot with antibodies specific for mutated NPM1 protein and genomic DNA fragment analysis showed absence of NPM1 mutant protein and gene, respectively, in either CD34+ or CD34- purified cell fractions from all 3 patients (e.g. in Figure 1). These data suggest CD34+ cells detectable after induction chemotherapy do not represent residual CD34+ LSC, but normal CD34+ cells indicative of a regenerating bone marrow. Importantly, evaluation of minimal residual disease (MRD) by real-time RT-PCR on correspondent bone marrow samples showed low copies number of NPM1 mutant in all cases (<0.1 NPM1/Abl %), confirming achievement of a good complete hematological remission.

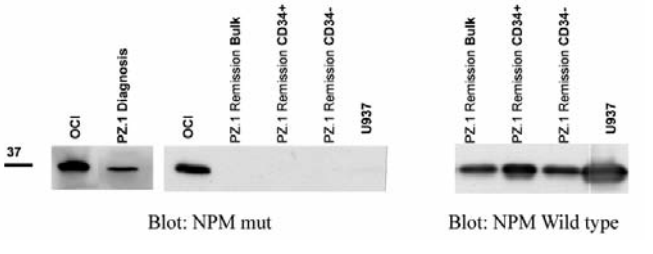

Figure 1.

C074

PURINERGIC SIGNALLING INHIBITS HUMAN ACUTE MYELOBLASTIC LEUKEMIA CELL PROLIFERATION, MIGRATION AND ENGRAFTMENT IN IMMUNODEFICIENT MICE

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Introduction. Extracellular nucleotides ATP and UTP are emerging as ubiquitous molecules involved in a wide variety of biological responses and their biological effects are mediated by specific plasma membrane receptors, P2 receptors (P2R). Previously, we showed that extracellular nucleotides stimulate the proliferation and engraftment potential of normal human hematopoietic stem cells. In this study, we assessed whether P2R are expressed on acute myeloblastic leukemia (AML) cells and whether their engagement modulates leukemic cell functions. *Methods.* Purinergic receptors transcript levels were evaluated with quantitative real-time PCR. Affymetrix Human HG-U95Av2 GeneChip arrays were used to investigate molecular profiling. Migration toward a nucleotide gradient was determined by a 24-well transwell assay. Growth of CFU-C was assayed using methylcellulose culture. Leukemic stem cells were isolated by cell sorting. Xenotransplant experiments were performed using NOD/SCID/IL-2Rγ-Null immunodeficient mice. *Results.* We show that AML cells expressed several and functional P2R subtypes at molecular and protein level. Stimulation of AML cells by extracellular nucleotides (ATP, UTP, BzATP) induced intracellular Ca^{2+} concentration increases. Furthermore we identified a number of genes significantly modulated by ATP treatment. Gene expression profiling revealed that leukemic cells stimulated with ATP underwent a down-regulation of genes involved in cell proliferation and migration whereas those involved in cell cycle inhibition were strongly up-regulated. At the functional level, purinergic signalling inhibited cell cycling and induced apoptosis. Moreover, the clonogenic efficiency of leukemic cells was significantly reduced by the addition of ATP and, to a higher extent, by the stable analogs INS415 and INS973. We also observed a pronounced inhibitory effect of triphosphate nucleotides on spontaneous migration of AML cells and in response to CXCL12. *In vivo*, xenotransplant experiments demonstrated that the homing and the engraftment capacity of human AML stem cells to NOD/SCID/IL-2RGamma-Null mice bone marrow was significantly inhibited by pre-treatment with ATP, UTP and INS415 and INS973 analogues. The same results was observed using purified CD34+/CD38- leukemic stem cell. *Conclusion.* Thus, our data show that, conversely to normal stem cells, purinergic signalling inhibits leukemic cell proliferation, migration and engraftment. Characterization of purinergic signalling in leukemia may help the better understading of the mechanism of neoplastic transformation and tumor progression.

C075

IDENTIFICATION OF A NOVEL P190-DERIVED BREAKPOINT PEPTIDE SUITABLE FOR PEPTIDE VACCINE THERAPEUTIC APPROACH IN PH+ ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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Introduction. Ph+ acute lymphoblastic leukemia (Ph+ALL) is a high-risk acute leukemia with poor prognosis, in which the specific t(9;22)(q34;q11) translocation results in a chimeric bcr-abl (e1a2 breakpoint) and in a 190 KD protein (p190) with constitutive tyrosine kinase activity. The advent of first and second generation tyrosine kinase inhibitors (TKIs) improved the short term outcome of Ph+ALL patients not eligible for allo-SCT; yet disease recurrence is almost inevitable. Peptides derived from p190-breakpoint area are leukemia-specific antigens that may mediate an antitumor response toward p190+ leukemia cells. Of note, Riva *et al.* (Blood, 2010) have recently found "natural" BCR-ABL breakpoint specific cytotoxic T lymphocytes (CTLs) in the bone marrow of Ph+ ALL patients treated with imatinib correlating with a better

response to this TKI. These recent evidences together with the encouraging results observed with therapeutic p210 derived peptide vaccine for CML reinforced the rationale of developing a breakpoint peptide vaccine in Ph+ ALL patients as immune target therapy synergistic with TKIs. In particular, we searched for p190 breakpoint peptides strongly inducers of a peptide-specific CD4+ T cell response, yet including in their sequences many class I epitopes for CTLs response. *Methods.* We employed standard peptide-HLA binding databases to investigate the p190 fusion region for novel 25mer peptides with strong HLA class II binding prediction and with class I epitopes in their sequence. All promising peptides were synthesized and purified by HPLC for *in vitro* use. The capability of identified peptides to induce peptide-specific CD4+ T cells was performed with CD4+ T cells freshly isolated from PBMC. CD4+ cells were cultured for 21 with autologous CD14+ cells and different peptides in the presence of IL-15. *Results.* Three promising 25mer long fusion peptides named p190-13, p190-15 and p190-17 were identified with strong HLA binding properties for HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*1101 and HLA-DRB1*0301(DR17). Only p190-13 peptide (PDNGEGAFHGDAEALQRPVASDFEP) was able to induce peptide specific CD4+ T cell proliferation in all 5 healthy donors tested as measured by standard 3HThymidine assay, with a stimulation index (SI) ranging from 2.0 to 2.5 (SI= cpm CD4+ T cells + test peptides/CD4+ T cells + control peptides; proliferation was considered positive for SI≥2). We tested p190-13 immunogenicity also in 2 Ph+ALL patients in complete remission during imatinib treatment and both showed an anti-p190-13 CD4+ T cell response. *Conclusions.* In conclusion novel identified p190-13 25mer peptide is able to induce *in vitro* a peptide-specific CD4+ T cell response in imatinib treated Ph+ALL patients. In addition this peptide includes all the epitopes for which p190-specific CTLs were naturally found in the same setting of patients. Thus it appears a good candidate for developing an immune target vaccine strategy possibly synergizing with TKIs for remission maintenance.

C076

A POLYMORPHISM IN THE CHROMOSOME 9P21 ANRIL LOCUS IS ASSOCIATED TO PHILADELPHIA POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA SUSCEPTIBILITY

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Introduction. Little is known about alterations of cyclin dependent kinase inhibitors p15INK4B, p16INK4A and p14ARF due to single nucleotide polymorphisms (SNPs) located within the CDKN2A/B genes and/or neighbouring loci. In order to investigate the potential involvement of such common DNA sequence variants in leukemia susceptibility, an association study was performed. *Methods.* 23 SNPs spanning the MTAP, CDKN2A/Band CDKN2BAS loci, as well as relative intergenic regions were genotyped in a case-control cohort made up of 149 leukemia patients, including Philadelphia positive (Ph+) acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) samples, and 183 healthy controls. 6 SNPs were selected on the basis of their previous association with several diseases, such as coronary artery disease (rs2891168, rs518394, rs564398, rs10757278), type 2 diabetes mellitus (rs564398), frailty (rs2811712). The remaining 17 SNPs were selected to deepen the SNPs coverage for the examined region. Genotyping was performed using iPLEX Gold technology and MassARRAY high-throughput DNA analysis with Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, Inc., San Diego, CA). *Results.* A total of 17 SNPs, spanning the 9p genomic interval that encompasses the MTAP, CDKN2A/B and CDKN2BAS loci, were successfully genotyped and used for investigating their potential associations with the leukemia phenotypes. Five SNPs (rs1012713, rs10965179, rs34011899, rs3731232, rs3218010) with MAF <0.05 in cases and controls, as well as one SNP (rs3931609) showing >20% missing call rates, were instead excluded from the association analysis and potential population stratification affecting the control sample was ruled out as its genotypes distribution satisfies the Hardy-Weinberg equilibrium criterion. Among the 17 SNPs, rs564398, mapping to the CDKN2BAS locus that encodes for ANRIL antisense non-coding RNA, showed a statistically significant correlation with the ALL phenotype, with a risk pattern that was compatible with an overdominant model of disease susceptibility and a OR of 2 (95% CI, 1.20 to 3.33; $P=7.1\times10^{-3}$). *Conclusions.* Since a co-ordinated regulation of ANRIL and p14/ARF, p16/CDKN2A, p15/CDKN2B transcription has been already observed in both physiologic and pathologic conditions, we hypothesized that rs564398 association reflects a condition of high linkage disequilibrium between such polymorphism and a causative variant that is able to alter CDKN2A/B expression profiles by changing ANRIL dosage, thus leading to abnormal proliferative boosts and consequent increased ALL susceptibility.

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C077

CYTOPLASMATIC PTEN LOCALIZATION IS ASSOCIATED WITH MUTATED NUCLEOPHOSMIN (NPMC+) IN ACUTE MYELOID LEUKEMIA

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Introduction. PTEN (phosphatase and tensin homologue deleted in chromosome 10) is a dual lipid and protein phosphatase with cytoplasmatic and nuclear localization. Its involvement in basic cellular functions appears to be ubiquitous in normal tissues. As integrant of signal transduction networks operating in human cells it exerts diverse functions according to its localization via the interaction with specific partners. PTEN has been described as lost or mutated in a number of human solid tumors, while it is seldom mutated or deleted in acute myeloid leukemia (AML). The localization balance between nuclear and cytoplasmatic PTEN clearly affects its tumor-suppressor function and can lead to the development of neoplasia. *Methods.* We investigated PTEN localization by immunofluorescence in 102 the novo AML and 30 APL diagnostic samples. The samples were characterized at molecular level for NPM1 and FLT3-ITD mutations. To evaluate a possible direct interaction between NPMc+ and PTEN, immunoprecipitation assays were done in OCI-AML3 cells (which carries an exon-12 NPM1 mutation A) and in Hek 293 cells after transient transfection with the plasmid construct pEGFP and pEGFP-NPM-Mut A. Moreover, we explored PTEN influence over the activity of the PI3K/Akt/mTOR signal pathway in 17 AML samples, 6 with mainly cytoplasmatic PTEN and 11 with both nuclear and cytoplasmatic PTEN by western blot phosphorylation levels of both p-Akt (Ser 308 p-Akt) and p-4EBP-1 (Thr37/46). *Results.* In normal bone marrow, CD34 positive cells have both cytoplasmatic and nuclear PTEN localization. Twenty-four/30 (80%) of APL samples had mainly cytoplasmatic PTEN. Among AMLs 46/102 (45%) of samples showed cytoplasmatic PTEN although, in NPMc+ AML patients, this percentage raised to 25/34 (73%). The association of mainly cytoplasmatic PTEN and NPMc+ was statistically significant (P<0.0001). As showed by immunoprecipitation and transfection assays, there was not direct interaction between the two proteins and the mechanisms responsible for PTEN localization in NPMc+ AML remains to be clarified. Phosphorylation of both p-Akt (Ser 308 p-Akt) and p-4EB-P1 (Thr37/46) showed lower levels in all samples with mainly cytoplasmatic PTEN, except 2 cases which carried a FMS-like tyrosine kinase (FLT3) internal tandem duplication (ITD). *Conclusion.* It is known that the influence of PTEN on crucial cell function apparatus depends on the protein localization, which in turn is determined by its conformational status. Our results suggest a consistent link between PTEN localization and the mutational status of NPM protein in AML cells. Because cytoplasmatic PTEN reduces the activity of

PI3K/Akt/mTOR pathway, we hypothesize that PTEN delocalization into the cytoplasm in NPMc+ AML contributes to the better response to therapy and more favorable outcome of this leukemia subset.

C078

PROGNOSTIC ROLE OF MINIMAL RESIDUAL DISEASE DETECTION AS DETERMINED ON AN AGE-DEPENDENT BASIS, IN ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Although remarkable progress has been made over the last 2-3 decades in the therapy of acute myeloid leukemia (AML), two thirds of young adults still die of their disease. Results for older adults, who represent the majority of patients with AML, are even more unsatisfactory. In fact, while 40-50% can achieve a complete remission (CR), less than 10% are long-term survivors. Based on this, age is universally recognized, together with performance status and cytogenetic/genetic characteristics of the leukemic clone, as a pivotal factor affecting the choice of treatment and prognosis. In consecutive series of adult patients with de novo AML, we have repeatedly demonstrated the prognostic role of minimal residual disease (MRD) detection, by flow cytometry. In particular, we have found that a level of MRD ≥ 3.5×10^{-4} leukemic cells at the end of consolidation is associated with a relapse rate of 70-80%. In the present study we evaluated whether the prognostic impact of MRD detection remained unaltered even in age-stratified (<60 and >60 years) populations of adult patients with de novo AML. For the purpose of this work, we analyzed 131 young adults (median age 46, range 18-71) and 53 elderly adults (median age 67, range 61-78). All patients under study, achieved CR after induction therapy of EORTC/GIMEMA protocols AML10, LAM99P and AML12 (for patients <60 years) or AML13, AML15A and AML17 (for patients >60 years) The two cohorts were well balanced in terms of FAB subtypes, MDR1 expression, frequency of FLT3-ITD and NPM1 mutated cases. A lower frequency of good-risk karyotypes was observed in elderly *vs.* young patients (4% *vs.* 20%, P=0.010). Significantly, the incidence of minimal residual disease (MRD) negativity was lower in elderly patients (13% *vs.* 32%, P=0.008). Among 46 MRD positive patients, 38 (83%) have relapsed and 6 (17%) have not; among 7 MRD negative, 4 (57%) have relapsed and 3 (43%) have not: thus, despite the lower incidence, post-consolidation MRD negativity might confer a survival advantage. In fact, MRD status after consolidation had a statistically significant impact both on OS and RFS (P=0.001 and P=0.022, respectively), with MRD negative patients projected to a long term survival >30% both for OS and RFS. On the opposite, Among 89 MRD positive younger patients, 51 (57%) experienced a relapse and 38 (43%) have not whereas among 42 MRD negative, 9 (21%) have relapsed and 33 (79%) are in continuous remission. In this subset also, we observed a significantly better outcome for post consolidation MRD negative patients both for OS (62% *vs.* 19%, P<0.001) and DFS (60% *vs.* 7%, P<0.001). In conclusion, among the category of elderly adults, flow-cytometric MRD negativity defines a subgroup of patients with a rate of relapse which is half that of MRD positive patients (17% *vs.* 43%). Nevertheless, elderly patients infrequently become MRD negative but even when MRD negativity is obtained, rate of relapse remains considerable as compared to the younger counterpart (57% *vs.* 21%), confirming that age represents by itself a poor-risk features in AML.

C079

JAGGED-1, JAGGED-2 AND DLL-1 ARE THE ELEMENTS OF NOTCH SIGNALING MAINLY RESPONSIBLE FOR THE ANTIAPOPTOTIC EFFECT ON B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA EXERTED BY BONE-MARROW MESENCHYMAL STEM CELLS

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Background. Notch signaling plays an important role in promoting self-renewal in hematopoietic stem cells, and has a crucial pathogenetic role in the t(7;9)(q34;q34.39) T-cell acute lymphoblastic leukemia (ALL). Its role in B-ALL, on the other hand, is widely unknown. *Aim.* to evaluate the role of the Notch pathway in the survival of B-ALL cells in coculture with bone marrow mesenchymal stem cells (BM-MSCs). *Methods.* MSCs were expanded *in vitro* from BM samples of healthy donors. At passage 3-4 they displayed a homogeneous mesenchymal immunophenotype and showed multipotent differentiation ability. B-ALL cells were obtained by density gradient centrifugation from BM samples of 5 newly-diagnosed patients with high blast count (mean purity: 91.2%, range: 82-97), and were cocultured with BM-MSCs at 10/1 ratio for 3, 7 and 28 days. Apoptosis of B-ALL cells was evaluated by Annexin-V/7-AAD staining, while proliferation was assessed with the CFSE method and flow cytometry. Shifts in the expression of all ligands and receptors of Notch signaling (i.e. Jagged-1, -2, DLL-1, -3, -4 and Notch-1, -2, -3 and -4) were monitored over time. *Results.* At each time point the number of surviving B-ALL cells was increased by the coculture with BM-MSCs (day 3: 56.5±1.6% *vs.* 73.5±0.2%, P<0.001; day 7: 15.8±2.7% *vs.* 58.0±1.4%, P<0.001; day 28: 4.6±1.5% *vs.* 30.6±2.1%, P<0.001). This finding was mostly due to a dramatic reduction in apoptosis, and was reproduced on B-ALL cells cultured alone by the addition of soluble recombinant Notch ligands Jagged-1, Jagged-2 and DLL-1. The expression of Notch-1, Notch-3, Notch-4 and Jagged-1 by B-ALL was markedly upregulated by the coculture with BM-MSCs. On the other hand, Notch-3 and -4 were upregulated on BM-MSCs. Notch-2, already at low levels at basal conditions on both cell types, became undetectable from day +3 onwards. The expression of Jagged-2, DLL-1, -3 and -4 did not significantly change in any condition. When we blocked downstream the Notch pathway using -secretase inhibitor XII, B-ALL cells were all dead at day +3 if cultured alone, while 42.5±0.5% of the total population was still alive in the coculture with BM-MSCs. We next blocked single Notch receptors and ligands through specific neutralizing antibodies: 61.2±0.4%, 51.4±0.5% and 30.8±0.8% of B-ALL cells were still alive with neutralizing anti-Notch-1, -3 and -4, respectively. The single blocks of Notch ligands Jagged-1, -2 and DLL-1 significantly reduced the survival of B-ALL cells either cultured alone and co-cultured with BM-MSCs, whereas the blocks of DLL-3 or DLL-4 had little effects on cell survival. We managed to obtain a level of inhibition of the antiapoptotic effect exerted by BM-MSCs comparable to the use of -secretase inhibitor only by using the combined blocks of Jagged-1, -2 and DLL-1 (Figure 1). *Conclusions.* Jagged-1, -2 and DLL-1 are the fundamental triggers of the anti-apoptotic effect exerted by BM-MSCs on B-ALL cells through activation of Notch signaling.

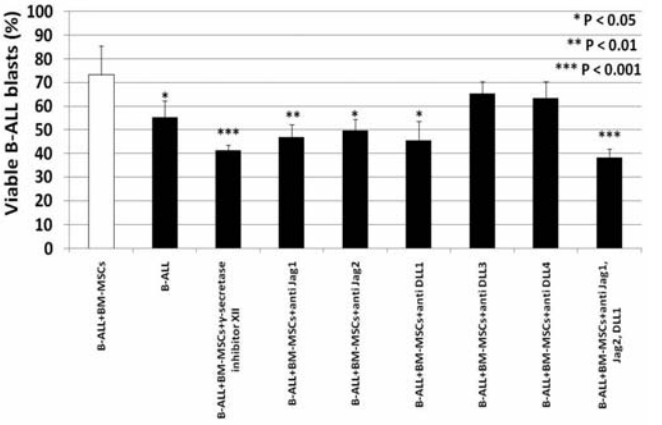

Figure 1. Coculture of B-ALL blasts and BM-MSCs (day+3).

C080

MINIMAL RESIDUAL DISEASE MONITORING BY QUANTITATIVE ASSESSMENT OF WT1 GENE EXPRESSION IN ACUTE MYELOID LEUKAEMIA

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WT1 over-expression is described in several oncological diseases

including acute myeloid leukemia (AML). Quantification of WT1 in bone marrow samples may be useful as a marker of minimal residual disease (MRD) and may predict the relapse of AML also after hematopoietic stem cell transplant (HSCT). *Methods and results.* The quantitative expression of WT1 was measured, using the WT1 ProfileQuant kit (Ipsogen), in 150 AML patients at diagnosis (median age 58, range 14-89), WT1 was over-expressed in BM above background (defined as 250 copies/10^4ABLcopies in BM according to Cilloni, JCO 2009) in 144/150 cases (96%) of AML at diagnosis, with a median value of 8097 copies WT1/ 10^4 ABL (range 500-5000000). There was no significant difference in WT1 expression value between AML aged over 65 years and the younger. Monitoring of MRD was evaluated in a cohort of 52 AML, we consider as MRD positive the detection of WT1 transcripts level exceeding the upper normal values. Twenty-four patients, valuable for MRD, were treated with conventional chemotherapy schedules: 9 patients relapsed and all of them showed MRD positivity and a significant increase in WT1 expression before relapse, 11 MRD negative patients maintained a stable complete cytologic remission (CcR) condition (mean follow up of 12 months) and 4 MRD positive patients are in CcR after a mean follow up of 9, 8, 8 and 6 months respectively. Only 10 patients, in the chemotherapy group, showed at diagnosis WT1 levels exceeding 20000 copies/10^4 ABL copies, allowing discrimination of at least a 2-log reduction compared with the pre-treatment level, 6 of them achieved over 2 log reduction after induction therapy obtaining a persistent CcR; 4 cases did not achieved 2 log of WT1 reduction post-induction, 3 of them relapsed and the last patient is actually MRD^+ in RC at 9 months of f.u. Twenty-eight patients underwent to HSCT, four of them received a double transplant , a total of 32 transplant procedures were evaluated (20 allogeneic and 12 autologous transplant). After HSCT, a rapid decline in WT1 expression levels was observed in all patients who attained or maintained a condition of CcR. Nine patients relapsed (2 of them extra B.M.) after HSCT and all of them had a significant increase in WT1 expression before relapse. We found a complete concordance between WT1 expression levels and other disease markers for both groups of therapy (when available). *Conclusions.* As demonstrated by Cilloni *et al.* (2009) WT1 is a useful early marker to predict risk of relapse after induction therapy in patients showing high copy number of WT1 at diagnosis. Moreover in our experience WT1 expression is useful for monitoring MRD in more than 95% of AML patients, even in patient showing less than 20000 copies/10^4 ABL at diagnosis; the persistence of MRD positivity or an increase of WT1 value is a confident parameter to predict AML clinical relapse.

Chronic Lymphocytic Leukemia

C081

THE GENETICS OF RICHTER SYNDROME IDENTIFIES DISEASE HETEROGENEITY AND IS AN INDEPENDENT PREDICTOR OF SURVIVAL POST TRANSFORMATION

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Background. A fraction of CLL transforms to Richter syndrome (RS), generally represented by diffuse large B-cell lymphoma (DLBCL). Knowledge of the genetic lesions driving RS transformation is scant. *Aim.* We aimed at exploring the pattern and prognostic impact of RS molecular lesions. *Methods.* Upon pathological review, all RS (n=86) were classified as DLBCL. Candidate genetic lesions were selected among those recurrently affecting de novo DLBCL and CLL. Mutational analysis of TNFAIP3/A20, CARD11, BLIMP1, CD79A/CD79B, EZH2, BCL6, c-MYC, PAX5, TTF, PIM1 and TP53 was performed by Sanger sequencing. Probes used for FISH were: i) LSI13, LSID13S319, CEP12, LSIp53, LSIATM, LSI IGH/BCL2, LSI BCL6, LSI IGH/c-MYC/CEP8, c-MYC breakapart, LSI N-MYC; BCL3 split signal; iii) 6q21/alpha-satellite; iv) BAC clones 373L24-rel and 440P05-BCL11A. Clonal relationship between CLL and RS was assessed by immunoglobulin gene analysis.

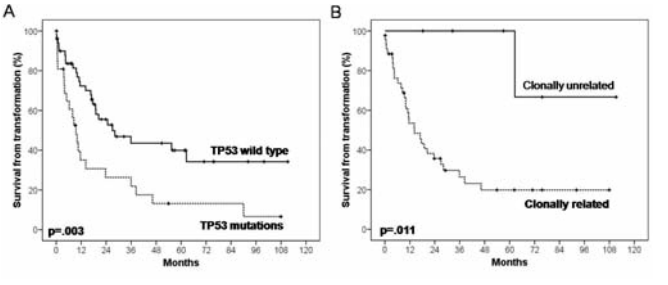

Figure 1.

Results. MUM1 was expressed in 78.3% RS, BCL6 in 28.0%, CD10 in 5.3%. IG were unmutated in 65.5% cases. TP53 mutations occurred in 31/85 (36.5%) RS, CARD11 mutations in 5.3%, BCL6-intron1 mutations in 7.1%, c-MYC mutations in 3.6%, PIM1 mutations in 10.7%, PAX5 mutations in 7.1%, and TTF mutations in 14.3%. Mutations of TNFAIP3/A20, BLIMP1, BCL6-exon1, CD79A/CD79B, and EZH2 were consistently absent in RS. c-MYC abnormalities occurred in 26.2% RS (translocation: 16.4%; amplification: 9.8%), BCL2 amplification in 10.5%, BCL6 amplification in 7.0%, and BCL6 translocation in 1.8%. 17p13 deletion occurred in 27.1% RS, 11q22-q23 deletion in 16.0%, +12 in 12.1%, 13q14 deletion (MIR15/16B) in 13.7%, 6q21 deletion in 8.3%, MYCN amplification in 3.8%, and BCL3 translocation in 2.1%. TP53 disruption by mutation and/or deletion occurred in 48.3% cases. Analysis of paired clonally related CLL/RS showed that TP53 disruption, c-MYC activation and CARD11 mutations are acquired at transformation. We then investigated the impact of molecular lesions on RS survival. At diagnosis, 64.1% RS were older than 60 years, 35.9% showed ECOG PS >1, 75.6% Binet stage B-C, 93.6% Ann Arbor stage III-IV, 43.6% B symptoms, 44.9% tumor size >5 cm, 30.8% involvement of >1 extranodal site, 62.5% LDH elevation, 69.2% Hb <11 g/dL, and 30.8% platelets $<100\times10^9$/L. Biological variables associated with poor RS survival were TP53 mutation (P=.003), del17p13 (P<.001), and MUM1 expression

(P=.047) (Figure 1A). Multivariate analysis selected TP53 mutations (HR:1.86 P=.040) as an independent predictor of RS survival along with ECOG PS (P=.001), tumor size (P=.001), and platelet count (P=.049). Clonally unrelated RS (7/81; 8.6%) differed from clonally related RS (45/81; 55.6%) because of lower prevalence of MUM 1 expression (33.3% *vs.* 82.2%; P=.010) and TP53 mutations (0 *vs.* 47.7%; P=.033). This difference translates into longer survival of clonally unrelated RS compared to clonally related RS (5-years survival: 66.7% *vs.* 19.8%; P=.011) (Figure 1B). *Conclusions.* This study documents that: i) the genetic pattern of RS differs from that of de novo DLBCL; ii) TP53 disruption and c-MYC activation are the most frequent RS genetic lesions; iii) TP53 mutations are an independent prognostic factor in RS; iv) clonally unrelated RS are biologically and clinically distinct from clonally related RS.

C082

THE VITAMIN NICOTINAMIDE ENHANCES THE ACTIVATION OF THE P53/MIR-34A/SIRT1 TUMOR SUPPRESSOR NETWORK IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Nicotinamide (Nam), the amide form of niacin, is the main precursor of nicotinamide adenine dinucleotide (NAD^+). It regulates availability of this co-enzyme and consequently activity of NAD^+-consuming enzymes linking them in a dynamic balance. This work took origin from the observation that pharmacological doses of Nam inhibit the physiological activation and proliferation of B lymphocytes, at least in mice. These seminal observations were transferred to the human system by hypothesizing that this vitamin may affect human B cell homeostasis comparing its effects on normal *vs*. leukemic B lymphocytes. Chronic lymphocytic leukemia (CLL) was selected as disease model for analyzing the effects of Nam and for testing its therapeutic potential. *Methods.* We studied a cohort of 40 CLL patients, with heterogeneous clinical and biological features, using a biochemical approach, real-time quantitative PCR, immunofluorescence assays for detecting apoptosis and proliferation and a fluorescent enzymatic assay to measure SIRT1 activity. CLL cells were treated with different pharmacological doses of Nam alone or in combination with etoposide, a DNA-damaging chemotherapeutic agent. Normal B cells were obtained from tonsils and spleens, and used as a control. *Results.* Pharmacological doses of Nam (5-10 mM) significantly inhibit proliferation and induce apoptosis of CLL cells. At earlier time points, Nam markedly reduces phosphorylation of multiple intracellular substrates, including the MAPK family member ERK1/2. Normal B lymphocytes were significantly less sensitive to the action of Nam. An hypothesis to explain the above data is that Nam exposure interferes with the activity of NAD^+-dependent enzymes. Attention was focused on SIRT1, a deacetylase that plays a critical role in cancer and that acts as a longevity factor. Nam exposure blocks the activity - and also the expression - of SIRT1 from nuclear extracts of CLL cells, but not of normal B lymphocytes. SIRT1 is a negative regulator of p53, and its function is to decrease p53-mediated apoptosis following DNA damage. The working hypothesis was that the link between these two molecules is represented by miR-34a, a direct transcriptional target of p53 that can bind SIRT1 mRNA and trigger its degradation. Combined treatment of CLL cells with Nam and etoposide is followed by i) up-regulation of miR-34a expression, ii) marked down-modulation of SIRT1 expression and function, and iii) induction of expression and acetylation of p53. This positive feedback loop is operative in the CLL model,(but not in normal B lymphocytes) with the final outcome of a significantly enhanced tumor cell apoptosis. *Conclusions.* These data demonstrate that CLL cells are uniquely sensitive to the pharmacological actions of Nam and prompt further studies to evaluate whether this vitamin can be added to the armamentarium of therapies for CLL patients, due to a well-established safety profile.

C083

A NEW CHIMERIC ANTIGEN RECEPTOR (CAR) TARGETING THE CD23 ANTIGEN EXPRESSED BY CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL) CELLS

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Background. B-cell chronic lymphocytic leukemia (B-CLL), the most common form of leukemia in adults in Western countries, is characterized by a progressive accumulation of mature $CD19^+CD5^+CD20dim$ B lymphocytes that over-express the B-cell activation marker CD23. Here we cloned and expressed in T lymphocytes a novel chimeric antigen receptor (CAR) that targets the CD23 antigen (CD23.CAR). *Methods.* Cytotoxic activity of control non transduced T cells (NT) and CAR^+ T cells was measured using a standard 51Chromium release assay. Co-culture experiments of NT and CAR+ T cells (1×10^6 cells/well) with viable LCLs (ratio 1:1) in the presence or in the absence of serial dilutions of B-CLL patients plasma enriched in soluble CD23 (sCD23) have been performed to assess sCD23 mediated inhibition of the $CD23.CAR^+$ T lymphocytes cytotoxic activity. The expansion of CAR^+ T lymphocytes in response to $CD23^+$ targets has been proved by weekly stimulation with allogeneic, gamma-irradiated (30 rads) LCLs (ratio 1:1), without addition of exogenous cytokines. Interferon(IFN)-γ, Tumor necrosis Factor(TNF)-α and TNF-β release was measured with a Flow Cytomix Assay, while Interleukin(IL)-2 production was measured using a specific Enzyme-Linked Immunosorbent Assay. Soluble CD23 levels of B-CLL patients-derived plasma samples have been detected using a human CD23 ELISA kit. *Results.* $CD23.CAR^+$ T cells showed specific cytotoxic activity against $CD23^+$ tumor cell lines (average lysis 54%, at Effector:Target (E:T) ratio 40:1) and primary $CD23^+$ B-CLL cells (average lysis 58%, at E:T ratio 20:1). This effect was obtained without any toxicity against normal B lymphocytes, differently from other CARs that target CD19 or CD20 antigens expressed by leukemic cells, but physiologically also by normal B lymphocytes. Moreover, $CD23.CAR^+$ T cells released inflammatory cytokines (4-fold more IFN-γ, 157-fold more TNF-α and 1445-fold more TNF-β) in response to $CD23^+$ target cells. IL-2 was also released (average release 2681 pg/mL) and sustained the antigen-dependent proliferation of $CD23.CAR^+$ T cells. *Conclusions.* Altogether these data suggest that gene modification of T cells to express the CD23.CAR represents a selective immunotherapy approach to eliminate $CD23^+$ leukemic cells, while sparing normal B lymphocytes, in patients with B-chronic lymphocytic leukemia.

C084

BAX/BCL-2 RATIO PREDICTS DISEASE PROGRESSION AND OVERALL SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Bcl-2 has emerged as the most important factor in predicting survival between other proteins that are implicated in the control of apoptosis, proliferation and differentiation in chronic lymphocytic leukemia (CLL). In fact, malignant cells are arrested in the G0/early G1 phase of the cell cycle, and inhibition of apoptosis with up-regulation of the anti-apoptotic protein bcl-2 and down-regulation of the pro-apoptotic protein bax may define clinical prognosis. Noteworthy, the today availability of bcl-2 antisense oligonucleotides and novel pro-apoptotic BH3 peptidomimetics stimulated us to evaluate the clinical impact of apoptosis pathways on CLL prognosis. The primary aims of our study were: 1) to determine progression-free survival (PFS) and overall survival (OS) upon bax/bcl-2 ratio, 2) whether bax/bcl-2 ratio and ZAP-70 show additive prognostic impact, and finally 3) whether bax/bcl-2 ratio is an independent prognostic factor. *Methods.* We investigated 360 pts, median age 65 years, 167 males and 193 females. With regard to modified Rai stages, 116 patients had a low stage, 232 an intermediate stage

and 12 a high stage. Bax/bcl-2 ratio was determined by flow cytometry, dividing mean fluorescence intensity (MFI) of bax by MFI of bcl-2 on $CD19^+CD5^+$ CLL cells. The threshold was set at the median value >1.45. *Results.* Two hundred-five patients were bax/bcl-2 ratio positive (57%). Higher bax/bcl-2 ratio was significantly associated with low Rai stage (P=0.003), lymphocyte doubling time >12 months (P=0.0003), beta-2 microglobulin <2.2 mg/dL (P=0.001) and soluble CD23 <70 U/mL (P=0.005). There were significant correlations between higher bax/bcl-2 ratio and IgVH gene mutated status (216 cases, P=0.015) or low risk (normal or del 13q) cytogenetics (255 cases, P=0.011). Noteworthy, a strict association was found between higher bax/bcl-2 ratio and lower ZAP-70 (P<0.0001). With regard to clinical outcome, significant shorter PFS and OS were observed in pts with lower bax/bcl-2 ratio (10% *vs.* 60% at 14 years; P<0.0001 and 51% *vs.* 74% at 16 years; P=0.005) as well as in ZAP-70+ pts (5% *vs.* 57% at 12 years, P<0.0001 and 30% *vs.* 85% at 16 years, P=0.0001). To further explore the prognostic impact of bax/bcl-2 ratio, we investigated its expression in combination with ZAP-70. As a matter of fact, higher bax/bcl-2 ratio plus ZAP-70 < 20% identified the subset of pts with the longest PFS (70% *vs.* 2% at 12 years; P<0.00001, Figure) and OS (92% *vs.* 33% at 14 years; P<0.00001). The discordant pts presented an intermediate outcome (Figure). In multivariate analysis of PFS, bax/bcl-2 ratio (P=0.02), FISH (P=0.02) and ZAP-70 (P=0.04), were confirmed to be independent prognostic factors. *Conclusions.* Bax/bcl-2 ratio, performed by flow cytometry, was very useful to identify pts at different clinical outcome and therefore it may be added to other well-known biological prognostic factors in order to identify early and to treat timely progressive pts.

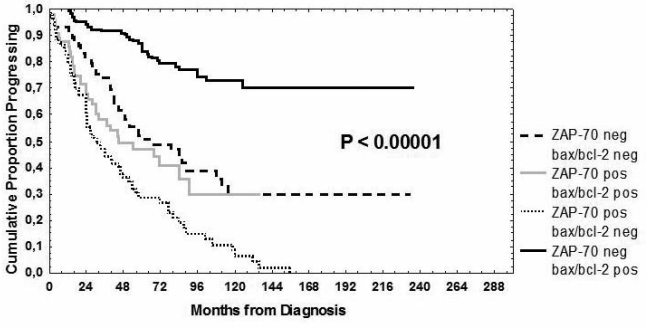

Figure. Progressio -free Survival by ZAP-70 and bax/bcl-2 ratio.

C085

CD69 AND CD79B OVEREXPRESSION PROGNOSTICATE A POOR OUTCOME IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Chronic lymphocytic leukemia (CLL) exhibits features of activated and antigen-experienced B-lymphocytes and CD69 up-regulation resembles B cells at an earlier and greater state of activation. Moreover, high B-cell receptor (BCR) signalling as detected by CD79b overexepression increases cell survival and cycle progression. Therefore, a hyperactivated and hyperstimulated B-CLL phenotype enhances intracellular signaling intermediates such as cyclin D2 and phosphorylated STAT1, STAT3 and ZAP-70 proteins, thus pinpointing a more aggressive and progressive disease. The primary endpoints of our study were: 1) to determine progression free survival (PFS) and overall survival (OS) upon CD69 and CD79b expression; 2) whether CD69 and CD79b expression had additive prognostic value and finally 3)whether CD69 and CD79b were confirmed to be independent prognostic factors. *Methods.* We investigated 458 pts, median age 65 years, 251 males and 207 females. With regard to modified Rai stages, 134 had a low stage, 302 an intermediate stage and 22 a high stage. CD69 and CD79b antigens were determined by multicolor flow cytometry, fixing a cut-off value higher than 30%. *Results.* $CD69^+$ and $CD79b^+$ CLL pts were 108/401 (27%) and 232/443 (52%), respectively. Of note, 393 pts were studied for both the antigens. CD69 and CD79b were significantly associated with modified Rai stages (P<0.0001), lymphocyte doubling time (P=0.00001 and P=0.008), β2 microglobulin (P<0.0001) and soluble CD23 (P<0.0001 and P=0.0001). A significant association was found between intermediate/high risk (trisomy 12, del11q, del17p) FISH cytogenetics and CD79b >30% (324 cases, P<0.0001). Moreover, there were significant correlations between IgVH status and CD69 (283 cases, P=0.0001) or CD79b (291 cases, P<0.00001). Equally, significant associations were found between ZAP-70 and CD79b (P<0.0001) or CD69 (P=0.008). With regard to clinical outcome, shorter PFS and OS were observed in $CD79b^+$ pts (15% *vs.* 54% at 14 years, P<0.0001; 38% *vs.* 86% at 18 years, P<0.0001) as well as in $CD69^+$ pts (9% *vs.* 49% at 14 years, P<0.0001 and 49% *vs.* 75% at 16 years, P=0.00002). Noteworthy, CD79b and CD69 showed additive prognostic properties, since CD79b <30% plus CD69 <30% identified a CLL subset at better prognosis with regard to PFS (66% *vs.* 3% at 14 years; P<0.0001) and OS (94% *vs.* 24% at 18 years; P<0.0001). The two discordant subsets ($CD69^+CD79b^-$ and $CD69^-CD79b^+$) showed an intermediate outcome (Figure). In multivariate analysis of PFS and OS, CD69 (P=0.0003 and P=0.006) and CD79b (P=0.0003 and P=0.01) were confirmed to be independent prognostic factors. *Conclusion.* CD69 and CD79b antigens, determined by flow cytometry, may be considered novel important prognostic parameters in B-CLL. Their easy and rapid laboratory evaluation could allow us to identify early progressive pts and to take timely therapeutic decisions.

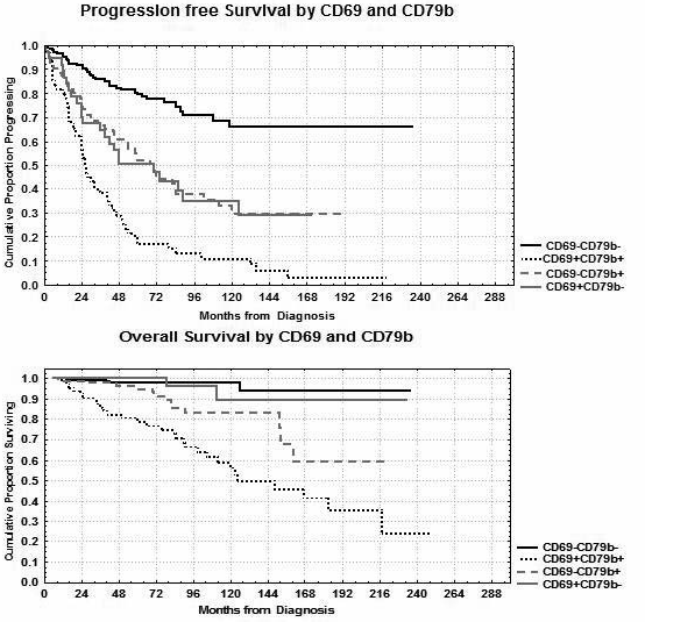

Figure.

C086

E2A IS A TRANSCRIPTIONAL REGULATOR OF CD38 EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. CD38, a surface molecule that functions as nucleotide-metabolizing ecto-enzyme and as receptor, is a negative prognostic marker for chronic lymphocytic leukemia (CLL) patients. *In vivo*, CD38 expression is higher in the bone marrow (BM) and in areas of intense CLL/T cell contacts than in the peripheral blood cells. *In vitro*, CD38 expression can be up-regulated during CLL cell activation by a number of different signals, including IL-2, the combination of CD40L and IL-4, CpG oligonu-

cleotides and co-culture with mesenchymal stem cells. Up-regulation of CD38 relies on de novo gene transcription and behaves as a polymorphic trait in the population. The human CD38 gene has a genetic single-nucleotide polymorphism (SNP), with a C G variation located in regulatory region in intron 1. We have recently reported that the frequency of the minor allele (G) is higher in a subset of CLL patients with poor prognosis. Because the C G SNP is located within a putative E-box [G(orA)CAXXTGG(orA)], it is possible that it may affect gene transcription through the E47 factor, the predominantly active isoform of the E2A gene in B lymphocytes and a critical element in B cell ontogenesis. *Methods.* 72 CLL patients molecularly and clinically characterized were included in the study. Normal B cells from PB, spleen and tonsils were used as control. The expression of E2A was tested by western blot analysis on cytosolic and nuclear fractions. The binding of E2A to the regulatory region of CD38 gene was evaluated by EMSA and ChIP assays. Nalm-6 and Raji cell line, both $E2A^+$, were used to silence the transcription factor by means of E2A shRNA lentiviral particles. *Results.* E2A was expressed by the majority of CLL cells analyzed (69/72), albeit with highly variable intensity. It was undetectable in normal B cells purified from spleen or peripheral blood. A positive direct association between E2A and CD38 was highlighted. $E2A^+$/G carriers displayed the highest CD38 levels, suggesting that E2A is i) associated with CD38 expression, and that ii) its binding is influenced by the CD38 genotype. Direct interaction of E2A with the CD38 regulatory region was confirmed by chromatin immunoprecipitation, which also indicated a stronger affinity in the presence of the G allele. Furthermore, E2A silencing led to a significant reduction of surface CD38. A functional interplay between E2A and CD38 was confirmed by exposing CLL cells to TLR-9 ligands and IL-2, both inducers of CD38 expression. Under these conditions, CD38 up-regulation was primarily conditioned by the presence of E2A and then by the G allele. *Conclusions.* Taken together, these results link E2A and CD38 expression in a common pathway, in which E-protein activity is required for the efficient induction of CD38 transcription.

C087

A PROTEOMIC STRATEGY IDENTIFIED ELEVATED LEVELS OF SERUM AMYLOID A PROTEIN IN THE PLASMA OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction. Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the western world and is characterized by the accumulation of relatively mature B cells. CLL is notable for variation in aggressiveness of disease. Many patients with low-risk disease at diagnosis can be followed expectantly, while others rapidly require therapy. This clinical heterogeneity appears to be sustained by different biologic parameters, such as the immunoglobulin variable gene (IgVH) mutational status, ZAP-70 expression and specific cytogenetic alterations. Many patients express a combination of all favorable or all unfavorable markers but there are also patients with different marker combinations. Unique easily accessible prognostic markers helping to predict the individual course of CLL are therefore highly warranted. *Methods.* Proteomic profiling of plasma or serum is a technique to identify new biomarkers in disease. The objective of this study was to identify new plasma biomarkers in CLL patients using surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry. To first establish the feasibility and specificity of the method using a case-control study design, plasma samples from 30 patients with a known CLL diagnosis were compared to samples from 30 age- and sex-matched healthy controls. All samples were run in triplicate and the obtained spectra aligned. Protein spectra were generated and the protein peak intensities normalized to the total ion current m/z values and analysed using Ciphergen ProteinChip software 3.2.0. Comparison of SELDI profiles using the Unsupervised Cluster analysis highlighted that the plasma profiles of patients with CLL could be clearly separated from control groups. Application of the BMV Program to these spectra identified the m/z 11,681 cluster as most significantly different. Preparative monodimensional electrophoresis was used to isolate this cluster from plasma that highly expressed or did not express the m/z 11,681 cluster. MALDI-TOF MS analysis identified the unknown protein as Serum amyloid A (SAA). We have previously demonstrated that plasma SAA SELDI-TOF intensities are correlated with SAA protein concentrations (r=0.41; P=.0001) determined by ELISA. Thus, plasma SAA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Biosupply Ltd., Bradford, United Kingdom) in a larger group of 86 newly diagnosed B-CLL patients (44males, 42 females median age of 62 years) and 30 age- and sex-matched healthy controls. *Results.* The analysis revealed statistically significant differences in SAA expression between the plasma of LLC patients and the plasma of healthy subjects (P=0.002). In addition, SAA levels are elevated in the plasma of patients with an unfavourable cytogenetic profile (try12; del17 del11) when compared to patients with normal or other cytogenetic status (P=0.02). In patients, SAA plasma levels are also positively correlated with peripheral lymphocyte doubling times of less than one year (P=0.009). *Conclusions.* Although preliminary, these data suggest that elevated SAA levels are associated with unfavorable known prognostic marker and support the view that inflammation is implicated in CLL development.

C088

HOST GENETIC BACKGROUND AND RISK OF RICHTER SYNDROME: THE GENOTYPE OF LRP4 IS AN INDEPENDENT PREDICTOR OF CHRONIC LYMPHOCYTIC LEUKEMIA TRANSFORMATION TO AGGRESSIVE LYMPHOMA

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Background. Richter syndrome (RS) represents the transformation of chronic lymphocytic leukemia (CLL) to aggressive lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL). Mechanisms and risk factors of CLL transformation to RS are known only in part. *Methods.* This study aimed at exploring the role of the host genetic background in RS transformation and was based on a consecutive series of 331 CLL, of which 21 had transformed to RS (all clonally related to the CLL clone). Twenty-eight additional cases of clonally related RS were also collected for validation purposes. 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, 45 SNPs from 45 genes (APOE, BARD1, BRCA2, CD5, CPA4, CPT2, CYP2C9, CD38, DUSP13, ENPP5, ERBB2IP, ERCC5, ERCC6 ESPL1, FHL5, FOXN1, GALNACT2, GTF2E1, HIPK4, IL16, IL19, INHBC, KLRC4, KRT1, LAMA2, LILRA4, LRP2, LRP4, MDM2, MGMT, MMP10, MYBPC3, PMS2, POLB, SEC23B, SEMA3C, THBS1, TOPBP1, TYR, USP47, XRCC2, ZNF169, ZNF527, ZNF573, IRF4) were genotyped by SNP-minisequencing. The primary endpoint of the study was cumulative probability of transformation measured from CLL diagnosis to RS transformation, death or last follow-up. *Results.* Univariate Cox analysis controlled for multiple comparisons by FDR testing 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 (Events/N: 12/79; 5-year risk: 13.5%) compared to patients carrying the LRP4 rs2306029 CT/CC genotypes that contained the wild type allele (Events/N: 9/252; 5-year risk: 4.4%) (Figure 1). Other variables at CLL diagnosis associated with an increased risk of RS were advanced Binet stage (P=.001), lymph node size =3 cm, LDH elevation (P=.001), CD38 expression =30% (P=.011), unfavorable genotype (P=.004), IGHV homology =98% (P=.002), and stereotyped HCDR3 (P=.001). None of the patient subgroups carrying the above risk factors was enriched with the LRP4 rs2306029 TT variant genotype (P=.050 in all instances), suggesting that LRP4 rs2306029 TT is not a surrogate of other RS risk factors. Multivariate Cox analysis selected LRP4 rs2306029 TT as an independent predictor of RS (HR: 3.54; P=.012), along with

stereotyped HCDR3 (HR: 4.09; P=.005). In order to confirm the enrichment of TT genotype in RS, LRP4 rs2306029 was genotyped in an independent validation RS series (n=33) matched for age, sex and geographical origin. Prevalence of LRP4 rs2306029 TT genotype did not significantly differ between the original RS series (12/21, 57.1%) and the validation RS series (13/33 39.4%; p=.202). LRP4 rs2306029 is a non-synonymous SNP leading to Ser1554Gly amino acid substitution. PolyPhen algorythm indicated LRP4 rs2306029 as a possibly deleterious variant (PSIC score=1.776). By flow cytometry, LRP4 expression =30% was documented in 63/64 CLL. By immunohistochemistry on bone marrow biopsies, LRP4 was expressed in 19/30 (63.3%) CLL. *Conclusions.* Our study documents that LRP4 protein is expressed in CLL and that LRP4 rs2306029 is an independent predictor of CLL transformation to RS. Since LRP4 is an antagonist of canonical Wnt/Beta-catenine signaling pathway, the LRP rs2306029 deleterious variant maight be pathogenetically implicated in RS development.

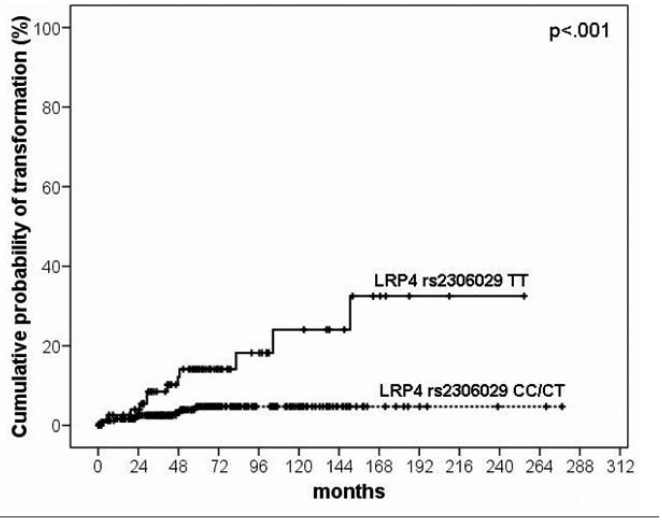

Figure 1.

Molecular Hematology and Cytogenetics

C089

C-MYB SUPPORTS ERYTHROPOIESIS BY TRANSACTIVATING KLF1 AND LMO2 EXPRESSION

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Introduction. The c-Myb transcription factor is highly expressed in immature hematopoietic cells and down-regulated during differentiation. c-myb is essential for the hematopoietic development, as c-myb-/- mice die at E15 due to failure of fetal hepatic erythropoiesis. To gain further insights into the role of c-myb during the hematopoietic lineage commitment, we studied the effects of c-Myb silencing in human $CD34^+$ hematopoietic stem/progenitor cells. *Methods.* c-Myb silencing in $CD34^+$ cells was performed by transfection of siRNAs using the Amaxa Nucleofector® Technology. In order to keep c-Myb expression silenced for all the commitment phase of $CD34^+$ cells, each sample was nucleofected 3 times, once a day. Moreover, to exclude non-specific effects of siRNA nucleofection, for each experiment, together with the sample transfected with the siRNAs targeting c-Myb, one sample electroporated without siRNAs and one transfected with a non-targeting siRNA were performed. c-Myb silencing effects on $CD34^+$ cells differentiation ability were studied by methylcellulose and collagen-based clonogenic assays and by morphological and immunophenotypic analyses after liquid culture. Furthermore, we investigated by microarray analysis the changes in gene expression induced by c-Myb silencing. *Results.* Methylcellulose assay revealed a remarkable increase of the percentage of monocyte (CFU-M) colonies and a decrease of the erythroid ones (BFU-E) in c-Myb-silenced $CD34^+$ cells. Moreover, collagen-based clonogenic assay demonstrated that c-Myb silencing strongly enhances the megakaryocyte commitment of $CD34^+$ cells. In agreement with these data, flow cytometric analysis showed an increase in mono-macrophage and megakaryocyte fractions in c-myb-silenced cells, while the erythroid population was strongly decreased. Morphological evaluation of May Grunwald-Giemsa stained cytospins further supported the conclusion that c-myb silencing forces the $CD34^+$ cells commitment towards the macrophage and megakaryocyte lineages at the expense of the erythroid one. Gene expression profiling of c-Myb silenced $CD34^+$ cells enabled us to identify new putative targets which can account for c-Myb knockdown effects. Indeed, Chromatin Immunoprecipitation and Luciferase reporter assay demonstrated that c-Myb binds to KLF1 and LMO2 promoters and transactivates their expression. Functional rescue experiments showed that the retroviral vector-mediated overexpression of KLF1 and LMO2 transcription factors in c-Myb silenced cells is able to rescue, at least in part, the impaired erythroid differentiation. *Conclusions.*Our data collectively demonstrate that c-Myb plays a pivotal role in human primary hematopoietic stem/progenitor cells lineage commitment, by enhancing erythropoiesis at the expense of megakaryocyte diffentiation. In particular, we identified c-Myb-driven KLF1 and LMO2 transactivation as the molecular mechanism through which c-Myb regulates erythroid versus megakaryocyte lineage fate decision.

C090

EXTREME VARIABILITY OF FIP1L1-PDGFRALPHA TRANSCRIPTS IN CEL: ANALYSIS OF 32 PATIENTS ENROLLED IN HES0203 ITALIAN CLINICAL TRIAL AND CORRELATION WITH MOLECULAR RESPONSE AFTER 5 YEARS FOLLOW-UP

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Introduction. Recent molecular studies of atypical Philadelphia negative chronic myeloproliferative disorders (CMPDs) have produced the

identification of more than 40 fusion genes. The presence of FIP1L1-PDGFRA (F/P, cytogenetically invisible) have been associated more frequently with diagnosis of CEL. As this is the second most common fusion gene targeted by imatinib (IM) after BCR-ABL1, it is expected to monitoring response at molecular level as in CML, but from the analysis of major series of cases, it emerges that the diversity of F/P fusion transcripts is much more complex. We conducted a prospective phase II multicenter study of HES to explore the activity and safety of IM 400 mg in the treatment of patients affected by HES, apart from molecular status. A total of 32 patients with F/P were enrolled and now we report on their clinical and molecular follow up. *Methods.* The primary endpoint of the study was to assess the clinical anti-proliferative activity of IM in HES. Patients received IM 400 mg once daily for the first year, than the dose could be modified. In patients with F/P rearrangement, the presence of the transcripts was assessed and monitored by nested RT-PCR every 3 months until negativity, and then every 6 months. After RNA extraction, RT-PCR was performed and direct sequencing was done using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 377 DNA Analyzer. *Results.* All 32 patients with F/P rearrangement had prompt responses to IM. Molecular and hematologic responses (MR, HR) were maintained with continuous therapy, molecular relapse were detected when IM was stopped for different reasons, but second and third MR was obtained with IM resumption. Deep molecular analysis revealed that all the 32 fusion transcripts were different at genomic level. Eight different FIP1L1 exons (exons 9 to 15 and 18) were found to be fused to PDGFRA exon 12 that was truncated in all cases to a variable degree. In 5 cases more than 1 band was evident for the same sample, and in these cases sequencing of c-DNA was performed for each band, identifing genomic variability also in the same patient, at different time point. Following the molecular classification proposed by Walz, we identified transcript type A in 4/27 cases(44%), type B in 11/27 cases (41%) and type C in 3/27 cases (15%). The distribution of genomic breakpoint reflects those reported by Walz *et al.*, with the majority of breakpoints located in FIP1L1 intron 10, 11 and 13. Others location were rare. All genomic PDGFRA breakpoints were found in exon 12 (55% in position 83/84, 30% in 100/101, 11% in 43/44, 4% in 25/26, where position +1 represents the first base in exon 12). *Conclusions.* After 5 years of follow-up, with this large series of patients we can confirm very high sensibility of F/P to IM, the necessity of continuous therapy and absence of acquired resistance. Primers and probes for quantitative RT-PCR have to be designed for every single patients for the complexity and variability in F/P transcripts. Clinical correlation between this heterogeneity and phenotype of disease and response to the imatinib therapy is not clear with present data and require largest studies.

C091

CHROMATIN COVALENT MODIFICATIONS IN THE RESPONSE OF CHRONIC MYELOID LEUKEMIA TO AURORA KINASE INHIBITOR MK-0457

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Introduction. MK-0457 is an Aurora kinase (AK) inhibitor active against the wild type (wt) and most Imatinib (IM)-resistant mutants of Bcr-Abl proteins and, in particular, T315I, which is also resistant to Nilotinib and Dasatinib. Accordingly, preliminary reports provided evidence for durable response to MK-0457 of chronic myeloid leukemia (CML) and acute lymbhoblastic leukemias (ALL) with the T315I mutation phenotype. We investigated a still elusive theme concerning MK-0457 impact on AK A and its targets driving the cell cycle progression and survival of Bcr-Abl-expressing cells. *Methods and Results.* MK-0457 *in vitro* cytotoxicity against Bcr-Abl$^+$ cells (K562 cell line, Bcr-Abl-transduced Ba/F3 and 32D cell clones and CD34$^+$ progenitors isolated from bone marrow samples of either IM-sensitive or -resistant CML patients) was first confirmed in clonogenic assays. It was mostly due to G2/M arrest and apoptotic death induction. The significant reduction of AK A expression and phosphorylation in response to MK-0457 (100 nanoM for 24 h, analyzed by mean of immunoprecipitation-immunoblottting analyses) almost revoked phosphorylation of serine 10 at the N-terminal tail of histone H3 (H3-S10, the critical residue for activation of a "condensation code" during mitosis and transcriptional regulation) associated with lysine 9 (H3-K9) tri-methylation and lysine 14 (H3-K14) acetylation. Chromatin immunoprecipitation (ChIP) revealed that H3-K9 tri-methylation with the contribution of histone metyl-transferase SUV39H1 promotes HP1 recruitment at a Bcr promoter region critical for Bcr-Abl transcription leading to fusion gene transcriptional repression. Moreover, the reduction of AK A enzymatic activity in response to MK-0457 significantly raised the expression of GADD45, a DNA damage-inducible gene implicated in the maintenance of genomic fidelity. GADD45 up-modulation proceeds from transcriptional events not mediated by HP1 recruitment at the gene promoter. Notably, GADD45 integrates MK-0457 effects through direct interaction with AK A and inhibition of AK A kinase activity. *Conclusions.* Our study proved that MK-0457 cytotoxicity on Bcr-Abl$^+$ cells either sensitive or resistant to IM arise from its inhibitory effects on AK A in addition to p210 Bcr-Abl tyrosine kinase. Covalent chromatin modifications involved in transcriptional regulation (namely Bcr-Abl down-modulation and GADD45 up-modulation) proceeding from AK A inhibition in response to MK-0457 contribute to the leukemic myelopoiesis exhaustion.

C092

12P, 1P, 1Q, 5Q, 11Q ABNORMALITIES AND IMMUNOPHENOTYPE IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE, MULTIPLE MYELOMA AND PLASMA CELL LEUKEMIA

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Introduction. Plasma cell disorders are characterized by the presence of recurrent genetic aberrations. We evaluated, by interphase Fluorescence In Situ Hybridization (FISH) and Flow Cytometry, chromosomal abnormalities and plasma cell (PC) immunophenotype in patients with Monoclonal Gammopathy of Undetermined Significance (MGUS), Multiple Myeloma (MM), and Plasma Cell Leukemia (PCL). *Methods.* A total of 565 patients (31 with MGUS, 34 with PCL and 500 with newly diagnosed MM) referred to our Hematology Department, were included in the study. FISH analysis was performed on bone marrow and peripheral blood PC purified using anti-CD138-coated magnetic beads. Slides containing PC fixed in Carnoy's solution were prepared for interphase FISH using standard methods. DNA probes were used to detect 13q14, 12p13, 1p36 and 17p13.1 deletions; t(4;14)(p16;q32), t(14;16)(q32;q23), t(11;14)(q13;q32); 11q23 (MLL), 1qter and 5q gain. PC immunophenotype was assessed using quadruple combinations of MoAbs for the detection of the following antigens: CD38, CD138, CD56, CD45, CD40, CD19, CD20, CD52, CD117, cytoplasmic kappa/lambda. *Results.* FISH results are shown in Table 1.

Table 1.

	MGUS	*MM*	*PCL*	
Genetic Pattern	Frequency (%)	Frequency (%)	Frequency (%)	P value
13q14-	48.1	53.9	75	0.05
17p13.1-	12	16.4	47.4	0.002
12p13-	8.3	14.3	28.6	ns
1p36-	16	17	36.7	0.05
1qter+	56	43.8	76.7	0.006
5q+	37.5	38	41.4	ns
11q23+	50	49.5	44.8	ns
t(11;14)	12.5	16.2	14.3	ns
t(4;14)	20.8	19	22.2	ns
t (14;16)	8.3	5.2	35.3	0.00001

At least one chromosomal abnormality was found in 86% MGUS, 96% MM and 100% of PCL patients. An higher frequency of deletion 12p13 and of at least one IgH translocation was observed among MGUS, MM and PCL patients but no significant statistical difference was noticed. However, these differences were significant (P=0.04) when MGUS/MM patients were compared with PCL patients (13.2% *vs.* 28.6% and 40.1% *vs.* 64.7%, respectively). A significantly different distribution of both 1p36 deletion (P=0.05) and 1qter gain (P=0.006) was

found among these three patients groups, with the highest frequency of chromosome 1 alterations in PCL patients. 5q gain was found with the same frequency in all groups. A significantly increasing frequency of deletion 13q14 and 17p13.1, was found from MGUS to PCL. PC immunophenotype showed a significantly higher expression of CD117 (31% *vs.* 8%; P=0.01), CD56 (69% *vs.* 47%; P=0.008) and CD19 (27% *vs.* 3%; P=0.008) in MGUS/MM compared to PCL patients. In the MGUS group, 10 patients showed disease progression to overt MM. When comparing "evolving" with "non-evolving" MGUS, we found that the evolving variant was characterized by a higher percentage of 13q14 (70% *vs.* 35%; P=0.05), 17p13.1 (22% *vs.* 6%; P=ns), 12p13 (22% *vs.* 0%; P=0.05), 1p36 (37% *vs.* 6%; P=0.05) deletions and 1qter gain (87% *vs.* 43%; P=0.04) and a lower percentage of 5q gain (25% *vs.* 46%; P=ns). *Conclusions.* Our results showed that poor-risk genetic abnormalities are more commonly seen in PCL, than in MM, than in MGUS. Moreover, this finding is emphasized when "evolving" MGUS are compared with "non-evolving" MGUS.

C093

INTERPHASE AND CYTOPLASMIC-IG FISH FOR REVEALING CHROMOSOMAL LESIONS IN 45 PATIENTS WITH PRIMARY AMYLOIDOSIS (AL)

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In AL the information provided by FISH in most plasma cells (PC) disorders is still lacking because of the rarity of the disease, the low PC burden and the low proliferative index. However, the few FISH studies performed with centromeric and IgH specific probes on PCs either immunoselected (iFISH) or recognized after cytoplasmic IgH staining (cIg-FISH) have revealed aneuploidies and/or IgH translocations in most AL patients and have suggested that AL and M-GUS share common pathways of karyotype instability. The aims of the present study were to establish which FISH approach is the most effective in revealing chromosomal lesions in AL patients, to evaluate the incidence of these chromosomal abnormalities, and to find their association with clinical parameters and outcome. The present study includes 45 patients who were diagnosed as primary AL and were submitted to FISH analysis. PCs were either isolated from marrow cell suspensions by using anti CD138 immunobeads and an auto-magnetic-activated cell sorter separation system (Myltenyi Biotec, Bergisch Gladbach, Germany) or recognized by immunostaining. iFISH and cIg-FISH were performed either on clinical diagnosis or during follow-up and were carried out with the FGFR3/IGH, the CCND1/IGH, the LSI D13S319/LSI 13q34, the LSI ATM/CEP11, the LSI p53/CEP17 probes (Vysis, Downers Grove, IL, USA) and with the ON MM 1q21/8p21.1 and the 4q21 tricolor probes (Kreated, Amsterdam, The Netherlands). In order to calculate the cut-off values, 1500 nuclei from five normal controls were examined and a one tail binomial distribution applied. For monosomies cut-off values were fixed at 8%, for trisomies at 1% and for translocations at 2%. iFISH and cIg-FISH revealed at least one chromosomal lesion in 80% and 73.3% of patients, a difference due to the fact that three patients did not provide a number of PC sufficient to perform cIg-FISH. However, in patients who yielded iFISH and cIg-FISH results the incidence of chromosomal defects as well as the percentage of positive cells were almost identical on each FISH approach. The t(11,14) translocation was the most common chromosomal defect (incidence 47.2%), followed by a 13q14 deletion (38.8%), a 1q21 amplification (36.1%), a CCND1 amplification (27.7%) and hyperdiploidy (27.7%). The second most common IgH translocation was the t(14;16) present in two patients. Moreover, the IgH locus was rearranged with an unknown and uncommon chromosomal partner in four patients Interestingly, a CCND1 amplification was moderately associated with cardiac involvement and a 13q14 deletion/1q21amplification with a high marrow PC percentage. In conclusion, iFISH and cIg-FISH are equally effective in revealing the chromosomal lesions in AL patients; an altered CCND1 regulation might be a relevant event in AL pathogenesis; 13q deletions and 1q21 amplifications might be late event in AL clinical course; CCND1 amplification seems to be associated with a cardiac involvement.

C094

WHOLE-EXOME SEQUENCING OF A PHILADELPHIA-NEGATIVE CHRONIC MYELOID LEUKEMIA PATIENT THROUGH THE EXON-CAPTURE TECHNIQUE

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Introduction. Atypical Chronic Myeloid Leukemia (aCML) is a myelodysplastic/myeloproliferative disease clinically resembling Philadelphia positive (Ph^+) CML. aCML, however, lacks the t(9;22) translocation, which is the molecular hallmark of Ph^+ CML. To investigate the molecular mechanisms involved in the development of aCML, we performed a whole-exome high-throughput sequencing analysis of an individual aCML patient with normal karyotype. *Methods.* The exon-capture protocol was performed by using an in-solution technique (SureSelect Human All Exon Kit) on myeloid leukemic cells and normal lymphocytes as control. The enriched genomic DNA was sequenced with a Genome Analyzer IIx (Illumina), using a 76 bases paired-end run protocol. The bioinformatic analysis was performed using the Galaxy framework; the cross-match analysis between leukemic and normal exomes was performed with dedicated in-house C# software. *Results.* The percentage of reads matching the reference hg18 (build 36) human genome was over 90%, with a mean exon coverage of over 40-fold for both the leukemic sample and the control. The percentage of nucleotides targeting exonic regions or exonic regions plus 100bp was 48% and 68%, respectively, with an overall 40-fold enrichment factor for exonic *vs.* non-exonic regions. The comparison between the leukemic and the control datasets led to the identification of 212 variants. Among them, 102 were single nucleotide variants (SNV; 60 transitions and 42 transversions, with a transition/transversion ratio of 1.43) and 110 were structural variants (STV, 57 insertions and 53 deletions). This dataset was filtered accordingly to the following rules: 1) Variants occurring in exons were annotated as tier1 mutations and were given the highest priority. 2) Variants occurring in non-exonic regions were annotated as tier2. 3) Silent variants and residual Single Nucleotide Polymorphisms (SNPs) were filtered out. Globally, 14 heterozygous mutations were annotated as tier1 variants and 77 as tier2. 7 silent variants and 4 SNPs were discarded. Tier1 mutations are now under validation by Sanger sequencing. Among them, mutation R140Q, occurring at the active site of the IDH2 gene, has been already validated. Notably, this mutation was recently identified in 7/78 AML samples (Ward PS *et al.* Cancer Cell, 2010) and leads to a gain-of-function ability to catalyze the conversion of alpha-ketoglutarate to 2-hydroxyglutarate, causing the intracellular accumulation of the latter. Additional lesions are being validated. The results will be compared with the data generated by the whole-exome sequencing of a second aCML patient, which is now ongoing and by RNA-SEQ analysis to identify fusion genes. *Conclusions.* Exon-capture is an efficient technique useful to analyze single nucleotide and structural variants in cancer cells. Using this technique, we identified the heterozygous mutation R140Q occurring at the active site of the IDH2 gene in an aCML patient.

C095

ITALIAN STANDARDIZATION OF BCR-ABL RQ-PCR AND QUALITY CONTROL METHODS: AN OVERVIEW OF FIRST 4 YEARS RESULTS

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Introduction. Quantification of BCR/ABL transcripts has proven to be the most sensitive approach to monitor response to standard treatment in chronic myeloid leukemia (CML). Variations in methods used to quantify BCR/ABL made it difficult to compare results between laboratories. In order to increase inter laboratories comparability of obtained results, the first effort to standardize RQ-PCR protocols was made by Europe Against Cancer (EAC) program. Subsequently, in the IRIS trial was established that the best approach for achieving comparable BCR/ABL values, is the use of an international reporting scale (IS) achieved by the application of laboratory-specific conversion factors, in which these values are anchored to major molecular remission (MMR). *Methods.* In 2006 three of the IRIS participating labs (Naples, Bologna and Turin) aligned their results to the IS after an exchange with Adelaide reference laboratory of 40 cells dilutions from the lowest levels of detection to 10% of MRD. In this way they receive its first Conversion Factors (CF) whose values are strictly controlled up to 2009 through more than 200 samples exchange. In 2007 we start first round of quality control by an italian Network (LabNet) of 13 labs in order to express the results of BCR/ABL in IS. The laboratory-specific conversion factors (CF) are calculated comparing the "RNA Reference Standard" (kindly provided by NanoGen) to results obtained from reference laboratories at Naples, Bologna and Turin. The Statistical analysis was based on the Bland & Altman algorithm where the antilogs of method mean bias provide each lab-specific CF. *Results.* The conversion to the IS greatly improve the accuracy of the results: indeed, the mean bias of participant labs passed from -0.22508 to <0.001 after conversion. In the second round of quality control in 2008, the number of participating increase to 24 and we checked over time stability of CFs and compared the possibility to calculate CF using standard RNAs against the use of 15 patients RNAs expressing different levels of MRD, with a Bias before conversion of 0.239 and a Bias after conversion <0.001. In 2009, 24 laboratories participate to the third round of quality control. In this phase the reference laboratories compare the results of 15 patients RNAs sent by each lab. *Conclusions.* The results of this round show a Bias before conversion of 0.268 and after conversion of 0.003. In conclusion we show that it is possible align to the international scale the measure of minimal residual disease of CML of a large number of labs, and that it is possible to maintain the alignment over a long period of time (four years). However, it is important to re-calculate lab-specific CFs in the case of reagents or instrumentation changes.

C096

ROLE OF ERK5 IN THE SURVIVAL OF MYELOID LEUKEMIA CELLS IN HYPOXIA

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Introduction. Bone marrow is a physiologically hypoxic tissue, where oxygen levels are likely decreased further in leukemic patients as a result of cell overcrowding as well as anemia, which are frequent consequences of leukemia cell growth. The effects of severe hypoxia on myeloid leukemia cells have been investigated in murine erythroleukemia (MEL) cells to characterize the interference of hypoxia with intracellular signalling pathways, MAPK in particular, relevant to leukemia cell survival and growth. *Methods.* Cells were incubated in 0.3% O2 or normoxia for several days. Cells were harvested at different time points and analysed as for their number and viability by the annexin V test, or lysed to undergo SDS-PAGE and western blotting.

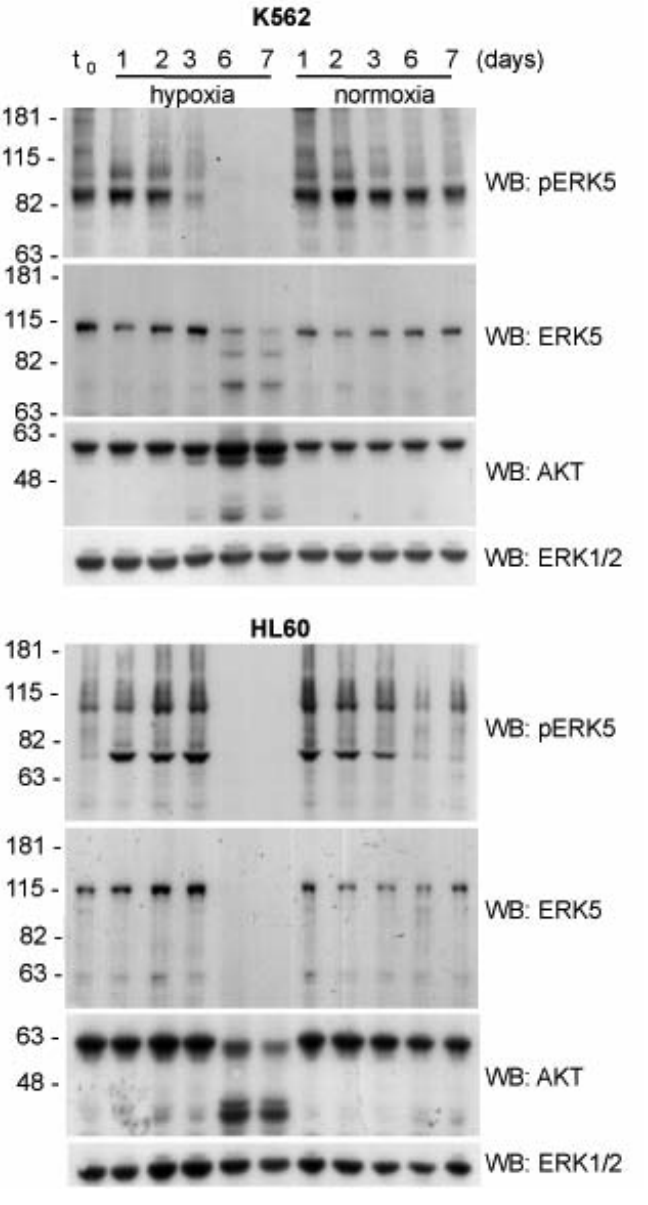

Figure.

Results. Hypoxia prevented the cell number increase which occurred in normoxia and determined early and massive apoptosis, as well as cell cycle arrest of surviving cells. Consistently, the AKT protein, an impor-

tant pro-survival signal, was cleaved in hypoxia. Hypoxia decreased the intensity and duration of ERK1/2, p38 and JNK phosphorylation/activation occurring in normoxia, without altering the expression of these proteins. On the other hand, hypoxia suppressed p120ERK5 constitutive activation and protein expression, unchanged in normoxia. ERK5 mRNA was not decreased in hypoxia. Phosphorylation of a p82 ERK5 form was also abrogated in hypoxia, but not normoxia, although the protein level massively increased. This down-modulation was also found in the HL60 and K562 human leukemia cells undergoing hypoxia-induced apoptosis. Disappearance of p120ERK5 and dephosphorylation of p82ERK5 were prevented by treatment with the pan-caspase inhibitor z-VAD. Accordingly, when a dominant/negative form of ERK5 was overexpressed in K562 cells, cell number was significantly decreased after 48-72 hours in hypoxia, but not normoxia, apparently due to cell cycle arrest of cells in G0/G1 and modest apoptosis. Moreover, silencing of ERK5 with shRNA determined massive apoptosis after 72 hours of incubation in hypoxia, but not normoxia. *Conclusions.* These findings are consistent with a role of ERK5 as a pro-survival signal which is suppressed in leukemia cells undergoing hypoxia-induced apoptosis.

POSTERS

Molecular Hematology and Cytogenetics

P001

A METHODOLOGICAL APPROACH TO MYELOPROLIFERATIVE DISORDERS

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Objective. By using molecular markers, it is possible to gain information on both the classification and the pathophysiology of the myeloproliferative neoplasms (MPNs). We studied three molecular markers the BCR/ABL fusion gene, the single point mutation of JAK2 (Val617Phe) and enumeration $CD34^+$ cells. Reciprocal t(9;22) BCR/ABL fusion gene is a balanced translocation, and the chromosome 22 breakpoints (Philadelphia chromosome-Ph^+) determine formation of different fusion genes that are associated with chronic myeloid leukaemia (CML), Ph^+ acute lymphatic leukaemia (Ph^+ ALL) and acute myelogenous leukaemia (AML). JAK2V617F, a somatic gain-of-function mutation involving the JAK2 tyrosine kinase gene, occurs in nearly all patients with polycythemia vera (PV) but also in a variable proportion of patients with other myeloid disorders; mutational frequency is estimated at approximately 50% in both essential thrombocythemia (ET) and myelofibrosis (MF), up to 20% in certain subcategories of atypical myeloproliferative disorder, less than 3% in de novo myelodysplastic syndrome (MDS) or acute myeloid leukaemia, and 0% in chronic myeloid leukaemia (CML). Circulating $CD34^+$ cell counts are typically elevated in patients with MFI, and this was found to correlate with prognosis in one study but not in another. The etiology of the high $CD34^+$ cell count in blood is not known, but 2 pathophysiologic mechanism have been hypothesized. The first is fibrosis tissue distortion of the marrow architecture resulting in the displacement of the hematopoietic precursors into the circulation. The second is an adhesion defect specific to clonal MFI stem cells and progenitors resulting in their displacement from peripheral-blood. In this work, we evaluated 39 patients with myeloproliferative disorders followed at U.O.C of Oncology. RESULTS JAK2V617F was found in 6 cases including 2 PV, 1 in patient with suspect of myeloproliferative disorders in LNH, 2 cases of MFI and 1 of AML. Patients with BCR ABL positive include 5 LMC and 1 LMA. No individuals with either mutation were identified. The most consistent relationship was that between JAK2 mutation and circulating $CD34^+$ cells count. All patients with JAK2 mutation had high livell of $CD34^+$ cells (>15elements/ul). *Conclusions.* In the diagnosis and classification of myeloproliferative disorders the newly identified molecular markers are still very important, in addition to the clinical and laboratory data.

P002

MOLECULAR ANALYSIS OF HEREDITARY HEMOCHROMATOSIS: CASE REPORT

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Introduction. Our previous study showed that the hereditary hemochromatosis, a recessive disorder that leads to iron overload, is characterized by a particular clinical behavior in the province of Matera (Basilicata). Studying the frequency of the main mutations related to the disease (in particular C282Y and H63D) we observed that the second mutation is primarily responsible for the hemochromatosis in the district. Furthermore, a high frequency of the H63D carriers was found. In contrast, in Italian population C28Y homozygous are more frequent and they manifest more severe iron overload than the other HFE genotypes. The aim of the present work was directed to detect and understand, in molecular key, the peculiar clinical appearance focusing on a particular case report. *Methods.* A case report is presented for a 62-year-old female with a pathologic value (62%) of the transferrin saturation, a sensitive parameter of iron overload. The genetic test for hemochromatosis was performed. The experimental design provided: DNA extraction from whole blood, DNA amplification by multiplex PCR, reverse dot-blot on nitrocellulose strips to analyze the presence of 11 HFE gene mutations (V53M,

V59M, H63D, H63H, S65C, Q127H, E168X, E168Q, W169X, C282Y, Q283P), 4 Transferrin Receptor mutations (Y250X, E60X, M172K, the AVA Q 594-597) and 2 Ferroportin mutations (N144H, V162). In a second step, we made the primer design, based on HFE gene sequences present in NCBI database to amplify DNA and to isolate the most significant fragments. After, HFE gene fragments sequencing was performed. The next bioinformatics analysis allowed to verify the homology among our obtained sequence and known HFE gene sequences. *Results.* The first step shows that the analyzed genotype is H63D heterozygous. We found an abnormal transferrin saturation value, as expected for the homozygous. There aren't additional factors that can explain this result, as evidenced by clinical history. In addition, the sequencing results put in evidence the presence of a genetic variation in HFE gene, reported in SNP database as rs2858996. *Conclusions.* The present study confirms the singular clinical behavior of the disease in Matera province. Furthermore, the polymorphic site could change a sequence required for intron excision, resulting in alternative splicing mechanism. The work emphasizes the importance of gene sequencing in diagnosis and management of the complex disease, such as the hemochromatosis. For an appropriate characterization of the disorder we should consider and investigate both the structural and functional gene aspects. Although extremely important due to its biological significance, the coding region should not be the only fraction investigated in a genomic analysis, because several important regulatory mechanisms involve the non-coding gene region, the UTR (UnTranslated Region) in particular.

P003

ANALYSIS OF CIRCULATING MICRORNAS EXPRESSION PROFILE IN PATIENTS TRANSPLANTED FROM MATCHED UNRELATED DONORS

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Introduction. Allogeneic haemopoietic-stem-cell transplantation (HSCT) is the treatment of choice for many malignant and non-malignant disorders. Despite the recent advances in post-transplant immunosuppressive therapy, Graft-versus-Host Disease (GVHD) still represents the major life-threatening complication, developing in a substantial number of HSCT patients and resulting in poor outcome. Recent studies have indicated that microRNAs (miRNAs) circulate in a stable, cell-free form in the bloodstream and that the abundance of specific miRNAs in plasma or serum can serve as biomarkers of cancer and other diseases. We examined plasma microRNA (miRNA) expression profiles from patients transplanted from matched unrelated donor (MUD) to assess their clinical application for diagnosing and monitoring GVHD. *Methods.* Having obtained an informed consent, we collected plasma samples from 6 patients who received unmanipulated HSCT from MUDs. After HSCT, 3 of 6 patients developed acute GVHD. Blood samples were collected serially at day +30, +60, +90, +150 after HSCT. MicroRNAs were isolated from the peripheral blood (PB) plasma using a modified mirVana™ miRNA Isolation Kit (Ambion Inc). The miRNAs expression profile was examined using a quantitative PCR-method (TaqMan ® Human microRNA cards, Applied Biosystems) that allows the analysis of 384 human miRNAs by low density array technology. Plasma samples of normal subjects have been included in the study. Relative quantification of miRNA expression was calculated with the 2- Ct method. The data were normalized respect to hsa-mir-16 and relative to a calibrator sample (average of normal subjects plasma samples). *Results.* Initial analysis showed that miRNAs are stable and detectable in all plasma samples from MUD transplanted patients. The plasma miRNA levels in patients without GVHD showed an heterogeneous expression pattern until day +150, when the miRNA levels reached those of the healthy controls probably reflecting a normal immune reconstitution. Among the 384 mirRNAs analyzed, we identified 4 mirRNAs that may have a predictive role for GVHD. mir203, upregulated in patients with GVHD, acts downregulating SOCS3, a protein involved in inflammatory responses. mir142-5p is upregulated in patients with GVHD but not in patients with a normal immune reconstitution, where the expression levels are similar to those of the healthy controls. Similarly, mir10b is upregulated in the GVHD group. mir155, involved in many biological processes such as inflammation and hematopoietic differentiation, is upregulated in all samples of patients with GVHD whereas in the other group it s expression profile is similar to the one of the healthy controls at day +150. *Conclusions.* Further analysis are required to specifically identify the significance of the microRNAs upregulated prior to the GVHD onset. Although preliminary, these results indicate that the detection of circulating miRNAs might provide new complementary markers of GVHD.

P004

HISTONE ACETHYLASE INHIBITORS VORINOSTAT AND ITF2357 EXERT A DEMETHYLATING EFFECT ON THE MYELOMA CELL LINE U266

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DNA methylation and histone modifications collaborate to the regulation of gene expression. Histone deacetylase inhibitors (HDACIs) demonstrated cytotoxic activity on several human tumor cell lines. We investigated whether two of the most potent HDACIs, vorinostat and ITF2357, have demethylating activity both on global DNA methylation and on single gene promoters. The human myeloma cell lines U-266, the human acute megakaryoblastic leukemia M-07e, the acute myeloid leukemia HL60, the human chronic myeloid leukemia in blast crisis K562, the human erythroleukemia cell line HEL, the human cervix carcinoma cell line HeLa, and the human melanoma cell line SK-MEL-30 were incubated with several increasing concentrations of vorinostat and ITF2357 for 12-48h. After detection of respective IC50 for each cell line by MTT assay, we performed the LUminometric Methylation Assay (LUMA) to evaluate the global DNA methylation. This method is based on a polymerase extension assay using the Pyrosequencing™ platform. DNA methylation is defined as the HpaII/MspI ratio. If DNA is completely unmethylated, the HpaII/MspI ratio would be 1.0, and if DNA is 100% methylated the HpaII/MspI ratio would approach zero. Moreover, we analysed specific DNA methylation level of promoters of SOCS-1, JUN-B, and PTEN by the Methyl-Profiler DNA Methylation qPCR Primer Assays kit (SABiosciences). All testes cell lines resulted partially methylated, with a mean HpaII/MspI ratio of 0.49, the K562 cells having the highest and HL60 the lowest methylation levels. Only HEL, U266, and M-07e resulted demetylated after treatment with both vorinostat and ITF2357. SOCS-1 was unmethylated in HL60, K562, KG1-a, with consequent no variations after both treatments. JUN-B was unmethylated in U266, KG1-a, K562, HEL, and M-07e. PTEN was unmethylated in SK-MEL30, U266, KG1-a, K562, and HL60. Vorinostat demethylated SOCS-1 promoter in U-266 and HeLa cell lines. ITF2357 exerted the same demethylating effect in SK-MEL30, U266, and HeLa. Methylation status of JUN-B and PTEN did no change after treatment with both HDACIs. This study shows that HDACIs vorinostat and ITF2357 could exert also a demethylating action that would be line-dependent. It could be particularly relevant the demethylation of SOCS1. Indeed, this gene negatively regulates the Janus kinase/signal transducer and activator of transcription (Jak/STAT) signaling pathway. SOCS-1 is hypermethylated in 62.9% of MM patient samples; thus, a compound able to demethylate it would be useful in this kind of haematological malignancy.

P005

HOW THE HISTONE DEACETYLASE INHIBITOR ITF2357 INTERFERES WITH APOPTOSIS AND INFLAMMATION PATHWAYS IN THE HL-60 MODEL: A GENE EXPRESSION STUDY

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Histone deacetylase inhibitors (HDACIs) have been recently developed as promising anticancer drugs. They are able to block the proliferation, induce differentiation and apoptosis in a wide variety of transformed cell in culture, to increase expression of the cyclin-dependent kinase inhibitor p21waf1, to reduce levels of cyclin A and D and inhibit the activity of thymidylate synthase. In addition to their anti-neoplastic activity, HDACIs seem to be very promising anti-inflammatory drugs.

ITF2357, a novel member of the hydroxamic acid family, has been shown to be potent in prevention of inflammation in LPS-induced shock and concanavalin A-induced hepatitis in mice. Moreover, survival of leukaemia-bearing SCID mice was significantly prolonged when they received ITF2357, so supporting the anti-leukemic activity previously shown by the *in vitro* experiments. On these bases, we decided to investigate the mechanism of action of ITF2357 in the HL-60 model, with particular attention to the apoptotic and inflammatory pathways, by performing gene expression studies (real-time RT-PCR and microarray assays). A dose- and time-dependent inhibition of cell growth and increased apoptosis were observed. ITF2357 reduced levels of Bcl-2, Mcl-1, and Bcl-X proteins, and increased levels of Bak. Both caspase 9 and 8 inhibitors reduced apoptotic rate of 73%. Exposure to ITF2357 did not abrogate the NF- B DNA binding. Among the pro-inflammatory genes analysed, ITF2357 significantly down-regulated ATF1, CFB, IL8, IRAK1, LTBR, RIPK1, and TLR4. Among the anti-inflammatory genes, ITF2357 increased expression levels of HMOX1 and NLRP12. After microarray assays, IL10, IL6, EGF, PPAR, TGF , P38MAPK, aryl hydrocarbon receptor, xenobiotic metabolism, PPAR/RxRa, NF-kB, apoptosis, LPS/IL1, G-protein receptor, T-cell receptor, and PDGF were the most de-regulated pathways. CD40, CD86, CDKN1A, CEBPB, IL1E, JUN, STAT3, and WT1 were down-regulated. On the contrary, resulted up-regulated: ADRB2, EDN1 and KLF2. In the culture supernatants, TNFalpha levels increased, whereas IL6 and TGFbeta levels decreased, while levels of ICAM1, IL1beta, and IL10 did not change. This is the first gene expression study concerning effects of ITF2357 on HL-60 cells; the deep impact that it seems to play on the cell proliferation by inducing apoptosis and on the inflammation appear particularly interesting for a possible use of it in different haematological clinical settings (during chemotherapy, but also for limiting complications arising after allogeneic transplantation, such as GVHD).

P006

NEW INSIGHTS INTO TET2 INVOLVEMENT IN HEMATOLOGICAL DISEASES

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Background. In myeloid malignancies rare acquired 4q deletions of different sizes are prevalently found in complex karyotypes. At 4q24 high resolution single nucleotide polymorphism (SNP) and array-CGH revealed TET2 microdeletions, uniparental disomy and mutations. *Aims.* As TET2 is a putative tumor suppressor gene we studied 12 patients with haematological diseases and diverse types of 4q rearrangements and screened an additional 129 patients for TET2 involvement. *Methods.* We selected 12 patients (5 with AML, 3 with MDS, 3 with CMML and 1 with transient pancytopenia and a history of clear renal cell carcinoma) with 4q abnormalities at karyotyping. FISH was performed with 4q21-q25 genomic clones and with 3 fosmids encompassing TET2 exons 1-5: G248P85971A1 (106279899 to 106280552), G248P81536D9 (106320855 to 106359050), and G248P88482A5 (106341804 to 106383916). Array-CGH was done with the CytoChip V2.01 BAC-Array (Bluegnome, Cambridge, UK). Bisulfite converted DNA were amplified with Methylation Specific PCR (MSP) using two pairs of primers spanning from nucleotide 106067752 to 106067858 of TET2 gene promoter. Biological samples included bone marrow, peripheral blood (PB) cultured with PHA, CD3-positive PB lymphocytes and buccal mucosa cells at diagnosis and during disease evolution. TET2 mutations were analysed by DHPLC and direct sequencing. We screened 73 males and 56 females (age range: 5-89, median: 67 years) with MDS/AML with normal karyotype (37), MDS/AML with del(5q) (22), JAK2V617F positive MPN (11), MDS/AML/MPN with -7/del(7q) (32), MDS/AML with 3q26-translocations (9), and CMML/atypical CML (18). *Results.* 12 patients with 4q rearrangements: FISH and array-CGH identified a minimal common deleted region of about 75kb, corresponding to clone RP11-16G16, encompassing the 3'TET2. TET2 mutations of the residual allele were detected in the 3 CMML and 1 AML whereas TET2 promoter CpG island methylation was not found in any case. Additional karyotypic or cryptic changes were found in 9 patients and were acquired in another case during disease progression. The most frequent recurrent changes were -5/del(5q), in 6 cases, and -7/del(7q) in 7. In the patient with severe transient pancytopenia constitutional karyotype and FISH indicated 50% germline mosaicism in all tissues. Screening: FISH detected TET2del in 1 AML patient with inv(3)(q21q26),-7 and TET2mut in 5/17 MDS/AML with monosomy 7. *Conclusion.* Abnormalities in the TET2 suppressor gene underlie haematological diseases in various ways. We observed for the first time that: - del(4)(q24)/TET2 may be germline or acquired; - the incidence of del(4)(q24)/TET2 is less than 1% in myeloid disorders; - concomitant residual allele mutations are present in CMML; - loss of TET2 and of tumor suppressor genes at 7q22-q36 delineate a specific clonal evolution into AML.

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P007

THE SQSTM1 GENE AT 5Q35 IS A NEW NUP214 FUSION PARTNER IN ADULT T-ALL

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Background. Rare fusions involving nucleoporins e.g. NUP98 and NUP214 are believed to predict poor prognosis in T-ALL. NUP214-ABL1 and SET-NUP214 have been identified to date and SET-NUP214 is closely associated with a specific gene profiling signature (GEP).[1] Remarkably, GEP analysis in 69 adults with T-ALL[2] identified one case with a slightly different profile. High expression levels of HOXA cluster genes, MEIS1 and NUP214 were present as in the SET-NUP214 positive samples but unlike them, SET levels were remarkably high. This 20-year old man with a chemo-resistant pre-T ALL had 46,XY,del(6p) karyotype. *Aim.* To determine whether NUP214 was involved in a new chromosomal rearrangement in this case of T-ALL. *Methods.* We set up FISH assays with RP1-112N13 for the 9q sub-telomere, RP11-143H20 flanking the 3'NUP214, RP11-544A12, spanning the gene, and two overlapping fosmids, G248P89801E11 (NUP214 ex. 25-31) and G248P8659A12 (NUP214 ex. 31-36). Nested RT-PCR was performed using primers SQSTM1_528F (5'-TGCCCAGACTACGACTTGTG-3') / NUP214_6543R (5'-AGTAATCATGCGCCTTGTGAGTT-3'), for the first amplification round, and SQSTM1_763F (5'-AATCAGCTTCTGGTCCATCG-3') / NUP214_6337R (5'-CAAAGCTGAACCCTCCTGTG-3') for the second. To establish the prevalence of the SQSTM1-NUP214 fusion we used nested RT-PCR to screen 67 adults with T-ALL. *Results.* FISH with RP11-544A12 hybridized with both 9 and an apparently normal 5. G248P89801E11 were detected on both normal 9 while G248P8659A12 was detected on normal 9 and on der(5), narrowing the 9q34/NUP214 breakpoint to 3' region of NUP214. The 5q35 breakpoint was telomeric to RP11-718N2, a ~1.7Mb region containing 15 candidate genes, with an appropriate centromere-telomere orientation. Since GEP revealed 3/15 genes telomeric to SQSTM1/5q35 were down modulated, they might have been included in the 5q deletion. SQSTM1 was first selected as putative NUP214 fusion partner. RT-PCR detected an amplification product of 852bp. Molecular cloning and sequencing identified the in-frame fusion between nucleotide 849 (exon 5) of SQSTM1 and nucleotide 6014 (exon 33) of NUP214. Nested-PCR screening of 67 T-ALL adults did not identify any additional case. *Conclusion.* SQSTM1 mutations and over-expression were described in congenital Paget's Bone Disease and solid tumours, respectively. No translocations involving SQSTM1 have as yet been reported. This case illustrates a cryptic unbalanced translocation der(5)t(5;9)(q35;q34) underlay a new SQSTM1-NUP214 fusion. As NUP214 behaves as promiscuous gene undergoing different types of rearrangements in T-ALL, we recommend applying specific FISH assays to detect NUP214 in the diagnostic work-up of T-ALL. The incidence and clinical impact of NUP214-rearrangements need to be investigated in large prospective studies. *Aknowledgements.* PRIN 20078C9NRT_003.

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P008

TP53 MUTATIONS ARE FREQUENT INVOLVED IN SPLENIC MARGINAL ZONE LYMPHOMAS HARBOURING DEL17P13

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Introduction. The molecular pathogenesis of splenic marginal zone lymphoma (SMZL) is largely unknown. TP53 inactivation by mutation and/or deletion is involved in several hematologic malignancies and frequently associates with poor outcome. Aims. The study aims at verifying whether TP53 inactivation through somatic mutations and loss of 17p13 is a genetic mechanism involved in SMZL. *Methods.* The study was based on 20 newly diagnosed SMZL harbouring TP53 deletion as documented by GeneChip Human Mapping 250K NspI SNP array (Affymetrix, Santa Clara, CA, USA). TP53 mutation status was assessed by direct sequencing of TP53 exons 2 to 10. *Results.* TP53 mutations (n=12) were observed in 11/20 (55%) SMZL and included missense mutations (6/12, 50.0%), non-sense mutations (1/12, 8.3%), short deletions (4/12, 33.3%), and splice site mutations (1/11; 8.3%). Among missense mutations, the median residual transactivation activity compared to germline TP53 was 16,95% (by http://www-p53.iarc.fr/MutationValidationCriteria.asp). TP53 mutations were distributed in exon 4 (1/12, 8.3%), exon 5 (2/12, 16.6%), exon 6 (2/12, 16.6%), exon 7 (2/11, 16.6%), exon 8 (4/12, 33.3%), and exon 10 (1/12, 8.3%). No mutations were found in exons 2, 3 or 9. Mutations were tested for distribution on TP53 functional domains. Nine of 12 (75.0%) mutations targeted the TP53 DNA-binding domain, 1/12 (8.3%) targeted codons directly involved in DNA contact, and no mutation targeted codons involved in the zinc-binding site. Highly conserved areas of the TP53 molecule were affected by 4/12 (33.3%) mutations. Mutational hot spots were affected by 1/12 (8.3%) mutations. *Conclusions.* TP53 mutations occur in half of SMZL cases harbouring TP53 deletion. This study prompted the investigation of the prevalence and prognostic relevance in a consecutive cohort of SMZL.

P009

EPIGENETIC MODIFICATIONS IN A T(8;21) ACUTE MYELOID LEUKAEMIA CELL LINE CAUSED BY ADMINISTRATION OF TWO HDAC INHIBITORS, VPA AND SAHA

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Introduction. The acute myeloid leukaemia (AML) cell line Kasumi-1 is characterized by the translocation t(8;21), manifested with the expression of AML1/ETO fusion protein, responsible for HDAC recruitment, determining transcriptional repression of target genes involved in myeloid maturation. We demonstrated that butyrates are able to restore histone acetylation and to induce granulocytic maturation, and we recently exhibited the selective anti-leukemic activity of low dose ITF2357 on AML1/ETO-positive cells. Transcriptionally active chromatin is characterized by histones with acetylated lysine tails. We investigated the acetylation of specific lysine residues on histone H4 and H3 after exposure to HDAC inhibitors (HDACi) members of different chemical family. We also analyzed the effects of these HDACi on the expression of Interleukine-3 (IL3) gene, target of AML1/ETO, and the amount of histones acetylation on IL3 promoter. *Methods.* Kasumi-1 cells were treated with VPA 2 mM or SAHA 1 M. Cells were lysed after different times of culture to extract cell proteins for the analysis by Western blotting and mRNA for the relative quantification in Real Time PCR or to immunoprecipates chromatin bound to acetylated H4 or H3. *Results.* VPA or SAHA treatment increased H4 and H3 total acetylation, with a more rapid kinetics for SAHA respect to VPA. Moreover, both HDACi were able to induce, even with a different trend, a marked increase of acetylation on 5, 8 and 16 lysine residues of H4 and lysine 9 of H3, but also in case of SAHA, on lysine 12 of H4. In particular VPA, even inducing transient modifications, presents a late effect but more lasting than SAHA, that acts at earlier times. These epigenetic modifications were paralleled by a resumption of transcriptional activity. In fact, we showed that VPA and SAHA induced the riexpression of IL3 gene, with a fast effect of SAHA and a delayed but more effective effect of VPA. This differential expression depended on a different amount of acetylated H4 and H3 on IL3 promoter. SAHA determined a rapid and simultaneous increase of acetylated H4 on 5, 8 and 12 lysine residues and acetylated H3 on 27 lysine residue, by which could be depend its rapid but transient effect on histones acetylation and IL3 expression. Whereas VPA induced a contemporary acetylation of H4 on 8, 12 and 16 lysine residues and H3 on 27 lysine residue, that could be justify its sustained effect for more times. *Conclusion.* These results suggest that VPA seems to have a more relevant effect respect to SAHA, inducing expression of gene silenced by AML1/ETO, such as IL3, and increasing H4 and H3 acetylation on IL3 promoter.

P010

SYSTEMIC SCLEROSIS AND CHRONIC GRAFT-VERSUS-HOST DISEASE SCLERODERMA-LIKE HAVE OTHER SIMILAR PATHOGENETIC MECHANISMS THAN STIMULATORY AUTOANTIBODIES TO PDGFR?

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Chronic graft-*versus*-host disease (cGVHD), a major complication of allogeneic hematopoietic stem cell transplantation, is a complex multisystem syndrome with overlapping features of immunodeficiency and several of the naturally occurring autoimmune diseases. In particular, scleroderma-like cGVHD may resemble systemic sclerosis (SSc) in skin and lung involvement. Recent studies suggested that both these diseases are driven by stimulatory autoantibodies to platelet-derived growth factor receptor (PDGFR), which in turn stimulates the production of reactive oxygen species and collagen by fibroblasts. Several case reports, case series and uncontrolled studies on patients with SSc and in sclerodermatous cGvHD describe regression of fibrosis and good tolerability after therapy with Imatinib mesylate, a compound that inhibits PDGFR kinases. We studied 11 female patients affected by SSc and 9 patients (5 male, 4 female) with scleroderma-like cGVHD to evaluate the role of T-cell clonality in the pathogenesis of both diseases. Clonality was assessed by analyzing the rearrangement of the T-cell receptor (TCR) gamma gene (region VJA and VJB). Additionally, the lysates prepared from peripheral blood mononuclear cells were studied to detect constitutive phosphorylation of ERK1/2 kinases. We analyzed also expression profiles of genes involved in apoptosis, including BCL2A1, CASP1, CASP6, EGR1, EGR2, FAS, FOS, TNF, TNFRSF1A, transduction signals (IKB, NFkB), intercellular adhesion (ICAM-1), and immune regulation (Fox-p3, CD52, CD83, CXCL1, CCL2, CCL5, IL-10, IL-17). Ten patients (90.9%) with SSc and 5 patients (55.6%) with cGVHD had oligoclonal T-cell expansion. ERK-autophosphorylation was detected in 5 of 10 (50.0%) patients with SSc and in 2 of 5 (22.2%) patients affected by cGVHD. Phosphorylation of ERK1/2 kinases in the other 3 patients with sclerodermatous cGVHD is not available. In addition, we found that expression profiles of several genes implicated in the Th1/Tregs/Th17 responses were similar in patients with SSc or cGVHD, while expression profiles of other genes, particularly those associated with the Th2 response, were different. In conclusion, we think that expanded clonal T-cells could play a critical role in the pathogenetic mechanisms of both SSc and scleroderma-like cGVHD, probably in some cases by the activation of the same gene expression profiles and the same signal transduction pathways. Further studies are needed to confirm these preliminary data and to better explain similarities and differences between these two clinical conditions.

P011

EXPOSURE OF CULTURED MESENCHYMAL STEM CELLS TO CHEMOTHERAPY: A SIMPLE AND RELIABLE PROCEDURE TO INVESTIGATE THE MOLECULAR MECHANISMS OF STEM CELL SENESCENCE

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Introduction. The regulatory mechanisms that prevent premature ageing of stem cells, and eventually cell senescence and apoptosis, have been recently studied with increased attention. The risk of cell ageing is of special concern when stem cells are taken from an organism, manipulated *in vitro* and finally transplanted *in vivo*. A good indicator of cell replication history is the length of telomeres (TL), the long tandem repeat sequences at the end of chromosomes. Telomeric DNA in humans decreases at each somatic cell division, and undergoes progressive shortening with increasing age. Two main factors might interfere on the steady state of stem cells and trigger the ageing process: an increased cell proliferation and the exposure to DNA-damaging substances. Aim of the present study was to investigate at which extent exposure to toxic agents may make older the BM-derived Mesenchymal Stem Cells (MSCs). *Methods.* Cultured human MSC from BM were exposed to low doses of Doxorubicin (Doxo) at 10 nM or Etoposide (Eto) at 500 ng/mL, for 2 hours; drug exposure was replicated four times at 7 day interval. TL was assessed by Flow Fish analysis. Differentiation assays were performed by culturing hMSC with osteogenic and adipogenic differentiating medium and visualized with Alizarin red and Oil red staining, respectively. ATM phosphorilation following exposure to chemotherapy was assessed by Western blot analysis; telomerase activation was tested as well. *Results.* A progressive decrease in TL was observed in cultured MSCs, with a shortening to 89% of TL at day 7 in culture compared to a 100 % value assumed at day 0 (T 0). When MSCs were exposed to chemotherapy, telomere loss was enhanced, with 82 % and 80% TL at day 7 following Doxo and Eto, respectively. TL further decreased after 2 weeks of culture (untreated 83% *vs.* Doxo 76% *vs.* Eto 73%); the different TL behaviour in drug-treated MSC was even more evident after 3 and 4 weeks in culture (untreted 82%, Doxo 68%, Eto 70% at week 3; untreated 73%, Doxo 54%, Eto 62% at week 4). To verify drug specificity, MSC were exposed to increasing doses of drugs and the reduction of TL occurred in a dose-response manner. Exposure to chemotherapy also influenced MSC differentiation *in vitro*, with enhanced adipogenic and osteogenic differentiation under appropriate induction compared to untreated cells. Western blot analysis indicates strong ATM activation after drugs exposure, meanwhile telomerase activity remain inactive. *Conclusions.* i. DNA damages induced by Doxo and Eto bring about an early cell ageing documented by TL reduction; ii. telomere loss is an early event following chemotherapy exposure, it can be detected at day 7, although it is maximally proven at week 3 and 4 following drug exposure; iii. the *in vitro* observations suggest that not only hematopoietic cells but also stromal cells, including MSCs, may suffer relevant DNA damages following treatments with chemotherapeutic drugs.

P012

CHROMOSOME 8P11 TRANSLOCATIONS: CYTOGENETIC AND MOLECULAR-CYTOGENETIC CHARACTERIZATION OF PATIENTS WITH FGFR1 REARRANGEMENT AND CORRELATION WITH CLINICAL-BIOLOGICAL FEATURES

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Introduction. Rearrangements of FGFR1 gene, associated with translocations of chromosome band 8p11 are described in rare myeloproliferative syndromes that are frequently characterized by a high incidence of T-lymphoblastic-lymphoma and eosinophilia (WHO 2008). So far, 10 partner genes have been reported; the most common are: ZNF198 (13q12), FGFR1OP (6q27), CEP110 (9q33) and BCR (22q11). Despite the small number of cases described in literature, clinical heterogeneity has been observed among patients with different translocations. Therefore it has been hypothesized that FGFR1 partner gene can influence the disease phenotype. The syndrome is aggressive and rapidly evolves to acute leukemia, usually myeloid. The aim of our study was to define molecular-cytogenetic and clinical-biological features of patients with FGFR1 rearrangements. *Methods.* The cases were selected according to the presence of abnormalities at the band 8p11 by conventional cytogenetics. Subsequently patients were analyzed by FISH, RT-PCR and SNPs-array. *Results.* At diagnosis, chromosome 8p11 abnormalities were identified in 12 patients. FISH analysis for FGFR1 rearrangement has been possible in 11 patients. Four patients showed FGFR1 rearrangement in association with the following translocations: t(6;8)(q27;p11), t(8;9)(p11;q33) t(8;13)(p11;q12), t(8;22)(p11;q11), involving FGFR1OP, CEP110, ZNF198 and BCR genes, respectively. Only in 1 case the 8p11 translocation was the sole aberration; indeed 1 patient showed trisomy 21, 1 patient monosomy 7 and the last one a complex karyotype. The patient with the t(8;13) showed the deletion of 5' FGFR1 on der(8) by FISH analysis. SNPs array analysis are still ongoing in order to highlight micro-alteration that can contribute to disease development and progression. At the disease presentation, the diagnosis were different: polycythemia vera (PV), chronic myelomonocytic leukemia (CMMoL), T-ALL and B-ALL. Two patients have undergone to allogenic bone marrow transplantation (BMT) and are still alive and well. The remaining 2 patients died within 1 year from diagnosis because of disease progression. Table 1 summarizes molecular-cytogenetic characterization and clinical features of these patients. *Conclusions.* Our cases are characterized by 4 different translocations associated with FGFR1 rearrangements and at diagnosis each patient presented different clinical features. Although the number of patients is limited, our results seem to confirm that the FGFR1 partner gene influence the disease phenotype. The occurrence of specific additional or secondary abnormalities can contribute as well in influencing the disease phenotype and eventual transformation. Our data confirm the rarity and the aggressive course of this disorder. So far, only allogenic BMT seems to be effective in eradicating the malignant clones and therefore it is the treatment of choice.

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Table 1.

	Age/ Sex	Karyotype	Molecular rearrangementi	Diagnosis	Outcome
1	44/F	46,XX,t(6;8)(q27;p12)(8)/45,XX, t(6;8)(q27;p12),-7(12)	FGFR1OP/ FGFR1	PV	dead
2	71/F	46,XX,t(8;22)(p11;q11)(10) 46,XX,del (3)(p11p21),t(7;9) (p12;q21),t(8;22)(p11;q11), der(8)t(8;9)(p11;q13)?,-9(6)/ 46.XX(4)	BCR/ FGFR1	B-ALL	dead
3	33/M	46,XY,inv(2)(p15q21),t(8;9) (p12q34)(30)	CEP110/ FGFR1	CMMoL	alive (allo-BMT)
4	30/M	46,XY,t(8;13)(p11q12)(15)/47,XY, t(8;13)(p11q12),+21(3)	ZNF198/ FGFR1	T-ALL	alive (allo-BMT)

P013

CBFA2T2 AND C20ORF112: TWO NOVEL FUSION PARTNERS OF RUNX1 IN ACUTE MYELOID LEUKAEMIA

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Introduction. RUNX1 encodes a transcription factor crucial for hematopoiesis. In leukemia, this gene is often the target of translocations generating chimeric transcripts. Chimeras are usually composed of the 5' of RUNX1 and the 3' of the partner gene. They retain the Runt Homology Domain (RHD; N-terminal) and lack the C-terminal transactivation domain, which is a leukemogenic factor acting as a dominant, negative

inhibitor of wild-type RUNX1 in transcription activation. *Methods.* Appropriate clones (BAC or fosmids) were selected by browsing the UCSC Human Genome Browser (http://genome.ucsc.edu/), March 2006 (NCBI36/hg18) release, and used in FISH experiments. PCR analyses were performed on samples in order to obtain fusion junctions. To detect putative protein-conserved domains, in silico translation and BlastP analyses were performed by using the ORF finder tool of the NCBI website (http://blast.ncbi.nlm.nih.gov). *Results.* The first case showed an insertion of chromosome 20 material within RUNX1, juxtaposing it to the CBFA2T2 gene (20q11.22). CBFA2T2 is a paralog of both CBFA2T1 (8q21.3) and CBFA2T3 (16q24.3) genes, which are known to form chimeras with RUNX1. The second patient displayed a balanced t(20;21), fusing RUNX1 to C20orf112 (20q11.21), a PAX5 fusion partner. All predicted protein products retained the RUNX1 RHD domain and lost the transactivation domain, which was replaced by either the C-terminal of CBFA2T2, recruiting transcriptional corepressors, or by C20orf112. Conversely, the reciprocal C20orf112/RUNX1 lost the RHD, but retained the transactivation domain. *Conclusions.* In summary, we report the characterization of two novel chimeric transcripts identified in AML with a t(20;21) translocation cases, involving CBFA2T2, an ETO homologous gene on chromosome 20, and C20orf112, a known partner of PAX5 in pediatric acute lymphoblastic leukemia.

P014

MYELODISPLASTIC SYNDROME WITH TRISOMY 21 IN A PATIENT WITH CONSTITUTIONAL SUBMICROSCOPIC 21Q22 DELETION

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Introduction. We describe a patient with syndromic thrombocytopenia, psychomothor delay, mycrocephaly and low stature, that developed a myelodisplastic syndrome (MDS) at seventeen years age. The analysis, performed on bone marrow and peripheral blood cells by conventional and molecular cytogenetic, showed a constitutional microdeletion, involving RUNX1 and the trisomy of chromosome 21 in the myelodisplastic clone. These data support a model in wich haploinsufficiency of RUNX1 causes a sporadic syndromic thrombocytopenia or Familial Platelet Disorder (FPD) and predisposes to the acquisition of additional chromosomal and genomic aberrations that evolves in Myelodisplstic Syndrome/Acute Myeloid Leukaemia (MDS/AML). Furthermore the myelodisplastic clone with trisomy 21 showes that other dosage-sensitive unknown genes, other than RUNX1, plays a role in MDS/AML associated with trisomy of chromosome 21. *Methods.* Cytogenetic analysis was performed on bone marrow cells and on peripheral blood lymphocyte, with standard techniques and evaluated with Giemsa-trypsin-Giemsa banding according to International System for Human Cytogenetic Nomenclature (ISCN 2009). Fluorescent In Situ Hybridization (FISH) experiments was performed on bone marrow samples with LSI AML1/ETO Dual Color, Dual Fusion Translocation and CEP 21 (Abbott); at the same time, the High-resolution oligo array-CGH (Agilent Human Genome CGH Microarray 44B) was performed on the DNA of the patient. *Results.* The bone marrow cells showed marked dysplastic morphology and the following abnormal karyotype: 46,XX[14]/47,XX,+21[3], the peripheral blood karyotype was normal. The High-resolution oligo array-CGH demonstrated a constitutional de novo microdeletion of one chromosome 21. The interstitial deletion was found to be approximately 4,4Mb (Megabases), extending from 32,29 Mb to 36,51 Mb on band 21q22.11-12, involving MRAP, IFNAR2, IFNGRR2, KCNE2, KCNE1 and RUNX1 genes. The FISH performed on bone marrow cells, revealed on metaphases one green signal for AML1 on 80% cells and two green signals in the remaining 20% cells. When we performed WCP probe 21 analysis, we find +21 in 8% out of 35 methaphases observed (three painted green signals). The first pattern of signals, is related to cells with karyotype 46,XX, while the second pattern of signals corresponds to cells with karyotype 47,XX,+21. These results indicate that in the myelodisplastic clone the third chromosome 21 are not deleted on band 21q22. *Conclusions.* Our results further support the fundamental role, in the pathogenetic mechanism of syndromic thrombocytopenia and MDS/AML, of the numerical abnormalities of chromosome 21 associated with submicroscopic rearrangement of RUNX1 and other dosage-sensitive unknown genes on chromosome 21.

P015

STIMULATION WITH CPG OLIGONUCLEOTIDES PLUS INTERLEUKIN-2 (IL-2) IMPROVES THE OUTCOME OF CONVENTIONAL CYTOGENETIC ANALYSIS IN SPLENIC MARGINAL ZONE LYMPHOMA

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Introduction. Splenic Marginal Zone Lymphoma (SMZL) is an indolent disease representing <2% of lymphoid neoplasms. In up to 40% deletion of 7q22-36 are found in this lymphoma and other recurrent abnormalities include +3q, +7, +12 and +18 and deletion of 6q. It has been suggested that the presence of 7q deletion and unmutated IGHV genes may be associated with unfavourable outcome. The advantage obtained with CpG oligonucleotides (ODN) cultures improved the success of Conventional Cytogenetic Analysis (CCA) and recently provided important advances in the risk stratification of chronic lymphoprolipherative diseases. We studied 18 SMZL cases by CCA with ODN and with traditional mitogens in order to evaluate quantitative and qualitative differences in the obtained metaphases. *Methods.* Lymphocytes harvested from 18 patients with hystologic/immunophenotypic diagnosis of SMZL were separately cultured using tetra-decanoyl phorbol acetate (TPA), E.Coli Lipopolysaccaride (LPS) and ODN plus IL-2. Karyotypes obtained from the TPA, LPS and ODN cultures were then compared. The evaluation took into account the following features: Mitotic Potential (i.e.: the number of metaphases/slide), Resolution Potential (i.e.: ≥400 evident chromosomal bands/karyotype), Mitogen Efficacy (i.e.: induction of mitosis in ≥1 pathologic cell), the profile of cytogenetic aberrations. *Results.* Mitogenic potential: TPA-cultures demonstrated the maximum growth inadequacy (0-5 metaphase/slide) in 7/18 cases (38,9%). In LPS-cultures an insufficient number of metaphases was seen in 22,2% of the cases, while all ODN-cultures showed at least 5 metaphases/slide. Resolution potential: ODN-cultures showed a satisfying resolution in 14/18 cases (77,8%). Good-quality metaphases in LPS-cultures were 8/18 (44,4%) with 3/18 (16,7%) with poor-quality metaphases. In TPA-cultures good-quality metaphases were obtained in 8/18 (44,4%). Mitogenic Efficacy: the absolute number of abnormal metaphases/patient was 14/18 (77,8%) in ODN-cultures, 9/18 (50%) in LPS-cultures and 3/18 (16,7%) in TPA-cultures. The greatest number of normal metaphases was observed in TPA-cultures (8/18; 44.4%). Cytogenetic Aberrations: aberrations of chromosome 14 were detected in 6/18 cases (33,3%); 5/18 cases (27,8%) showed structural and numerical aberrations of chromosome 3; +12 and deletion of 7q were detected in 4/18 cases each (22,2%), rearrangements of chromosome 13 were present in 3/18 cases (16,7%). Other recurrent abnormalities were detected with lower incidence involving chromosome 1, 5, 8, 9, 17 and 22. *Conclusions.* ODN cultures showed the better mitogenic potential and mitogenic efficacy, providing the higher number of metaphases and the lower number of failures. Moreover ODN cultures revealed abnormal metaphases in 77.8% of cases analyzed *vs.* ≤50% with other mitogens, revealing some previously unreported chromosome aberrations. Therefore we suggest that ODN cultures may represent a useful tool in the evaluation of SMZL cytogenetics.

Phenotype and Molecular Disorders

P016

ANALYSIS OF CEREBROSPINAL FLUID FROM PATIENTS WITH HEMATOLOGIC NEOPLASM: COMPARISON OF FLOW CYTOMETRY AND CYTOMORPHOLOGY WITH RETROSPECTIVE CLINICAL ASSESSMENT IN 227 SAMPLES

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Background. The diagnostic potential of flow cytometry (FC) and cytomorphology (CM) in analyzing cerebrospinal fluids (CSF) from patients with hematologic neoplasm (HN) according to different clinical settings, and the so-called quantity not sufficient for FC are undetermined. *Methods.* Among CSF analyzed by four-color FC between 2002 and 2010, we selected those with (i) suspected or known HN at withdrawal, (ii) CM performed on the same sample, and (iii) availability of follow-up findings for independent retrospective clinical assessment (RCA). The most accurate cut-off of phenotypically abnormal events acquired by FC was determined on 2/3 of samples, randomly selected (testing population), and confirmed on the remaining samples (validating population), by ROC curve analysis. FC and CM results, positive (more than 5 events with abnormal phenotype for FC; positive/uncertain results for CM) or negative for neoplastic cells, were compared to RCA (positive or negative). *Results.* Two hundreds twenty-seven CSF submitted for suspected (3%) or disclosed HN (prior to treatment: 24%, during follow-up: 73%) were selected for analysis. During a median follow-up of 7.7 (interquartile range: 2.3-13.3) months, 64 CSF (28.2%) were found RCA-positive. Overall, FC specificity (93.8%) was lower than that of CM (98.7%). However, 9 out of 10 false positive results by FC were related to either occult central nervous system disease at diagnosis (n=7) or minimal residual leptomeningeal disease under effective intrathecal treatment (n=2); thereafter, actual FC specificity was 99.4%. Overall, FC and CM sensitivities were 74.6% and 55.9%, respectively. Intra-method FC sensitivity did not significantly differ in the analysis of screening and follow-up samples (75.7% *vs.* 72.7%, P=.8), while CM sensitivity was significantly lower in the latter than in the former (39.1% *vs.* 67.4%, P=.046). Intra-method FC and CM sensitivities did not significantly differ in (i) B- and T-cell lymphoproliferative disease (LPD)(75.7% *vs.* 72.7%, P=.8 and 55% *vs.* 43%, P=.6, respectively), (ii) differentiated and precursor LPD (77.1% *vs.* 76.9%, P=.9 and 53% *vs.* 55%, P=.9), (iii) precursor LPD and acute myeloid leukemia (76.9% *vs.* 63.6%, P=.7 and 55% *vs.* 66.7%, P=.5, respectively). FC was observed to retain 100% sensitivity when acquiring more than 220 (corresponding to the third tertile of distribution of) $CD45^+$ cell events, the correspondent sensitivity displayed by CM being 65.7%. When acquiring 220 events or less, however, FC sensitivity (31.8%) was lower than that displayed by CM (43.5%). *Conclusions:* FC is the best diagnostic tool for detecting neoplastic cells in CSF from patients screened for HN, regardless the clinical setting. Quantity not sufficient should be defined, for FC, when approximately less than 250 $CD45^+$ cell events are acquired on diagnostic panels; in this setting, CM seems to retain diagnostic advantage.

P017

G-CSF ALONE OR IN COMBINATION WITH CHEMOTHERAPY DOES NOT MOBILIZE ENDOTHELIAL PROGENITOR CELLS INTO PERIPHERAL BLOOD

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Introduction. Several drugs can increase the number of EPCs into the peripheral blood (PB); however, there is insufficient data concerning the frequency of EPCs during hematopoietic stem/progenitor cells mobilization and leukapheresis procedures for transplantation purposes in patients and in healthy donors (HD) undergoing different regimen of $CD34^+$ cell mobilization. *Methods.* We studied 19 patients after they received chemotherapy and G-CSF (5 μg/Kg), 19 healthy donors (HD) after they received G-CSF alone (5 μg/Kg), and 24 healthy non-mobilized adults (CTRLs). EPCs were detected at the end of mobilization, both in the PB and in the leukapheresis samples. By flow cytometry, we evaluated: $CD34^+CD45^-$ cells, including EPCs but also mature endothelial cells (ECs); $CD34^+$ $CD45^-CD133^+VEGFR2^+$, a population of putative EPCs; $CD34^+CD45^-CXCR4^+$ or $CD34^+CD45^+$ (hemopoietic cells) co-expressing CXCR4. Results are shown as median (range) of electronically gated $CD34^+$ cells. We assessed the number of Endothelial Colony Forming Cells (ECFCs) grown *in vitro* according to Ingram *et al.* (Blood 2004;104:2752). Results are expressed as median number (range) of ECFC/107 mononuclear cells. *Results.* The percentage of $CD34^+CD45^-$ cells in the PB of HDs (11.6%, 1.1-25.3) was higher (P<0.0002) than that of patients mobilized with cyclophosphamide and G-CSF (0.6%, 0.1-7.3), and comparable to that of CTRLs (13.8%, 0-54.7). The percentage of $CD34^+CD133^+$ $VEGFR2^+CD45^-$ cells was increased in the PB of HDs receiving G-CSF (16.0%, 0-37.3) with respect to that found in the PB of patients receiving cyclophosphamide and G-CSF (0.0%, 0-25.9), and to that of CTRLs (0.0%, 0-83.4) but the difference was not statistically significant. The percentage of $CD34^+CD45^-$ PB cells expressing CXCR4 in mobilized patients was higher than that of $CD34^+CD45^+$ hemopoietic cells (P=0.0048). Similar results were obtained in the leukapheresis samples (*data not shown*). The functional analysis of EPCs showed that the frequency of ECFCs, progenitors faithfully belonging to the endothelial lineage, was comparable both in the PB and in the leukapheresis of either groups, with a median value of 0/107 cells (0-1.47), and not statistically different from that found in the PB of the CTRLs (0/107 cells, 0-0.5). *Conclusions.* Our results indicate that the frequency of $CD34^+CD45^-$ cells is significantly higher in subjects receiving G-CSF than in those receiving cyclophosphamide and G-CSF, and comparable to that of CTRLs. The functional characterization of EPCs showed no significant difference within the two pharmacological treatments and CTRLs, suggesting that the increase of $CD34^+CD45^-$ cells in HD compared to patients is mainly due to mature ECs still able to respond to a chemoattractive cytokine such as SDF-1 through CXCR4. These findings suggest that the mobilization of EPCs into the PB is regulated by pathways different from those involved in hematopoietic stem/progenitor cells.

P018

MONITORING OF MINIMAL RESIDUAL DISEASE BY QUANTITATIVE WT1 GENE EXPRESSION AND HEMATOPOIETIC CHIMERISM ANALYSIS FOLLOWING REDUCE INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA

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WT1 is well-known to be a panleukemic marker that is expressed in 90% of acute myeloid leukemias (AML). Quantification of WT gene expression in bone marrow (BM) samples may be useful as a marker of minimal residual disease (MRD) during and after treatment for early prediction of relapse. We evaluated the validity of this AML-MRD marker after reduce intensity conditioning (RIC) allogeneic stem cell transplantation (SCT). The quantitative assessment of WT1 expression by real-time quantitative PCR (according to the Leukaemia NET method) was measured in 25 patients (pts) with AML at diagnosis, at the time of RIC-SCT and after transplant at precise time points. All pts showed high WT1 levels at diagnosis with a mean of 4895 (SD 4462) and a median of 3679 (range 454-16853) copies WT1/104 ABL. At transplant, 18/25 pts (72%) were in Complete cytologic Remission (CcR) and 7/25 (28%) had refractory AML. At the pre-SCT evaluation, BM samples from pts transplanted in CcR showed significantly lower WT1 expression levels compared to the samples from pts with refractory AML (P 0.002). Median follow-up after RIC-SCT was 18 months (range 2-54). On 18 pts transplanted in CcR, those (17/18) who maintained CcR after RIC-SCT displayed a WT1 copy number persistently low and a full donor chimerism (FDC) during all the follow-up period. In patients who received RIC-SCT with active disease obtaining a sustained CcR after transplant (3/25), WT1 levels decreased to normal range in the first two months after RIC-SCT and remained low through the entire study period. All pts who relapsed after RIC-SCT (4/25) had a high WT1 copy number before the cytologic relapse; in 50% of these cases, an increase in WT1 expression was documented before molecular chimerism decreasing. With this experience, taking into account the limited number of cases, we con-

firmed a concordance between WT1 expression levels (measured by RQ-PCR at precise and sequential time points) and status of AML before and after RIC-SCT and we found a concordance between WT1 expression levels and hematopoietic chimerism status. Our data suggest that, in the RIC-SCT setting, the sequential and quantitative analysis of WT1 combined with hematopoietic chimerism evaluation, may be useful for monitoring MRD and as a predictor of overt AML cytologic relapse.

P019

WT1 IS A USEFUL MINIMAL RESIDUAL DISEASE MARKER TO EVALUATE ACUTE MYELOID LEUKEMIA OUTCOME: RELATIONSHIP BETWEEN BONE MARROW, PERIPHERAL BLOOD AND HARVEST

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Introduction. In healthy patients,WT1 expression in bone marrow (BM) and PB is low or not detectable by PCR, while is high in AML. Recent data suggest that WT1 may be a useful tool for evaluating the minimal residual disease (MRD) in AML. We monitored WT1 expression from AML patients, both at the onset of the disease and during the follow up (F.U.). Patients and methods: In 41AML patients (19M/22F, median age: 71, range 23-86) WT1 expression was evaluated by RQ-PCR, normalizing WT1 by gene ABL with the following calculation: WT1/ABL*104.WT1 values>180 were considered abnormal. *Results.* 1. WT1 is highly expressed at diagnosis but not in all patients: 35 of 41 patients (85%) showed high levels of WT1 at diagnosis. The median value was 1136 (range 190.2-9176). BM blasts percentage at onset was 58%(range 14-95); as regards cytogenetic profile, 4 patients had favorable karyotype, 13 intermediate, 6 unfavorable and 12 unevaluable;10 had favorable molecular profile, 14 intermediate, 8 unfavorable, 3 unevaluable. Among the 6 AML patients(15%) with low levels of WT1,the median value was 78.8 (range 52.5-168.3), BM blasts percentage (BC) was 69%(8-85);5 had normal karyotype, 4 intermediate molecular profile. 2. WT1 is useful to monitor relapse of patients with AML. At F.U., we analyzed WT1 in 19 patients (median F.U. 4 months, range 1-18). Three pattern of patients have been identified: a) patients achieving complete remission (CR) with reduction of WT1 under the starting value; b)patients with refractory AML, never showing a reduction of WT1; c) patients showing reduction of WT1 as response to treatment and then showing an increased WT1,predicting the relapse 58 days (range 30-119) before the morphological evidence. There was a relationship between WT1>180 and BC>50%,but WT1 levels were high also in 3 CR patients (BC<5%) who relapsed, confirming that WT1 is a sensitive marker of MRD. 3. WT1 is expressed in leukemic blasts and not in normal immature $CD34^+$ cells: We found low WT1 values in 5 aphaeretic products(median 3.9 range 1.15-15.2) from 5 non-AML patients (without BM involvement) although the high count of immature cells (median $CD34^+$ 3760/uL, range 2250-13650),suggesting that WT1 expression is not related to the content of normal immature progenitors. 4. WT1 levels in BM and PB are covariated: in 26 cases we correlated WT1 values in BM and PB; in 81% of cases WT1 levels were lower in PB than in BM; in 19% PB levels were superior (overall ratio: r^2=0.87). *Conclusions.* Our data confirm that WT1 is over-expressed in high percentage (85%) of AML-patients. Its expression is only partially related to the BC. In a small percentage of patients with normal WT1 levels at onset, the role of this marker after treatment needs to be refined: preliminary data suggest that also in this subset we can observe WT1 variations related to the course of disease. Moreover WT1 could be useful for MRD evaluation also in the aphaeretic products and in PB. Larger series of patients are needed to confirm this aspect.

P020

SUSTAINED LONG TERM COMPLETE HAEMATOLOGICAL RESPONSE WITH LOW DOSE RITUXIMAB IN A PATIENT WITH REFRACTORY WARM-TYPE AUTOIMMUNE HAEMOLYTIC ANAEMIA

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Introduction. 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. The optimal schedule has not been established yet and standard dose might be an overtreatment. Low dose rituximab (LD-R) (100 mg weekly for 4 weeks) has been tested in patients with idiopathic thrombocytopenic purpura and in a case with warm-type autoimmune haemolytic anaemia (AIHA). In that latter case, a partial response was reported but no further details on degree and duration of response has been described. We report here the effects of 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. *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, 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 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 (21 months from the first course of therapy and 15 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 and a sustained CR can be obtained with a maintenance therapy. Considering the efficacy and the good safety profile, LD-R could be tested in a larger series of patients with warm AIHA.

P021

ACTIVATION OF PURINERGIC RECEPTORS BY ATP MODULATES $CD4^+$ CELLS AND TREG FUNCTION

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Introduction. Adenosine triphosphate (ATP) is emerging as an extracellular signalling molecule playing pivotal roles in several cellular processes through specific cell membrane purinergic P2 receptors (P2Rs). Under physiological conditions, ATP is present in the extracellular space at low concentrations (1-10 nM), whereas during inflammation and tumor cell growth high concentrations of ATP are present in the extracellular space when 5-10 mM ATP are quickly released from cytoplasm following plasma membrane damage or membrane stretching. *Methods.* $CD4^+$ were immunomagnetically purified and activated by anti-CD3 and anti-CD28 Abs. After activation Tregs were immunomagnetically isolated. The presence and function of purinergic receptor was investigated in $CD4^+$ and Tregs. $CD4^+$ and Tregs were cultured with ATP in order to evaluate their apoptosis, proliferation, cytokine secretion, migration, phenotype and adhesion. *Results.* Activated $CD4^+$ express all P2Rs subtypes, whereas Tregs do not express P2X6 and P2Y2. At a functional level, low concentrations of extracellular ATP do not modulate $CD4^+$ functions. An increase in ATP concentration (250 nM) stimulates $CD4^+$ during activation: activated $CD4^+$ enhance their proliferation and the secretion of several cytokines critical for T-cell function (IL2, IL1β, IFNγ, IL8). Tregs seem to be unaffected by 250 nM of ATP. In contrast, high concentrations of ATP (1 mM) "turns off" activated $CD4^+$ and "turns on" Tregs. 1 mM of ATP inhibits activation of $CD4^+$ stimulated by anti-CD3 and anti-CD28, enhancing apoptosis and diminishing proliferation, cell-adhesion and the release of pro-inflammatory cytokines. Conversely, 1 mM of ATP attracts Tregs and strongly stimulates their proliferation.

Tregs cultured with 1 mM ATP seems to be more efficient in inhibiting T cell proliferation. *Conclusions.* The present *in vitro* data might explain how, *in vivo*, ATP balances the involvement of activated $CD4^+$ or Tregs in case of inflammation or tumor cell growth. Intermediate concentrations of ATP alert $CD4^+$ to the presence of a possible damage not yet requiring Tregs involvement. High concentrations of ATP inhibit the activation and functions of stimulated $CD4^+$ and enhance Tregs functions. Thus, during inflammation high ATP prevents a further involvement of activated $CD4^+$-cells and promotes Tregs recruitment avoiding hyperinflammation; during tumor cell growth high ATP might be a tumor-escape mechanism, by killing activated $CD4^+$-cells and by attracting Tregs to surround the tumor.

P022

IL-4 IS REQUIRED FOR THE INHIBITORY EFFECT OF RAPAMYCIN ON ANTIGEN-PRESENTING CELLS *IN VITRO*

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Background. APCs are essential to induce immune responses. Monocytes are the most important antigen-presenting cells (APC) precursors both *in vivo* and *in vitro*. Rapamycin is a new immunosuppressive agent which is known to alter the function and survival of APC. In this study we tested the effects of rapamycin on the differentiation of monocytes to distinct APC subtypes *in vitro*. *Methods.* Purified monocytes from healthy donors were cultured *in vitro* with GM-CSF (50 ng/mL) with or without IL-4 (800 U/mL) or IFN- (1000 U/mL). Rapamycin was added at the start of culture. Cells were then harvested and checked for their (1) recovery, (2) apoptosis, (3) expression of costimulatory and DC-differentiation molecules, (4) allostimulation and (5) production of TNF-α and IL-12. *Results.* Rapamycin decreased the recovery of APC cultured in the presence of IL-4 in a dose dependent fashion, due to increased apoptosis, while monocytes cultured in GM-CSF with or without IFN- were not affected, even at doses as high as 100 ng/mL. Rapamycin decreased the expression of the costimulatory molecules CD86 and CD80 on all APC subtypes, although the effect was more marked in IL-4 containing cultures. Interestingly, rapamycin increased the expression of CD1a in cells cultured with IL-4, and even induced its novel expression in cells cultured with GM-CSF with or without IFN-α . Moreover, rapamycin blocked the secretion of IL-12 and TNF-α by monocytes cultured with IL-4 (by 90±7.3% for TNF-α and 93±8% for IL-12, n=10) and, albeit to a lesser extent IFN- and GM-CSF only. However, kinetic studies showed that the effect of rapamycin occurred earlier (starting on day 2) in the presence of IL-4. Blockade of IL-4-mediated STAT6 activation by pre incubation of cells with IFN-α blocked apoptosis of APC induced by rapamycin and by pharmacological inhibition of PI3K as well. *Conclusions.* These results suggest that IL-4 is required for maximum sensitivity to rapamycin. Inhibition of APC survival by rapamycin appears more strictly dependent on IL-4 than inhibition of APC function possibly due to the involvement of distinct signalling pathways.

P023

A NEW ENTITY OF CHRONIC/RELAPSING BENIGN LYMPHADENOPATHY ASSOCIATED WITH HHV-6B INFECTION OF FOLLICULAR DENDRITIC CELLS

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Introduction. Several studies have suggested an association between HHV-6 infection and malignant lymphoproliferative disorders, but the wider use of immunohistochemical (IHC) examinations has subsequently shown that the expression of viral antigens is restricted to reactive cells, arguing against a direct major role of HHV-6 in lymphoid malignant transformation. By contrast, active HHV-6 infection has been correlated to either infectious mononucleosis-like syndrome, or to Rosai-Dorfman disease in which viral antigens have been detected in both the histiocytes and the follicular dendritic cells (FDCs). We have further investigated whether HHV-6 infection may have a pathogenetic role in benign/reactive lymphadenopathies, which are not related to other well-recognized etiologies and tend to recur without evolving into lymphoma. *Methods.* We report on 9 adult patients (age 18-76 years) with either localized or generalized lymphadenopathies, in whom the most frequent either reactive or malignant well-recognized causes of lymph node enlargement have been repeatedly excluded. Of interest, some patients presented with recurrent lymphadenopathies (one to 3 recurrences) with a follow-up up to 13 years. Extensive histological, IHC, serological and molecular examinations were performed in order to study a potential involvement of HHV-6 active infection or reactivation. *Results.* A common lymph node histological pattern at presentation revealed follicular hyperplasia and paracortical expansion. At least some cases showed features consistent with progressive transformation of germinal centers. IHC analyses revealed HHV-6B positive staining of FDCs together with scattered positivity of interfollicular cells. The IHC reactions for both HHV-6A and HHV-6B, performed on further 70 lymph node tissues from patients with benign lymphadenopathies induced by other known etiologies, resulted negative. Serology was positive for both IgM and IgG suggesting viral reactivation/reinfection. However, the molecular analyses failed to detect HHV-6 viremias in cell-free-serum samples of all the 9 patients with positive HHV-6B IHC staining, while positivity for HHV-6B DNA was disclosed by PCR analyses in 7 out of the 7 lymph node tissues studied to date. Constitutional symptoms were absent in all but a 67-year old patient, who developed AITL eleven months later. Of note, this latter patient was the only one, presenting with serological features consistent with HHV-6 primary infection. *Conclusions.* We show for the first time that HHV-6B should be considered among the possible causes of benign lymphadenopathies, with chronic/relapsing behavior. IHC is the method of choice for investigating the presence of HHV-6 infection in such cases. It is conceivable that HHV-6 might indirectly modulate and trigger the proliferation of lymphocytes, by locally affecting the antigen presentation mediated by FDCs and the lymph node microenvironment.

P024

CHARACTERIZATION OF ASPERGILLUS-SPECIFIC T CELL RESPONSES TO DIFFERENT ASPERGILLUS ANTIGENS AT DISTINCT PHASES OF THE INFECTION IN HEMATOLOGIC PATIENTS WITH INVASIVE ASPERGILLOSIS

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Introduction. Invasive Aspergillosis (IA) shows a dismal outcome in a high percentage of hematologic patients. The reasons of this elevated mortality rely also on several drawbacks affecting diagnostic methods and reducing the opportunities for an earlier treatment. We have investigated whether the detection and the characterization of Aspergillus-specific T-cells may be diagnostic of IA and contribute to identify times of therapeutic intervention. *Methods.* 145 patients have been studied. They were classified according to the new Mycosis Study Group criteria as follows: 16 proven, 30 probable, 17 possible IA and 82 controls. This latter were either patients with a proven infection other than IA (75%) or patients with no clinical and microbiological features of IA (25%). Enzyme linked immunospot (ELISPOT) and cytokine secretion (CS) assays have been harnessed to detect Aspergillus-specific T cells. Heated conidia, crude hyphal extract and 4 Aspergillus recombinant proteins (CRF1, PEP1, GEL1, a1-3 glucan) have been used as antigens. *Results.* ELISPOT resulted positive in 14 out of 16 for Aspergillus-specific inteleukin-10 producing (Asp-IL10) T-cells; in 2 out of 16, also for Aspergillus-specific interferon-gamma producing (Asp-IFNγ) T-cells, and

in 1 out of 16 only for Asp-IFNγ T-cells. The patient positivity rate was 93.75% in proven, 45.1% in probable, 35.3% in possible IA and 2.4% in the controls. The sample positivity rate of the assay resulted 83.78% in proven, 35.7% in probable, 50% in possible IA and 5.9% in the controls. In 3 proven IA patients ELISPOT detected almost exclusively Asp-IL10 T-cells versus all the four recombinant proteins at the onset of the infection, while, as IA improved, a progressive increase of Asp-IFNγ T-cells was detected versus Gel1 and Pep1 firstly, and versus all the four proteins, afterwards. In 3 more patients, by expanding the ELISPOT findings, CSA showed that a predominat T helper type 2 response characterizes the onset of IA, as: 1. no Asp-IFNγ T-cells could be detected, 2. almost all Asp-IL10 T-cells were $CD4^+$ cells of central memory (CM) phenotype, given the absence of specific T-cells of regulatory phenotype, 3. Aspergillus-specific IL4 producing T-cells either of CM or effector memory (EM) phenotype were present and 4. also Aspergillus-specific IL17 producing $CD8^+$ T-cells, either CM or EM, could be demonstrated. *Conclusions.* Our data show the potential of the ELISPOT in the diagnosis of IA. By a biological point of view, the demonstration of Asp-IL10 T-cells versus all the recombinant antigens, when almost exclusively Asp-IFNγ T-cells are reported to occur to the same proteins in healthy subject, and the detection of a predominant T helper type 2 response at the onset of the disease, seem to identify a stated time point for therapeutic interventions. Larger perspective studies are needed to confirm these results, to establish the variables possibly affecting the assay and the optimal sampling strategy.

Chronic Lymphocytic Leukemia I

P025

DUAL ROLE OF HS1 IN B-CHRONIC LYMPHOCYTIC LEUKEMIA: LYN SIGNALLING MODULATOR AND CYTOSKELETON-CONNECTED MOLECULE

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Introduction. The kinase Lyn is overexpressed, active and abnormally distributed in leukemic B cells of Chronic Lymphocytic Leukemia (CLL) patients. It is also part of an anomalous cytosolic complex involving hematopoietic lineage cell-specific protein 1 (HS1) and other molecules. These aberrant properties of Lyn could partially explain the defective apoptosis of CLL cells, directly or through its substrates, i.e. HS1, that has been associated to apoptosis in different cell types. The aim of the present study was to verify the hypothesis of whether HS1 is involved in Lyn-mediated leukemic cell survival. *Methods.* We investigated the levels and the role of HS1 in 61 untreated CLL patients and 26 healthy controls. Expression and subcellular localization of HS1 were assessed by western blotting analysis, confocal microscopy and differential ultracentrifugation; in particular, confocal microscopy was performed to verify HS1 co-localization with cytoskeletal molecules. Coupled to western blotting and Real time-PCR analysis, *in vivo* and *in vitro* experiments were performed to define fludarabine (FLU) and cyclophosphammide (Cy) effect on HS1. *Results.* We found that HS1 is overexpressed in leukemic cells as compared to normal B lymphocytes (1.38±0.54 *vs.* 0.86±0.22; $P<0.01$). The levels of HS1 significantly decreased and were subjected to cleavage in leukemic cells obtained from responsive patients to FLU-Cy therapy and cells cultured for 24h with the same drugs. Furthermore, abnormal localization of HS1 was also observed in malignant versus normal B cells, being particularly present in the nucleus of leukemic but not normal B lymphocytes. Since it is known that HS1 interacts with cytoskeleton, additional experiments have been performed to investigate if HS1 could interact with cytoskeletal components. We found that HS1 co-localizes with actin both in normal and leukemic B cells; in addition, HS1 co-localizes with tubulin only in CLL but not normal B cells and, in particular, with the centrosome which is recognize to represent the microtubule-organizing center. *Conclusions.* Overexpression of HS1 in leukemic B cells and its down-regulation by *in vivo* and *in vitro* use of FLU and Cy allowed us to hypothesize the involvement of HS1 in the maintenance of neoplastic B cell survival. An important observation coming from this study is the evidence that HS1 co-localizes with cell centrosome in leukemic but not in normal B cells. Considering that it has been demonstrated that centrosome deregulations lead to a poor prognosis for CLL patients, HS1-centrosome interaction is likely to contribute to the maintenance of leukemic cells proliferative activity. In conclusion, HS1 might represent a new target for therapy in order to block the proliferation of leukemic cells and make them responsive to drug-induced apoptotic stimuli. The suggestion of a putative therapeutic use of HS1 once again focuses the attention on BCR-Lyn-HS1-cellular activation and apoptosis resistance pathways.

P026

INCREASED BONE MARROW LYMPHOPLASMACYTOID CELLS AND PRODUCTION OF SDF1 AS POSSIBLE PREDICTIVE MARKERS OF THE RESPONSE TO IMATINIB MESYLATE THERAPY IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Introduction. In the last years, imatinib mesylate (formerly known as STI571) has become the first line treatment of Chronic Myelogenous Leukemia (CML) and of a rare form of gastroenteric stromal cancer. In the latter case, the response to the drug is also due to immunocompe-

tent cells, able to produce cytokines with antineoplastic activity. Methods. In this study, 40 CML patients, treated with imatinib mesylate (400 mg/die) underwent bone marrow (BM) aspirates at diagnosis, after 3 months and every 6 months, for morphologic, phenotypic, cytogenetic and biomolecular analysis. Plasma from BM and peripheral blood (PB) was also recovered for evaluation of cytokines able to induce B lymphocyte differentiation, such as interleukin (IL4, IL6, IL3, IL10, IL21, SDF1 and BAFF (by ELISPOT and Q-RT-PCR): moreover, the expression of MCP1, BMP4, BMP7, IP10 and IL8 were also measured. Results. We report that in 32 out of 40 CML patients a significant increase in the percentage of BM lymphoplasmacytoid cells was observed upon treatment with imatinib mesylate, with more than 10% (range 8-12%) of $CD20^+CD126^+$ cells. Among this population, two third of cells coexpressed IgM and one third was IgD^+, while a smaller fraction of $IgM^+CD126^+CD20^-$ (3-4%) or $IgD^+CD126^+CD20^-$ (2-3%) cells was also found. In all these patients SDF-1 increased in the BM plasma after imatinib (from 10-80 pg/mL to 150-450 pg/mL) and its receptor CXCR4 was up-regulated on $CD20^+CD126^+$ cells. In some cases also IP10 and its receptor CXCR3 were up-regulated. SDF1 increase partially coexisted with that of BAFF and of the bone morphogenetic proteins (BMP)2 and BMP7. No significant increase in transcription and secretion of IL3, IL4, IL6, IL10, IL21, IL8 or MCP1 was observed. The remaining 8 patients had less than 5% of $CD20^+CD126^+$ lymphocytes (range 2-4%), 2/3 coexpressing IgM and 1/3 coexpressing IgD. All patients with increased number of $CD126^+$ B lymphocytes underwent hematologic remission, most of them with complete molecular and cytogenetic remission. On the other hand, among the patients with low or undetectable $CD20^+CD126^+$ cells, only 4 underwent hemathological remission and none of them displayed stable cytogenetyc and molecular remission. Exposure of BM cells, obtained from CML patients at diagnosis, to STI571 *in vitro* led to transcription and secretion of SDF1, and to a lesser extent, of BAFF. Conclusions. These data suggest that BM lymphoplasmacytoid B cells depend on, or contribute to, the pharmacological response. The increased production of SDF1, partially associated with the production of BMP4 and BMP7, that follows imatinib administration might be responsible for the observed increase in BM lymphoplasmacytoid cells, possibly due to the double proliferative/chemotactic effect of the cytokine on B cells, leading to both redistribution and *in situ* differentiation of $CD20^+CD126^+$ lymphocytes. Finally, this phenomenon might help in monitoring the outcome of disease and the response to treatment.

P027

SERUM- AND STROMA-FREE IN VITRO CULTURE OF PRIMARY B-CLL CELLS

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Introduction. In chronic lymphocytic leukemia (B-CLL), B lymphocytes gradually accumulate and survive thanks to the lack of apoptosis and the interactions with tissue microenvironment. However, B-CLL cells rapidly die *in vitro* when cultured in standard medium without bone marrow stroma cells, even by using serum or cytokines. We established a reproducible serum- and stroma-free B-CLL culture system, by employing a non conventional medium, i.e. Neurobasal medium that is used for neural stem cell culture. *Methods.* mononuclear cells from 15 untreated B-CLL patients, upon informed consent, were isolated through density gradient centrifugation. Cells were seeded in 75 cm^2 flasks at 1×10^6/mL concentration and incubated at 37°C with 5% CO_2 for 4 weeks. Eight samples (group 1) were seeded in two conditions: a. RPMi medium added with 10% FBS (RPMi10%); b. Neurobasal Medium (NS) supplemented with growth factors (GF) of epidermal (EGF) and fibroblastic (FGF) origin (NS+GF). Seven samples (group 2) were maintained in three conditions: a. RPMi without FBS (RPMi0%), b. NS medium alone, c. NS+GF. Cell vitality of each sample was planned to be evaluated weekly by Annexin V-Propidium Iodide protocol. *Results.* The mean percentage of alive cells in culture at day +1 and day +2 was, respectively: 31.8±28,25% and 24.22±20.66% in RPMi0%; 57.57±18.37% and 68.92±12.82% in NS; 67.95±5.93% and 69.16±5.93% in NS+GF. The differences on day +2 were statistically significant between RPMi0% and the other media (P=0.002, P=0.001, respectively; Anova Test), but not between NS and NS+GF. Considering the long-term culture, the mean percentage of alive cells at weeks +1, +2, +3 and +4 was, respectively: 2.54±2.23%, 0.91±1.36%, 0.15±0.27% and 0.01±0.01% in RPMi 0%; 29.28±12.11%, 11.03±8.63%; 3.98±6.23%, 1.59±2.53% in RPMi 10%; 45.75±11.28%, 28.23±14.73%, 26.61±17.72%, 13.96±13.08% in NS; 49.83±13.84%, 25.94±15.82%; 23.12±15.98%; 15.66±14.33% in NS+GF. At weeks +1, +2, and +3, culture with NS medium allowed cells to survive longer than that with RPMi0% (P<0.0001; P=0.001; P=0.003, respectively; Anova test). Similar results were found by comparing RPMi0% with NS+GF (P<0.0001; P=0.001; P=0.004; P=0.023; Anova test). At +3 weeks of culture, cell vitality was significantly higher in NS and in NS+GF than in RPMi10% (P=0.011; P=0.014, respectively; Anova test). No significant differences were found either between NS and NS+GF at any culture time or between RPMi0% and RPMi10% from +2 weeks on. *Conclusions.* Neurobasal Medium (with or w/o supplements) is capable of sustaining long survival of B-CLL cells in culture without using serum or stroma cells. With this method, it is possible to set up experiments to provide biological informations on B-CLL cells with culture lasting up to 3-weeks.

P028

SIGNIFICANT GLOBAL HYPOMETHYLATION OF DNA REPETITIVE SEQUENCES IN CELL CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH 17P DELETION

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Introduction. 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 chronic lymphocytic leukemia (CLL). Gene-specific hypermethylation and global hypomethylation have been previously reported in CLL. However, the relationship between aberrant global DNA methylation and clinical and biological risk factors remained unclear. Herein, we investigated the methylation levels of repetitive DNA elements in CLL, their correlation with the major cytogenetic and molecular features, and clinical relevance in predicting therapy-free survival (TFS). *Methods.* A quantitative bisulfite-PCR pyrosequencing method was used to evaluate methylation of Alu, long interspersed nuclear elements-1 (LINE-1) and satellite- (SAT-α) sequences in 77 untreated early-stage (Binet A) CLL patients. Clinical data were available for 65 patients. The median follow-up was 2 years (range 1-10 years), and 39 patients had received treatment by the end of the study. Peripheral B-cells from 7 healthy donors were used as controls. *Results.* In the present study we evaluated the methylation patterns of LINE-1, Alu and SAT- in 77 B-CLL representative of different molecular subtypes. Specifically, 13q14.3 deletion was present as a sole abnormality in 21/33 patients while in the remaining cases it was combined with 17p13.1 (n=4) or 11q22.3 deletions (n=7) or both (n=1). The 11q22.3 and 17p13.1 deletions were detected as sole abnormality in 6 and 7 patients, respectively. Trisomy 12 occurred in 17 patients, as a sole abnormality in all cases. ZAP-70 and CD38 expression resulted positive in 29 and 35 cases, respectively, whereas IgVH genes were found to be unmutated in 48 patients. We found that global DNA methylation levels (median %5 mC) were lower in CLLs compared with controls (21.4 *vs.* 25.9; 66.8 *vs.* 85.7; 84.0, *vs.* 88.2 for Alu, LINE-1 and SAT-α, respectively) (P<0.001). Among CLL patients, a significant association was observed with 17p13.1 deletion (16.8 *vs.* 22.4; 51.2 *vs.* 68.5; 52.6 *vs.* 85.0, for Alu, LINE-1 and SAT-) but not with other major genetic lesions, IgVH mutation status, CD38 or ZAP-70 expression. Importantly, follow-up analyses showed that lower SAT- methylation levels appeared to be an independent prognostic marker significantly associated with shorter TFS. *Conclusions.* Our study extended previous limited evidences in methylation of repetitive sequences CLL. In addition, it provided evidence that the hypomethylation of specific repetitive sequences may have clinical relevance CLL and may be used as a novel prognostic indicator of unfavorable disease progression.

P029

INCIDENCE OF CYTOGENETIC ABNORMALITIES IN NEWLY DIAGNOSED BINET STAGE A CLL AND RELATIONSHIP WITH PROGNOSTIC BIOMARKERS: UPDATED RESULTS ON 344 PATIENTS INCLUDED IN THE PROSPECTIVE O-CLL1 GISL STUDY

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Introduction. CD38 and ZAP-70 expression, IgHV mutational status and genetic abnormalities have been integrated in clinical prognostic evaluation of CLL. *Methods.* We investigated, by FISH, the incidence of the major cytogenetic alterations (+12 and 13q14, 17p13, 11q23 deletions) in 344 out of 384 Binet A CLLs enrolled in the prospective multicenter O-CLL1 GISL trial. *Results.* At least one abnormality was found in 225/344 (65.4%) cases. The most frequent abnormality was del(13q14), detected in 173 CLLs (50.3%) followed by +12 (44/344, 12. 8%) (one case harboring 17p13 deletion), del(17p13) (9/344, 2.6%) and del(11q23) (18/344, 5.2%). 13q14 deletion was found as a sole abnormality in 155 (45%) patients; in the remaining cases, it was combined with +12 (3 pts) and 17p13 (4 pts) or 11q23 deletions (11 pts). The 13q deletion was found as a monoallelic deletion in 139/173 (80.3%); in the remaining 34 cases the presence of a biallelic deletion was found in >20% of interphase nuclei. No acquisition of new cytogenetic aberrations was evidenced among the 13 CLLs developing progressive disease (range, 6 to 32 months; median, 20 months). In only one case, the proportion of nuclei with 17p13 and 13q14 deletions increased from the time of diagnosis (from 33% to 92%). Biomarkers data were available in all of the patients. CD38 percentages (mean value ± sem) were 7.9±1.3, 15.1±1.9, 51.7±5.5, 22.0±7.8,40.8±13.2, 39.8±7.3 for del(13q14), normal karyotype, +12, del(11q23), del(17p13) and multiple alterations, respectively (P<0.0001). The percentages of IgVH mutations significantly correlated with cytogenetic alterations; namely, 5.7±0.2 for cases with del(13q14), 4.7±0.4 for normal karyotype, 2.3±0.5 for +12, 0.05±0.05 for del(11q23), 2.0±1.1 for del(17p13) and 1.0±0.4 for multiple alterations (P<0.0001). Similarly, a significant correlation was found for ZAP-70 expression: namely 32.9±1.6 for cases with del(13q14), 38.5±2.1 for normal karyotype, 46.4±3.6 for +12, 67.0±8.3 for del(11q22), 41.0±12.8 for del(17p13) and 50.7±5.4 multiple alterations (P<0.0001). Finally, cytogenetic abnormalities were clustered in 3 risk groups [i.e. low del(13q14) and normal; intermediate (+12); and high risk 17p13 and 11q23 deletions] and correlated with a scoring system in which patients were stratified in 4 different groups according to the absence or presence of 1 2 or 3 biomarkers (Morabito *et al*., BJH, 2009). Notably, 166/175 cases scoring 0, gathered in the low FISH group, whereas 21/26 high FISH risk cases clustered in scoring 2-3 (P<0.0001). *Conclusions.* Our data indicate that cytogenetic lesions predicting unfavorable prognosis show a relatively low incidence in newly diagnosed Binet stage A CLLs and are significantly associated with negative prognostic biomarkers predictive of disease progression. Furthermore, preliminary results in a limited number of cases indicate that the acquisition of new abnormalities seem to be an infrequent event during disease progression.

P030

MESENCHYMAL STEM CELLS PROMOTE SURVIVAL AND BONE MARROW LOCALIZATION OF LEUKEMIC CLONE OF B-CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The localization of neoplastic B cells in the bone marrow (BM) and/or lymphnodes (LN) is a crucial step for CLL-B cells survival, since bidirectional interactions between malignant lymphocytes and bystander cells may led to establishment of an abnormal microenvironment which confers a growth advantage and extended survival to neoplastic clone. Among several accessory cells, we focused our attention on mesenchymal stem cells (MSCs), in order to clarify whether they play an active role on survival and accumulation of leukemic B cells. *Methods.* MSCs isolated from the BM of 47 B-CLL patients were expanded ex vivo and characterized through flow cytometry analysis and differentiation coltures (adipocytes and osteocytes). Fresh isolated CLL peripheral blood mononuclear cells were co-coltured with CLL-MSC or stromal cells and apoptosis were measured by Annexin V test and western blotting analysis. To assess the production and release by MSC of soluble molecules involved on marrow compartimentalization of neoplastic B cells, chemotactic tests were performed using conditioned media derived from CLL-MSC primary coltures. *Results.* While MSCs from B-CLL patients exhibited normal phenotype and differentiation capacities, when co-coltured with neoplastic B cells they exherted an anti-apoptotic effect reducing lymphocyte apoptosis. After 7 days of colture in presence of CLL-MSC, we observed a relevant extended survival of leukemic cells (60%±17.3 in presence of MSC *vs.* 14%±11.7 with medium alone), but not of normal B lymphocytes (30.20%±15 with MSC *vs.* 6.20%±5 with medium alone). Using a transwell system, we observed that the survival of CLL-B cells in presence of MSCs was reduced, suggesting that the anti-apoptotic effect should be mediated by direct physical contact between MSCs and leukemic cells. Finally, chemotaxis tests showed the ability of MSCs to produce molecules promoting migration and localization of neoplastic B cells in bone marrow. *Conclusions.* MSCs derived from patients with B-CLL, despite an apparent normal phenotype and normal differentiation ability, provide survival signals to neoplastic cells, extending their lifespan and producing chemotattic factors favouring their accumulation in bone marrow. These interactions are specific for malignant cells, not for normal B cells, suggesting that only leukemic cells respond to signals coming from CLL microenvironment. Interrupting the cross-talk between the malignant clone and accessory cells within microenvironment should be an attractive novel strategy for treating CLL.

P031

FRONT LINE THERAPY WITH LOW DOSE ORAL FLUDARABINE AND CYCLOPHOSPHAMIDE IN ELDERLY PATIENTS AFFECTED BY CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Elderly patients represent the largest group of CLL patients but the attendance of comorbidities influences the treatment strategy and limits the enrolment in clinical trials. Front-line chemotherapy with Fludarabine (FLU) did not showed statistical differences in terms of progression-free and overall survival in elderly CLL respect to Chlorambu-

cil. However low dose oral Flu- Cyclophosphamide (CTX) has been reported as a good cycle in terms of event free survival and toxicity. We evaluated the efficacy and toxicity of low dose of the oral formulation of FLU and CTX in untreated CLL patients older than 65 years. *Methods.* We enrolled 22 untreated patients with a median age of 70,5 years (range 65-75). Nineteen patients were in progressive stage B/II, 1 in stage C/III, and 2 in stage C/IV. Patients were given oral FLU 30 mg/m^2/die and oral CTX 250 mg/m^2/die in an outpatient regimen for three consecutive days every 4 weeks for six cycles. Prophylaxis against herpes zoster and pneumocystis carinii was provided by administration of acyclovir 800 mg tid, and trimetoprim-sulfametoxasol 960 mg bid for 2 consecutive days every week, during the entire treatment period and 2 months after. Eleven patients had unmutated and 8 had mutated immunoglobulin variable region heavy chain (IgVH) genes. Five patients had the 'high risk' cytogenetic abnormality del(11q22.3) or del(17p13.1). *Results.* Twenty one patients were evaluable for response to treatment. One patient died after the second cycle of therapy due to pneumonia in the setting of grade IV neutropenia. Eight patients obtained a complete response and 8 a partial response with an overall response rate of 73%. Four patients showed stable disease; two of them had unmutated IgVH and one showed del(17p13.1); one patient, with both del(17p13.1) and del(11q22.3), experienced progressive disease after 4 cycles. Median progression free survival was 23 month, median time to re-treatment was 31 months. Median overall survival was not reached after 67 months of follow up, in the same period 9 patients died for progressive disease. Eight patients (36%) developed grade IV neutropenia during treatment and received G-CSF; excepted for patient who died for pneumonia during treatment no patients experienced fever. Two patients developed grade III and IV anemia that required red blood cell transfusions. No patients experienced grade III/IV piastrinopenia. Extra-hematological toxicity consisting of nausea (grade I-II) was detected in 9 patients; four of these patients also experienced grade I-II vomiting. No evaluable patients required hospitalization. *Conclusions.* Oral FLU plus CTX as front-line therapy for elderly patients affected by CLL, was a good cycle able to achieve established clinical outcomes with mild toxicity.

P032

CD38 IS PHYSICALLY ASSOCIATED WITH CD49D AND POTENTIATES CD49D-MEDIATED ADHESION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Introduction. CD49d, a recently identified negative prognosticator in chronic lymphocytic leukemia (CLL), is an adhesion molecule mediating microenvironmental interactions of CLL cells in bone marrow and lymph nodes. Through CD49d-dependent adhesion toVCAM-1 and fibronectin (FN), CLL cells receive survival support that renders them less susceptible to conventional therapies. The closely associated expression of CD49d with CD38, another negative prognosticator in CLL, and the propensity of CD38 to associate and functionally cooperate with other molecules, prompted us to investigate whether: i) CD38/CD49d physical interactions may also occur on CLL cell membranes; ii) CD38 may potentiate the CD49d-mediated adhesion in CLL. *Methods.* Confocal microscopy and biochemical approaches were used to study the membrane organization of CD49d and CD38 in primary CLL cells. The CLL-derived CD49d$^+$CD38$^-$ cell line Mec-1, and a subclone in which CD38 expression was induced by viral transduction (Mec-1/CD38) were utilized for adhesion experiments. *Results.* Co-capping experiments in CD49d$^+$/CD38$^+$ CLL cells demonstrated a membrane relationship between CD38 and CD49d. Anti-CD49d monoclonal antibodies (mAbs) induced capping in approximately 75% of CLL cells, with a 80% redistribution of CD38 in the context of the capping area. The CD38/CD49d lateral association was confirmed at the biochemical level by immunoprecipitation experiments with anti-CD49d mAbs and subsequent painting of immunoprecipitates using anti-CD38 mAbs. CD38/CD49d association was also maintained after engagement of CD49d with its natural ligands, as witnessed by co-localization of CD49d and CD38 in uropods formed by CLL cells adhered and spread onto VCAM-1 and FN. To investigate whether the CD38/CD49d association had also a functional meaning, adhesion assays on VCAM-1-coated plates were performed with the Mec-1 cell model. Mec-1/CD38 showed a marked increase in VCAM-1 adhesion compared to Mec-1 (mean values of adhered cells relative to control=5.4 *vs.* 2.3 and 5.4 *vs.* 1.9 after 15 and 30 minutes respectively). Moreover, phase-contrast and immunofluorescence microscopy highlighted clear differences in the morphology of adhered cells, with Mec-1/CD38 cells characterized by a more complex pattern of uropods than Mec-1, and a clear colocalization of CD38 and CD49d in adhesion sites. Notably, pre-treatment of Mec-1/CD38 cells with the non-agonistic anti-CD38 mAb SUN-4B7 resulted in adhesion values and cell morphology comparable to those observed in CD49d$^+$CD38$^-$ Mec-1 cells. *Conclusions.* CD49d and CD38 are physically associated on CLL cell membranes, and CD38 directly contributes in enhancing CD49d-dependent adhesion on VCAM-1, likely through the involvement of specific molecular epitopes. This suggests a cooperative activity of both molecules in the adhesion-mediated chemoresistance of CLL cells. CD49d and CD38 targeting may be ways of overcoming stroma-mediated chemoresistance in CLL.

P033

IN CHRONIC LYMPHOCYTIC LEUKEMIA THE AMOUNT OF PROLIFERATION CENTERS CORRELATES WITH UNFAVORABLE CYTOGENETIC ABNORMALITIES

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Introduction. In CLL molecular cytogenetic abnormalities may identify specific disease entities and may be associated with distinct prognosis. Currently, peripheral blood is the preferred source to asses the biological risk. However, FISH on paraffin-embedded fixed tissues (PEFT) may also be used to study cytogenetic abnormalities in CLL lymph-node where the "proliferation center" (PC) is the histopatologic hallmark. PCs probably represent the sites where T-dependent immune responses to unknown antigens promote clonal B cell selection and expansion. *Aims.* i) to analyze the sensitivity and reproducibility of FISH on PEFT, ii) to correlate specific chromosome lesions to PC extension; iii) to estimate the frequency of chromosome lesions on lymph node samples. *Methods.* 183 CLL cases (including 21 Richter syndrome, RS) arranged in 5 tissue macro-arrays (TMAs) were analyzed by FISH for deletions involving 11q23/ATM, 13q14 and 17p13/p53; trisomy 12; and translocations involving the IgH gene on band 14q32. Cytogenetic features were correlated to 2 histologic patterns: 1) PCs-rich group including CLL cases with confluent and large PCs and RS cases and 2) typical group with scattered, well-distinct PCs. *Results.* Assessable data with the complete 5 probe-panel were obtained in 101/183 cases (55.1%); in 24 cases no data were collected for all the probes mainly because of insufficient numbers of cells on TMA samples. Chromosomal aberrations were detected in 77/101 cases (76.2%). The most frequent abnormality was 13q- (36.7% of the cases), followed by 14q32 translocations (30.8%), 11q- (24.7%), +12 (19.5%) and 17p- (15.6%). Ten cases showed 14q32/IgH gene extracopies (3-5 signals in 10-30% of the nuclei). 17p-, +12 and 14q32/IgH translocations were more frequently encountered in the PCs-rich group (P<.001, .030 and .043 respectively). The PCs-rich group was significantly associated to "high-risk" cytogenetic abnormalities (11q- and/or 17p; P=.001) and to a higher number of abnormalities (≥2; P=.001). The difference persists even if RS cases were excluded. *Conclusions.* FISH on TMAs is a reproducible and a feasible tool for the evaluation of cytogenetic abnormalities in CLL allowing for a decreased both time consumption and experimental variability. The incidence of cytogenetic abnormalities in our series resembles that of historical series with exception of 14q translocation and 17p- possibly due to a higher number of patients with resistant or progressive disease. The higher occurrence of unfavourable cytogenetic features in PCs-rich lymph nodes suggest that

PCs may be sites where, due to an antigenic stimuli, B cells are induced to proliferate thus conferring genetic instability. Further studies will clarify if cytogenetic abnormalities occurring in lymph-node and bone marrow PCs precede their appearance in the peripheral blood thus supporting the idea that at tissue level PC is crucial for triggering clonal proliferation nourishing the accumulation compartment.

P034

PERSISTENT IMMUNOPHENOTYPIC COMPLETE REMISSION PREDICTS FOR LONGER RESPONSE DURATION AND OVERALL SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Today the treatment goal of chronic lymphocytic leukemia (CLL) is the attainment of maximal disease control combining purine analogs with monoclonal antibodies. This approach have produced more complete immunophenotypic remissions and longer response duration (RD), often remaining a minimal residual disease (MRD) detectable only by flow cytometry. *Methods.* We treated in first line 130 CLL symptomatic patients (pts), median age 63 years, with six monthly courses of intravenous (25 mg^2) or oral fludarabine (30-40 mg^2) at conventional doses and then, after a median time of 30 days, with four weekly doses (375 mg^2) of rituximab (rtx). Fourteen pts had a low Rai stage, 113 an intermediate stage and 3 a high stage. We defined as high risk pts having at least two of these markers: unmutated IgVH, CD38>30%, ZAP-70>20%, intermediate/unfavorable cytogenetics (trisomy 12 or del11q or del17p). Fifty-four pts (41.5%) belonged to the high risk subset. For MRD flow cytometric study, the threshold was set at >1% CD19$^+$CD5$^+$CD79b$^-$ bone marrow (BM) CLL cells.

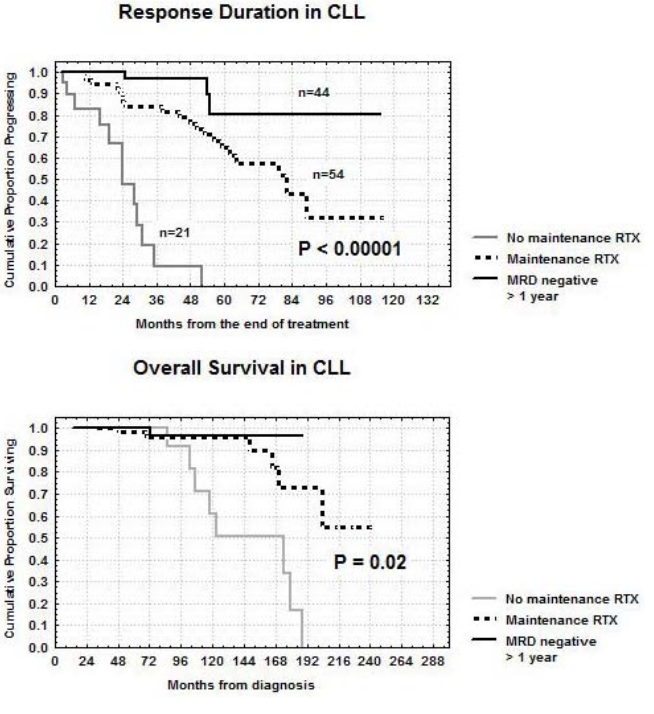

Figure.

Results. Based on NCI criteria, 98/130 (75%) pts achieved a CR, 28/130 (22%) a partial remission (PR) and 4/130 (3%) no response or progression. Immunophenotypic CR (CD19$^+$CD5$^+$CD79b$^-$ BM cells <1%) was achieved in 72/129 (55.8%) pts. Ten pts underwent grade 3 (WHO) infective lung toxicity, 1 patient acute fatal B hepatitis and 2 pts progressed towards Richter's syndrome. Hematologic toxicity included mainly neutropenia (grade 3 and/or 4 in 60 pts) and thrombocytopenia (grade 3 and/or 4 in 8 pts). Fifty-four pts either in CR with CLL BM cells >1% (MRD$^+$, n=16 pts) or in CR MRD$^-$, but with B-CLL peripheral cells going up >1000/microl within 2 years after induction (n=22 pts) or in PR (n=16 pts), underwent consolidation and maintenance therapy with four monthly cycles of rtx at 375 mg^2 followed by twelve monthly low doses of rtx (150 mg^2). The median follow-up duration was 54 months. All treated pts experienced a long progression-free survival from the end of induction treatment (40% at 9 years). Overall survival (OS) was 37% at 10 years from the start of treatment. Noteworthy, both persistently MRD negative (>1 year) pts (n=44) and pts undergoing consolidation and maintenance therapy (n=54) showed a longer RD *vs.* MRD$^+$ not consolidated pts (n=21) [97% *vs.* 76% *vs.* 10% at 4 years; P<0.00001, Figure]. Equally, OS was shorter in MRD$^+$ not consolidated pts in comparison with the other subsets (17% *vs.* 73% *vs.* 97% at 15 years; P=0.02, Figure). Noteworthy, within the high risk subset (n=54), both pts in persistent immunophenotypic CR (n=13) and consolidated pts (n=18) showed a longer RD (88% *vs.* 60% *vs.* 0% at 2.6 years, P=0.007) *vs.* MRD$^+$ not consolidated pts (n=14). *Conclusions.* In our experience, mainly a persistent MRD negativity but also the rituximab maintenance therapy may improve RD and OS in B-CLL, also within the high risk subset.

P035

PARP1 AND CHE1 IMPAIRMENT IDENTIFY A SUBSET OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH DEFECTIVE IR-INDUCED APOPTOSIS

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Introduction. PARP1 and Che1 are two proteins involved in response to DNA damage, as well as in apoptosis. Given the role of apoptosis in chronic lymphocytic leukemia (CLL), we first evaluated the gene expression profile of some DNA repair and apoptosis genes in a cohort of untreated cases, focusing our attention on these 2 transcripts. Secondly, protein changes of PARP1, Che1, as well as p53, were evaluated by *in vitro* tests on primary CLL cells upon exposure to ionizing radiation (IR). *Methods.* We carried out microarray analysis on 93 untreated CLL cases using the HGU133 Plus 2.0 Affymetrix arrays. In addition, freshly isolated primary cells from 21 CLL patients were analyzed at different time points (0, 8 and 24 hours) of exposure to 5 Gy IR: PARP1, CHE1 and TP53 mRNA expression levels were evaluated by Q-PCR and their protein levels were evaluated by western blot. PARP activity assay and apoptosis analysis by the Annexin-V technique were also performed. *Results.* Gene expression profiling showed that among the genes involved in DNA damage and apoptosis response, PARP1, CHE1 and TP53 had a high degree of correlation among each other in untreated CLL cells. Moreover, when CLL patients were subdivided into IGHV mutated (n=57), IGHV unmutated (n=25) and TP53-mutated (n=11) samples, PARP1 and TP53 displayed a significantly lower expression in the TP53-mutated group compared to IGHV mutated cases (P=0.019 and 0.012, respectively); at variance, no significant differences were observed for the expression of these genes between TP53-mutated and IGHV unmutated patients. Next, based on the *in vitro* IR-response, CLL samples were subdivided into two subgroups: responders and non-responders. Responders were characterized by: 1) complete cleavage of PARP1; 2) decrease of PARP activity; 3) reduction of Che1 protein levels; 4) p53 induction; 5) significant increase in IR-induced apoptosis. Non-responders showed: 1) incomplete PARP1 cleavage; 2) maintenance of PARP activity; 3) lack of Che1 reduction; 4) impaired p53 induction; 5) resistance to IR-induced apoptosis. When the response to IR was coupled to CLL biologic features, all but one IGHV mutated cases (n=7) were considered responders; contrariwise, non-responders included 6/9 of IGHV unmutated patients: among these, 3 cases carried TP53 mutations, 1 case harbored an ATM alteration and in the remaining cases further investigations are currently ongoing. *Conclusions.* In untreated CLL cells, microarray analysis showed significantly lower expression levels of PARP1 and TP53 in TP53-mutated cases and, to a lesser extent, in IGHV unmutated samples. In *in vitro* experiments, PARP1 and Che1 deregulation, beyond p53, allows to identify a subset of CLL samples

with apoptotic resistance to IR, the majority carrying known adverse biologic features, i.e. IGHV unmutated status or TP53 or ATM mutations. Overall, these findings pinpoint PARP1 and Che1 as potential therapeutic targets in CLL.

P036

INCREASED PLASMA LEVELS OF ANGIOPOIETIN-2 IDENTIFY CLL PATIENTS WITH ADVERSE CLINICAL OUTCOME INSIDE MUTATED IGHV AND LOW RISK KARYOTYPE SUBSETS

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Introduction. Angiopoietin-2 (Ang2) is secreted by chronic lymphocytic leukemia (CLL) B cells and induces increased angiogenesis. Moreover, abnormal Ang2 expression characterizes CLL patients with progressive disease. Ang2 seems to be involved in CLL pathobiology and may be a useful parameter for the refinement of patients' prognosis. *Methods.* Plasma samples were collected from peripheral blood of 316 CLL patients enrolled from several institutions and Ang2 glycoprotein levels were measured by ELISA method. *Results.* One hundred of 316 CLL (31.6%) showed Ang2 levels higher than 2459 pg/mL cut off. We found that high Ang2 was predictive of reduced TTFT (P<0.001) and OS (P=0.002). Significant association was found between high Ang2 and unmutated IGHV status (n=268, P<0.001). Concordance between Ang2 levels and IGHV mutational status was observed in 185 of 268 cases (69%), whereas discordance was observed in 83 of 268 cases (31%). In particular, 42 CLL had IGHV mutated genes and high Ang2 levels whereas 41 cases showed IGHV unmutated genes and low Ang2. By further analyzing these discordances, we found that increased frequencies of other adverse variables such as advanced stage (P=0.009), high CD38 (P=0.037), high B2 microglobulin (P=0.007) differentiated IGHV mutated/high Ang2 from IGHV mutated/low Ang2 CLL. Interestingly, lower frequency of 13q deletion was observed in IGHV mutated/Ang2 high subset (36% *vs*. 58%, P=0.021). Moreover, unmutated IGHV CLL showed reduced TTFT independently from Ang2 levels (median TTFT, 35 and 39 months in low and high Ang2 groups, P=0.58). Instead, mutated IGHV CLL harbouring low Ang2 levels showed indolent clinical course (median TTFT, 179 months), whereas discordant CLL with mutated IGHV/high Ang2 had a more aggressive disease (median TTFT, 79 months) (P=0.002). Then, correlations between Ang2 levels and genomic aberrations were sought. Two-hundred ninety-seven CLL were available for comparison. Eighty-seven CLL had intermediate/high FISH risk comprising trisomy 12 (n=43), deletions of 17p (n=23), 11q (n=20) and 6q (n=1), whereas 210 had low risk FISH comprising normal karyotype (n=101) or del13q only (n=109). Significant association was found between high Ang2 and intermediate/high FISH risk (P=0.005), even if several discordant cases (36%) were present. In particular, CLL with low FISH risk and high Ang2 were more frequently characterized by advanced stage (P=0.005), unmutated IGHV genes (P=0.016), high CD38 (P=0.034), high B2 microglobulin (P<0.001) and reduced TTFT (median, 87 months *vs.* not reached, P<0.001) compared with low FISH risk/low Ang2 subset. Among the cytogenetic subsets, we found that CLL with 13q deletion had the lowest Ang2 plasmatic levels (median,1949 pg/mL, P=0.018), whereas CLL subset with 11q deletion showed the highest levels (median, 2531 pg/mL, P=0.013). *Conclusions.* CLL patients with mutated IGHV genes or low cytogenetic risk show different clinical course by virtue of plasma Ang2 levels.

P037

DIAGNOSTIC POTENTIAL OF CD38 AND ZAP-70 EXPRESSION ALONE OR IN COMBINATION IN PREDICTING MUTATIONAL STATUS OF IMMUNOGLOBULIN HEAVY-CHAIN VARIABLE REGION (IGHV) IN BINET STAGE A CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS: PRELIMINARY RESULTS OF A PROSPECTIVE, MULTICENTER O-CLL1- GISL STUDY

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Background. Ten years ago, two different groups reported the prognostic impact of the IGHV mutational status in CLL. Then, CD38 and ZAP-70 were proposed as surrogate markers of this parameter; but subsequent studies failed to confirm these data. *Aims.* To assess the diagnostic power of CD38 and ZAP-70 alone or in combination in predicting IGHV status in Binet stage A patients included in the prospective multicenter O-CLL1 GISL study. Methods 383 cases were characterized for IGHV status, CD38 and ZAP-70 by western blot (ZAP-70wb: ZAP-70neg, ZAP-70weak and ZAP-70strong) and by flow cytometry protocols, in which the percentage of ZAP-70$^+$ CLL cells was determined in respect to an isotypic control (ISO-method) or residual ZAP-70+ T cells (T-method) and as a MIF ratio between gated T and CLL cells (T/B ratio-method). *Results.* The AUC of ROC curves of CD38, ZAP-70wb, ZAP-70 ISO-method, ZAP-70 T-method and ZAP-70 T/B ratio-method to predict IGHV status were significantly higher (P<0.0001) than that of diagnostic indifference (0.81 for CD38, 0.82 for ZAP-70wb, 0.85 for ZAP-70 ISO-method, 0.82 for ZAP-70 T-method, 0.75 for ZAP-70 T/B ratio-method). The diagnostic potential of CD38 (categorized by best cut-off=10%), ZAP-70wb [categorized in negative (negative and weak expression) and positive (strong expression)], ZAP-70 ISO-method (best cut-off=40%), ZAP-70 T-method (best cut-off=40%) and ZAP-70 T/B ratio-method (best cut-off=2.15) to predict IGHV status was as follows: sensitivity (73%, 82%, 78%, 79% and 76% respectively), specificity (84%, 79%, 78%, 69% and 65%), positive predictive value (67%, 63%, 60%, 52% and 48%), negative predictive value (88%, 91%, 89%, 88% and 86%), accuracy (81%, 80%, 78%, 72% and 68%). Kappa statistics revealed that the agreement between IGHV status and CD38, ZAP-70wb, ZAP-70 ISO-method, ZAP-70 T-method and ZAP-70 T/B ratio-method were of moderate degree (K=0.56, P<0.001; K=0.56, P<0.001; K=0.52, P<0.001; K=0.42, P<0.001 and K=0.35, P<0.001, respectively). Akaike analysis showed that the model based on ZAP-70wb has 99% probability of being the best model to predict IGHV status over the other candidate models based on flow cytometry meth-

ods. Thus, combining CD38 and ZAP-70wb, we devised patients into Group1 ($CD38^-/ZAP\text{-}70^-$), Group2 ($CD38^+/ZAP\text{-}70^-$), Group3 ($CD38^-/ZAP\text{-}70^+$) and Group4 ($CD38^+/ZAP\text{-}70^+$). Logistic regression analysis showed that the risk (odds ratios, OR) of being IGHV germline was high in patients in Groups 2 (OR=6) and 3 (OR=8), achieving the highest value in Group4 cases (OR=88;P for trend<0.0001). Notably, only 11/195 (6%) of cases were unmutated in Group1, while a substantially higher number of unmutated cases were accounted in Group4 (74/88, 84%). *Conclusions.* ZAP-70 detected by wb seems to better predict the IGHV status compared to ZAP-70 evaluated by flow cytometry. The combination of CD38 and ZAP-70 is a potentially relevant diagnostic tool for distinguishing between IGHV mutated and unmutated cases.

P038

EPRATUZUMAB/SAPORIN-S6 AN ANTI-CD22 IMMUNOTOXIN FOR SELECTIVE DEPLETION OF B-CELLS NEOPLASMS

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Introduction. CD22 specifically marks mature and neoplastic B cells and efficiently internalizes after mAbs binding. It represents a good molecular target for B-cell neoplasm immunotherapy. Epratuzumab is a humanized anti-CD22 mAb, which has induced tumor regression in preclinical and phase I/II clinical evaluations in patients with indolent or aggressive lymphoma. The results obtained in clinical studies encourage the attempts to improve the Epratuzumab effectiveness, i.e. by conjugation to toxic molecules (immunotoxins). In this work, Epratuzumab was chemically conjugated to saporin-S6, a plant toxin with RNA N-glycosidase activity. *Methods.* The cytotoxicity of the Epratuzumab/saporin-S6 immunotoxin was evaluated by protein synthesis inhibition, cell viability and inhibition of clonogenic growth in B cell lines. The caspase activities were assessed by luminescent assays. Apoptosis induced by immunotoxin was also assessed in cells obtained from B-cell chronic lymphocytic leukemia (B-CLL) patients; positivity to Annexin V was evaluated by flow cytometry. *Results.* After conjugation with Epratuzumab, saporin-S6 enhanced its protein synthesis inhibitory activity on five different target B cell lines by at least 3 logs with IC50 (concentration giving 50% inhibition) in the pM range. Protein synthesis of $CD22^-$ cells was not affected by the immunotoxin at concentrations up to 10 nM. The immunotoxin resulted specifically cytotoxic for cell lines, being able to completely eliminate target cells, sparing instead not target cells. These results were confirmed by another cytotoxicity assay, based on MTS reduction. Moreover, as the final target of an anti-tumour therapy is the total eradication of the disease, the clonogenic growth of target cell lines was determined after exposure to the anti-CD22 immunotoxin. A complete elimination of clones was reached with a 3 h exposure to the conjugate, at 10 nM concentration *vs.* a 15% inhibition of clonogenic growth reached with saporin-S6 alone. To demonstrate that the immunotoxin kills target cells by apoptosis, we studied the caspase activation pattern. Both caspase-8 and -9 strongly increased their activity in treated cells. An even stronger activation was reported for caspases-3/7 (more than 20-fold over control, after 48 h). In B-CLL cells, Epratuzumab/saporin-S6 was able to induce apoptosis (Annexin V^+ cells more than 88%), thus confirming and extending the results obtained on cell lines. *Conclusions.* This work point out that it is possible to specifically increase the Epratuzumab toxicity on target cells by linking it to saporin-S6. A good therapeutic index was achieved with Epratuzumab/saporin-S6 in a preclinical model of B-CLL. All these results may encourage further *in vivo* experiments for a possible future use of this immunotoxin for therapy of B-CLL patients.

P039

ABNORMAL IMMUNOGLOBULIN SERUM FREE LIGHT CHAINS (SFLC) ARE ASSOCIATED WITH CELLULAR AND MOLECULAR MARKERS AND PREDICT THERAPY REQUIREMENT (TFS) IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (CLL) is a disorder characterized by a highly variable clinical course. The most widely used biologically defined prognostic factors range from the expression of surface CD38 or cytoplasmic ZAP-70 to the more complex recognition of the mutational status of immunoglobulin (Ig)VH genes. The possibility to recognize precociously difference in clinical outcome, which are undoubtedly relevant for clinical managing, has been guiding towards a thorough search for additional and hopefully more easily measurable biomarkers in CLL. In this respect, the finding that an abnormal sFLC ratio maybe observed also in CLL. *Patients and methods.* Three-hundred and six previously untreated CLL patients (Binet stage A=225) from several Italian Institutions were enrolled in a collaborative study. Median age was 63 years; 186 patients were males. TFS was measured from diagnosis to first line treatment or last follow-up. The median follow up was of 3.2 years and 97 patients had received treatment at the end of the study. Levels of FLCs were determined in all study samples using a particle enhanced, high-specificity, homogeneous immunoassay FLC assay (Freelite; The Binding Site, Birmingham, United Kingdom) performed on a Delta Nephelometer (Radim). *Results.* ZAP-70, CD38 and IgVH mutational analyses were performed in 293, 302 and 255 cases, respectively. Eighty-four patients had unmutated IgVH genes; ZAP-70 and CD38 were positive in 98 and 60 cases, respectively. An abnormal sFLC ratio was found in 104 out of 306 cases (92 κ and 12 λ) In order to examine the potential prognostic relevance of sFLCs patients were divided into two groups based on their normal or abnormal ratio. The analysis revealed a significantly higher risk of starting treatment for the entire group of patients with abnormal sFLCs (HR=2.6, 95% C.I. 1.7-3.9, P<0.0001). Moreover, univariate Cox analysis showed that the patients who were CD38 positive (HR=2.2, 95% C.I. 1.4-3.5, P =0.001), ZAP-70 positive (HR=4.3, 95% C.I. 2.8-6.7, P<0.0001), and IgVH unmutated (HR=3.5, 95% C.I. 2.2-5.5, P<0.0001) and those cases with advanced Binet stage (B+C) (HR=2.0, 95% C.I. 1.3-3.0, P=0.002). Notably, sFLCs (HR=2.3, 95% C.I. 1.4-3.7, P=0.001) retained an independent association with TFS even when adjusted for CD38, ZAP-70 expression, IgVH mutational status and Binet stage. In these models, only the ZAP-70 expression (HR=3.0, 95% C.I. 1.7-5.1, P<0.0001), IgVH mutational status (HR=2.6, 95% C.I. 1.4-4.7, P 0.002) and Binet stage (HR=2.8, 95% C.I. 1.6-4.7, P<0.0001) retained a statistically significant association with risk of therapy requirement, whereas CD38 expression was no longer statistically significant. *Conclusion.* The correlation with ZAP-70 and CD38 cellular expression, and IgVH mutational status could show that an abnormal FLC ratio is a marker of a biologically different subtype of CLL. The prediction power of sFLC showed here needs prospective studies in order to confirm whether it has any practical utility in addition to, or in place of, the traditional prognostic markers in this disease. The definitive clarification of the value of sFLC as a prognostic marker in CLL is of additional relevance given that the sFLC assay is a simple, serum test in contrast to determining some prognostic markers, such as IgVH mutation status which involves DNA sequencing or ZAP-70 status where significant inter-laboratory variation exists.

P040

WHITHDRAW BY THE AUTHOR

Chronic Lymphocytic Leukemia II

P041

AN ADENOSINERGIC LOOP CONTRIBUTES TO THE GENERATION OF A MICROENVIRONMENT FAVORING GROWTH AND EXPANSION OF CLL CELLS

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Introduction. CD39 (ecto-nucleoside-triphosphate-diphosphohydrolase-1) and CD73 (5'-nucleotidase) are surface enzymes with catalytic sites located in the extracellular compartment. CD39 hydrolyses ATP/UTP and ADP/UDP to AMP. In turn, CD73 degrades AMP to adenosine. Adenosine released in the extracellular milieu may re-enter the cell or engage different types of adenosine receptors (A1, A2A, A2B, A3), eliciting potent immunosuppressive responses. The hypothesis behind this work is that leukemic cells expressing CD39 and CD73 generate adenosine, thereby influencing the extracellular environment and generating conditions favorable to growth. These effects are obtained through the inhibition of the host immune response. *Methods.* Expression of CD39 and CD73 was tested by immunofluorescence in 245 chronic lymphocytic leukemia (CLL) patients with defined clinico-molecular characteristics and by immunohistochemical analyses in bone marrow (BM) and lymph node (LN) biopsies. The ability of CLL lymphocytes to generate adenosine was tested by means of a HPLC-based assay. B and T lymphocytes were purified from CLL patients and healthy donors. RNA was extracted, retro-transcribed and samples analysed by RT-PCR. *Results.* Results indicate that CD39 is constantly expressed by leukemic B lymphocytes. CD73 is highly variable within the cohort (range: 1-99%, mean value: 27%) and significantly associated with CD38 and ZAP-70. This finding may indicate that the molecule is expressed by CLL cases with an aggressive behavior. Differential CD73 expression in CLL patients is confirmed by immunohistochemical analyses of BM and LN biopsies. Intensities and percentages of expression scored the highest values within the lymph nodes, where the proliferative core of the leukemia resides. Furthermore, CD73 appears to be expressed by CLL cells with a pro-lymphocyte morphology and surrounded by $CD2^+$ T lymphocytes. $CD39^+/CD73^+$ CLL cells produce adenosine *in vitro* following incubation with ADP, in a time- and dose-dependent fashion. No adenosine production was observed when testing $CD39^+/CD73^-$ CLL cells. The results of RT-PCR indicate that T lymphocytes from CLL patients express the A2A receptor, which does not appear the same cells purified from age-matched healthy controls. These findings confirm the hypothesis that CLL cells are characterized by a significant imbalance in the purinergic pathway. *Conclusions.* The first results of this study are suggestive of the existence of an adenosinergic loop active in selected CLL patients. Next steps are the analysis of its functional role in the development, maintenance and progression of CLL.

P042

IGHV GENE ANALYSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) is characterized by an extremely variable clinical course. Mutational status of the immunoglobulin heavy-chain variable (IGHV) defines two disease subsets with different prognosis. A skewed repertoire of IGHV/D/J genes has been reported in CLL, more than 20% of the cases having closely homologous (stereotyped) heavy chain complementary-determining region 3 (HCDR3) sequences. Several different subsets of stereotyped IGHV/D/J have been identified in large multicenter international studies, suggesting that stereotypy may have a geographic pattern distribution. In this study we report on IGHV genes status of CLL patients admitted and investigated in our Institution. *Patients and methods.* Of the 200 CLLs sequenced at diagnosis for IGHV genes, we considered 124 (56%) patients (pts) for whom clinical and biological information were available. IGHV/D/J genes sequencing analysis and stereotypy subsets identification have been performed as previously reported (Stamatopoulos *et al*., 2007). *Results.* Median age of the 124 pts was 68 years (range 36-86);M/F ratio was 1.7; 104 (85%) pts were in Binet A stage. Median follow-up was 41 months (36-140) for Binet A and 51 months (11-144) for Binet B-C pts; 41 Binet A and 18 Binet B-C pts underwent treatment after a median follow-up of 19.3 and 8 months respectively. Four pts were lost to follow-up, 7 deceased for CLL complications (4 Binet A, 3 Binet B or C). Fifty-four (46%) and 52 (49.5%) pts were respectively CD38 and ZAP70 positive by flow-cytometry analysis. Deletion of 13q14, 11q23 and 17p13 occurred in 55 (45%), 14 (13%) and 13 (9%) pts, respectively and trisomy 12 in 20 (15%) pts. A total of 122 IGHV/D/J rearrangements were analyzed: 63 (52%) pts had a mutated IGHV (57/63 were in Binet A). IGHV/D/J stereotypy was present in 25/122 pts (20%); 21 were in Binet A stage, and notably, only 4 patients showed mutated IGVH(16%). The most frequent stereotypy subset identified in our panel was 1 (8 pts). The remaining represented subsets were type 2 (1 pt), 3 (2 pts), 4 (4 pts), 7 (6 pts), 10 (1 pt), 13 (1 pt), 24(1 pt), 36 (1 pt). No cases of stereotyped IGVH3-21 were observed. As regards to Binet A, unmutated patients showed a significant shorter time to first treatment (TTFT) in comparison to mutated ones (P<.0001); notably, Binet A pts in subset 1 had shorter TTFT in comparison to both mutated and unmutated pts, as well to those in subset 7 (P<.0001). *Conclusions.* As clonal expansion of leukemic B cells may be triggered by unidentified foreign or self antigens, geographical analysis of IGHV/D/J usage may be important in identifying environmental and/or ethnic contribution. Our single Institution data are in line with those of other Mediterranean countries, where IGVH3-21 is infrequent. Even if most of the pts with stereotyped IGHV/D/J were unmutated, the presence of subset 1 confers adverse prognosis, underlining the biological and clinical importance of these data.

P043

INCREASED REGULATORY T-CELL NUMBERS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH PROGRESSIVE DISEASE

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Introduction. Regulatory T-cells (Treg) actively maintain immunological self-tolerance and play a significant role in the progression of cancer, likely through suppression of tumor specific immunity. Several studies highlight that the immune system in patients with chronic lymphocytic leukemia (CLL) is deficient. Methods. We have evaluated circulating Treg cell numbers, by means of a multiparametric flow cytometric approach ($CD4^+/CD2^{bright+}/CD127^{low}$), in 80 patients with previously untreated CLL and in 40 normal healthy age- and sexmatched volunteers. *Results.* CLL patients displayed a significantly lower percentage of Treg cells than in controls (P 0.03). However, CLL patients showed a higher absolute number of Treg cells compared to controls (P 0.02). The absolute number of Treg cells also positively correlated with moreadvanced Rai clinical stage (P<0.0001), peripheral blood B-cell lymphocytosis (P<0.0001), absolute $CD38^+$ B-cell number (P 0.001), and more elevated LDH levels (P 0.037). No correlation was found with ZAP-70 expression, IgVH mutational status and cytogenetic abnormalities. Conclusions. Taken together, our data show that Treg cells are higher in CLL patients than in controls, where they correlate with disease status. This subset of T-cells is likely involved in the crucial mechanism of pathogenesis and progression of CLL and a therapeutic intervention targeting these cells needs to be explored.

P044

CHRONIC LYMPHOCYTIC LEUKEMIA CELL APOPTOSIS INDUCED BY VERBENA OFFICINALIS ESSENTIAL OIL AND ITS COMPONENT CITRAL IS DUE TO PROCASPASE-3 ACTIVATION

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Introduction. Verbena Officinalis L. (Verbenaceae), commonly known as vervain, is a medicinal plant whose essential oil is mainly constituted by isoprenoids. The plant and/or its essential oil have been widely used in different traditional medicines. The plant was approved as a herbal medicine and dietary supplement by several regulatory acts of many Countries. Despite its widespread use, the mechanisms of pharmacological actions of the herb or the volatile oil are still unclear. Purpose of this study was to evaluate the pro-apoptotic activity of vervain essential oil and of its main component, citral, on lymphocytes collected from normal blood donors and patients with untreated chronic lymphocytic leukemia (CLL), a disease entity in which derailed apoptosis is still retained one of the primary pathogenic event causing the accumulation of neoplastic B-cells. *Methods.* We evaluated the pro-apoptotic activity of Verbena officinalis essential oil and of its main component citral, on lymphocytes collected from 10 normal blood donors and 15 patients with chronic lymphocytic leukemia (CLL), by adding to treated mononuclear cells, annexin V, propidium iodide, and CD19-APC-Cy7. Apoptosis was also evaluated using anti-active-caspase-3 monoclonal antibody. *Results.* The number of apoptotic cells was greater in CLL patients than in healthy subjects at all different times of incubation (4, 8 and 24 hours) for samples treated with Verbena officinalis essential oil (A) and citral (B) as well *vs.* controls at different concentrations (0.1% and 0.01%). The greater pro-apoptotic ability was showed by both essential oil of Verbena officinalis and citral at lower concentrations (after 4 h A 0.1%: 17.8% *vs.* 37.1%; A 0.01%: 15.8% *vs.* 52%; B 0.1%: 18.4% *vs.* 46.4%; B 0.01%: 15.8% *vs.* 54.2%; after 8 h A 0.1%: 23% *vs.* 38%; A 0.01%: 22.2% *vs.* 55%; B 0.1%: 32% *vs.* 42.2%; B 0.01%: 22% *vs.* 54.3%; after 24 h A 0.1%: 5% *vs.* 20.7%; A 0.01%: 25.8% *vs.* 47.2%; B 0.1%: 18.4% *vs.* 46.4%; B 0.01%: 15.8% *vs.* 54.2%). Patients carrying deletion 17p13 (p53 mutation) showed a reduced ability to undergo apoptosis with respect to patients with other genomic aberrations or normal karyotype. The proapoptotic activity of Verbena officinalis essential was shown to be due to a direct procaspase 3 activation . *Conclusions.* This data further support evidences that indicate natural compounds, such as Verbena Officinalis, as possible lead structure to develop new therapeutic agents.

P045

IMMUNE THROMBOCYTOPENIA COMPLICATING CHRONIC LYMPHOCYTIC LEUKEMIA TREATED WITH RITUXIMAB AS SINGLE AGENT: A RETROSPECTIVE ANALYSIS OF 12 PATIENTS

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Introduction. The clinical course of chronic lymphocytic leukemia (CLL) may be complicated by hematologic autoimmune disorders. It has been estimated that immune thrombocytopenia (IT) can complicate the course of CLL in approximately 2% of patients. There are no stanrd therapies for CLL-associated IT. More recently, patients treated with monoclonal antibodies rituximab and alemtuzumab have been reported, despite anecdotally, with encouraging results. *Methods.* We report the results of rituximab treatment in 12 patients (10 M; 2 F) with CLL-associated IT seen at our Institutions. The mean age at CLL diagnosis was 65 years (range 51-75 years) and 69 years (range 58-75 years) at IT diagnosis. IT developed at a a mean time of 17 months (range 0-96 months) from the diagnosis of CLL. In 4 cases IT was diagnosed at the same time as CLL. For 3 patients IT was considered fludarabine-related. All patients received steroids at therapeutic dosages as first-line treatment for IT. As second-line therapy, intravenous Ig (3 patients), vincristine (1 patient) and cytoxan (1 patient) were also given without beneficial effect. After a mean time of 37 days (range 1-86 days) from the diagnosis of IT all patients were scheduled to receive rituximab at a dosage of 375 mg/mq/weekly for 4 consecutive weeks. *Results.* Nine patients completed the scheduled 4 cycles of rituximab. Only 1 patient had irrelevant side effects (fever and chills) during the first dose infusion. In 2 patients rituximab was discontinued after the third cycle because of no-response (NR). In 1 patient broncopneumonia occurred after the third rituximab dose Ig serum levels were detected low and therapy was discontinued. This patient showed a NR after the last cycle. Overaal, 7 patients (58%) obtained a complete response (CR), 2 patients (17%) a partial response (PR). And 3 patients (25%) a NR. No responding patient started a maintenance program with rituximab while 2 unresponsive patients underwent splenectomy with increase in plateletes to the normalization. Finally, at a mean follow-up of 33 months (range 3-60 months), all patients but one (a NR patient died of broncopneumonia) were still alive, 7 of them in CR and 2 in PR. Conclusions. This retrospective analysis prove that the anti-CD20 monoclonal antibody rituximab is an effective and well-tolerated alternative treatment for CLL-associated IT.

P046

AMD3100 SYNERGIZES WITH FLUDARABINE INCREASING THE APOPTOTIC RATE IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The interaction between leukemia cells and bone marrow microenvironment has been proved to be fundamental for the development of the desease and for resistance to the treatment with conventional chemotherapy. Stromal cell derived factor 1 (SDF-1) together with its receptor CXCR4 constitute the main axis implicated in homing and retention of leukemic cells in the bone marrow and lymphoid organs. We studied the effect of the inhibition of SDF-1/CXCR4 interaction by using AMD3100, a bicyclam antagonist of CXCR4, and the effects resulting on chemotherapy sensibility to standard treatment with fludarabine in B cells chronic lymphocytic leukemia (B-CLL). *Methods.* We collect bone marrow (BM) cells from 4 patients with newly diagnosed B-CLL. Mononucleated cells were isolated via Ficoll density gradient centrifugation and cultured in RPMI 1640 with 10% FBS, 1% L-glutamine, 1% penicillin-streptomicin in absence of drugs, with fludarabine (3 ug/mL), with AMD3100 (4 ug/mL), with fludarabine and AMD3100 simultaneously, and with fludarabine after AMD3100 incubation. Annexin V - propidium iodinate assay was performed to determine the apoptotic rate by FACS analysis after 24 hours and 48 hours of incubation on CD5/CD19 positive cells. *Results.* Fludarbine plus AMD3100 cultures showed significantly the highest apoptotic rates after 24 hours (71.1%) and 48 hours (80.3%) when compared to fludarabine alone (61.5%) or AMD alone (54%). Interestingly the apoptotic rate is even higher when B-CLL cells were previously incubated with AMD3100 and then treated with fludarabine (76.5% after 24h and 86,2% after 48h), suggesting how the disruption of SDF1/CXCR4 binding is itself the main mechanism able to increase the sensibility to the fludarabine action. AMD3100 cultures showed the 24-hours-apoptotic rate higher (54%) than control (42,3%) but lower than fludarabine (61.5%), with no differences after 48 hours. It is questionable whether a direct cytotoxic effect could be attribute to AMD3100 directly or that the SDF1/CXCR4 disruption could kill the leukemic cells. We also demonstrated that the addiction of AMD3100 induces the down-regulation of the CXCR4 as showed by the complete disappearance of the signal in FACS analysis already after 24 hour of incubation. *Conclusion.* AMD3100 synergizes with conventional cytotoxic agents such as fludarabine increasing apoptosis rate in B-CLL cells. We hypothesize that, analogously to SDF-1, AMD3100 is able to induce receptor internalisation leading leukemic cells to death for the loss of stromal microenvironment interactions resulting in the enhanced responsivity to fludarabine. Furthermore our data show how the association with a CXCR4 antagonist may also reduce the dose of conventional chemotherapeutic agents.

P047

CLINICO-BIOLOGICAL IMPLICATIONS OF ADIPONECTIN SERUM LEVELS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The correlation between well-established biological parameters of prognostic relevance in B-cell chronic lymphocytic leukaemia (CLL) [i.e., mutational status of the immunoglobulin heavy chain variable region (IgV(H)), ZAP-70- and CD38-expression] and adiponectin serum concentration was evaluated in a cohort of 69 previously untreated Binet stage A CLL patients. Adiponectin levels inversely correlated with absolute peripheral blood lymphocyte count (r=-0.254; P=0.03), $CD38^-$positive CLL cells (r=-0.294; P=0.04) and ZAP-70 (r=-0.285; P=0.03). The univariate Cox proportional hazard model demonstrated that, in addition with lower serum levels of adiponectin (P=0.01), the unmutated IgV(H) condition (P=0.002) and ZAP-70-positivity (P=0.02) were associated with a shorter time to first treatment (TFT). However, in multivariate analysis only ZAP-70 positivity emerged as predictor of the TFT (P=0.008). Furthermore, adiponectin serum levels were retrospectively correlated with bone marrow (BM) microvessel area, serum levels of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiogenin, PECAM-1 (CD31), matrix metalloproteinase-9 (MMP-9), interleukin-8 (IL-8) and syndecan-1. The positive correlation between serum levels of adiponectin and VEGF (P=.03) does not translate into an increase of the extent of BM angiogenesis (P=.404), FGF-2 (P=.348), angiogenin (P=.402), and CD31 (P=.248) serum concentrations. Accordingly, IL-8 (P=.175), syndecan-1 (P=.06), and MMP-9 (P=.144) circulating levels were not likely to reflect adiponectin concentration. The levels of adiponectin in CLL were evaluated in 60 patients from an independent cohort investigated by gene expression profiling. Adiponectin gene expression was invariably low suggesting a limited (if any) role of leukemic cells in the production of circulating adiponectin levels. In contrast, both adiponectin receptor 1 (AdipoR1) and AdipoR2 mRNA were highly expressed by CLL cells with a degree of inter-patient variability. Interestingly, VEGF transcript was highly expressed in our patient cohort. Our results, although preliminary, lend support to the idea that adiponectin secretion by bone marrow adipocytes might be involved as an antiangiogenic factor in B-CLL.

P048

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

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Introduction. The pathogenetic mechanisms underlying chronic lymphocytic leukemia (CLL) transformation to Richter syndrome (RS) are poorly understood and might involve antigen stimulation. In B-cell neoplasia, analysis of intraclonal diversification (ID) of immunoglobulin (IGHV) genes provides information on tumor immunogenetics. We investigated ID in RS (all DLBCL), in order to i) define RS clonal evolution; and ii) assess the role of antigen stimulation in clonally related *vs.* unrelated RS. *Methods.* The study was based on 49 samples: i) 11 clonally related CLL/RS pairs; ii) 7 clonally unrelated RS; and, for comparison, iii) 20 de novo DLBCL. Fifty IGHV subclones were analysed per sample. Mutations observed in only one subclone were defined unconfirmed (UCM). Partially shared mutations were defined as confirmed (CM). The normalized mutation frequency (NMF) was calculated according to the formula: (CM+UCM)/number of subcloned sequences x sequence length. Phylogenetic analyses was performed with MEGA4. In order to define the load of oncogenic molecular lesions, RS were analysed for: i) mutations of TP53, CARD11, TNFAIP3/A20, BLIMP1, BCL6, PIM1, PAX5, TTF, c-MYC, CD79A/B, EZH2; ii) chromosomal abnormalities of BCL2, BCL3, BCL6, c-MYC, MYCN, ATM, TP53, 6q21. *Results.* Clonally related RS were classified as unmutated (100% homology; 6/11), minimally mutated (99.0-99.9% homology; 2/11) or borderline mutated (98.0-98.9% homology; 3/11). Stereotyped HCDR3 occurred in 5/11 cases (subset 8: 2/5; subset 2: 1/5; subset 6: 1/5; subset 7: 1/5). The NMF of IGHV ongoing mutations significantly decreased from CLL (mean: 0.89×10^{3}) to RS (mean: 0.19×10^{3}) (P=0.04). All CLL phases displayed ID (CID: 7/11; UID: 4/11), that, at RS transformation, was switched off in 5/11 cases. ID-positive RS carried a significantly higher number of genetic lesions (mean: 3) than ID-negative RS (mean: 1) (P=0.045). Genealogic trees showed that RS and CLL clones originated from a common progenitor cell in 10/11 cases, whereas in 1 single case RS evolved from a later CLL subclone (Figure 1). Clonally unrelated RS were classified as unmutated (3/7), minimally mutated (1/7) or borderline mutated (3/7), and carried stereotyped HCDR3 in 1/7 cases (subset 1). All 7 clonally unrelated RS carried CID. Accordingly, the NMF was significantly higher in clonally unrelated RS (mean: $0.96v10^{3}$) compared to clonally related RS (P=.001). Finally, ID in RS was similar to ID of de novo DLBCL sharing the same non-GCB profile (p greather than .050). *Conclusions.* The conclusions of our study are multifold. First, differences in ID reinforce the notion that clonally related and clonally unrelated RS are distinct biological entities. Second, clonally related RS originate from the same common progenitor of the CLL clone, rather than evolving from a later CLL subclone. Third, among clonally related RS, the association between ID and high load of oncogenic molecular lesions might reflect the predisposition to genetic instability of RS cases targeted by somatic hypermutation. In this setting, accumulation of genetic lesions might favor transformation. Fourth, in clonally related ID-negative RS, the tumor clone carries few genetic lesions and is selected to maintain the B cell receptor, suggesting a role of high affinity antigen stimulation in disease development.

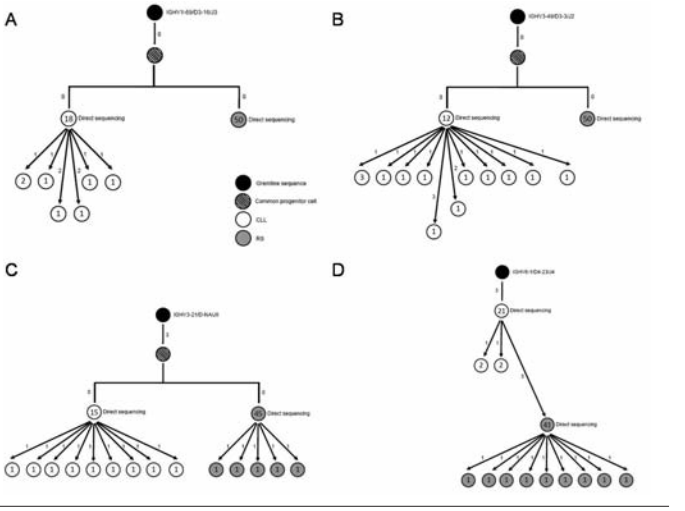

Figure 1.

P049

IN VITRO APOPTOGENIC EFFECT OF NEW SMAC MIMETIC COMPOUNDS ON CHRONIC LYMPHOCYTIC LEUKEMIA

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Defective apoptosis is commonly considered to be an important event both in development and progression of chronic lymphocytic leukemia

(CLL). The expression of inhibitor of apoptosis proteins (IAPs) is up-regulated in this disease. Among IAPs, the X-linked IAP (XIAP) is the most potent inhibitor of the apoptotic process. XIAP is characterized by three highly conserved tandem BIR domains (BIR1-3) which are able to bind and block some members of the family of cysteine proteases (caspases), that are the main effectors of apoptosis. BIR3 domain selectively targets caspase 9, an initiator caspase, while the linker region between BIR1 and BIR2 binds with executioner caspases 3 and 7, thus inhibiting the activity of both initiator and effector of apoptosis. The activity of XIAP is antagonized by Smac-DIABLO (Second Mitochondria-derived Activator of Caspases - Direct IAp Binding protein with LOw pI), a protein released from the mitochondria. Small molecules mimicking Smac (Smac-mimetics) can induce apoptosis in tumor cells by displacing the interaction of XIAP with caspases 3, 7 and 9, therefore promoting the apoptotic process. Here we describe the *in vitro* activity of newly synthesized Smac-mimetics (monomers and dimers) in human CLL and normal lymphocytes. Smac-mimetic compounds were evaluated for their ability induce apoptosis in mononuclear cell (PBMCs) isolated by density gradient centrifugation from peripheral blood samples of 28 CLL patients and 5 healthy donors. Apoptosis was evaluated by flow cytometric analysis of samples treated with Smac-mimetics following annexin V and propidium iodide staining. The samples were treated for 16 hours at 37°C and 5% CO_2 with low micromolar concentrations (1-10 microM) of the new Smac mimetic compounds alone or in combination with the proteasome inhibitor bortezomib (10 nM) or with Tumor necrosis factor–Related Apoptosis–Inducing Ligand (TRAIL) (50 ng/mL). Our compounds bind to XIAP with high affinity and are cell permeable. After 16 hours of treatment with the monomer compound Smac66 at a 10 microM concentration we have reached an apoptosis rate in CLL samples up to 72,5% (median value 44% ranging from 11% to 72,5%). The cytotoxic effect of Smac-mimetics was significantly higher (P=0.014) in CLL than in healthy donors samples, where the median rate of apoptosis was 6% (ranging from 2% to 18%). The monomers were more cytotoxic than the dimeric compounds on leukemic cells. No significant additional or synergistic effect was observed in combined treatment with bortezomib nor with TRAIL. In conclusion, while being very active in leukemic cells, our Smac-mimetics have modest effects on normal lymphocytes, suggesting a promising therapeutic potential as a new class of anticancer drugs in onco-hematology. Further molecular studies are currently ongoing to better elucidate the mechanism of action of our new Smac-mimetics as well as *in vivo* studies in animal models.

P050

BCL3 TRANSLOCATION IN CLL WITH TYPICAL PHENOTYPE: ASSESSMENT OF FREQUENCY, ASSOCIATION WITH CYTOGENETIC SUBGROUPS, AND PROGNOSTIC SIGNIFICANCE

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BCL3 encodes a co-activator of the nuclear factor kappaB (NF- B) family of transcription factors. B-cell malignancies activate BCL3 through juxtaposition with regulatory elements of the immunoglobulin heavy chain gene in the t(14;19)(q32;q13) translocation. Few series of CLL harbouring BCL3 translocation have been so far reported in the literature. All these reports agree in stating that CLL cases harbouring BCL3 translocation preferentially associate with atypical morphology and phenotype, and show an increased risk of transformation to aggressive lymphoma. Though BCL3 translocation has been described in typical CLL, a comprehensive study aimed at defining the prevalence of BCL3 translocation in this setting is lacking, and represents the aim of this study. We analysed the prevalence of BCL3 translocation in a consecutive series of 225 CLL harbouring a typical phenotype (Matutes score >3). BCL3 translocation was investigated by FISH (Dako, Glostrup, Denmark). Median age at diagnosis was 70 years. Male:female ratio was 126:99. Binet stage at diagnosis was A in 177/225 (78.7%) cases, B in 27/225 (12.0%), and C in 21/225 (9.3%). Median absolute lymphocyte count was 11.0×10^9/L. CD38 >30% was observed in 61/225 (27.1%) cases, ZAP70 >20% in 60/202 (29.7%), IGHV homology >98% in 74/221 (33.5%). FISH karyotype was positive for 13q14 deletion in 120/225 (53.3%) cases, +12 in 47/225 (20.9%), 11q22-q23 deletion in 15/225 (6.7%), and 17p13 deletion in 24/225 (10.7%). BCL3 translocation was observed in 4/225 (1.8%) CLL with typical phenotype. The partner chormosome was 14q32 (IGH) in all cases, as determined by FISH. BCL3 translocation was restricted to cases harbouring trisomy12 as the sole cytogenetic abnormality (4/40; 10.0%), while all CLL cases belonging to other genetic subgroups, including cases with 13q14 deletion, normal FISH karyotype, 11q22-q23 deletion or 17p13 deletion, scored consistently negative for BCL3 translocation (0/185) (Fisher's exact test P=.001). In order to validate these observations, BCL3 translocation was investigated in a second independent cohort (n=32) of typical CLL (Matutes score >3) harbouring trisomy 12 as the sole cytogenetic abnormality. This analysis confirmed that BCL3 translocation occurs in a significant fraction of CLL belonging to the trisomy 12 genetic subgroup (3/32, 9.4%). We then assessed whether BCL3 translocation might refine the prognostication of CLL with trisomy 12. CLL carrying both trisomy 12 and BCL3 translocation showed a risk of progression, transformation to Richter syndrome and death similar to that of CLL carrying trisomy 12 but lacking BCL3 translocation. These results document that BCL3 translocation is rare in CLL showing a typical phenotype and does not carry clinical relevance. Accordingly, in the context of typical CLL, FISH analysis for BCL3 translocation should not be routinely performed.

P051

SECRETOME ANALYSIS OF JVM-13 CELL LINE FOR THE IDENTIFICATION OF NEW CHRONIC LYMPHOCYTIC LEUKEMIA BIOMARKERS

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Introduction. B cell Chronic Lymphocytic Leukemia (B-CLL) is the most common hemopoietic malignancy of the elderly in western countries. Microenvironmental interactions play a significant role in CLL pathogenesis, providing survival factors and cell-cell interactions resulting in extended survival and apoptosis resistance. The analysis of the secretome of a CLL cell line could provide a model to study tumoral microenvironment signals for biomarkers discovery. *Methods.* Cell culture conditions were optimized to obtain 5% spontaneous mortality as assessed by trypan blue exclusion assay, adequate to the study of secretome. Secretome was harvested after 24h of serum-starvation and analyzed through nano-LC-MS/MS and OFFGEL fractionator technology followed by monodimensional SDS-PAGE and MALDI-TOF mass spectrometry. Identified proteins were entered in a proprietary, annotated, non-redundant database for logical filtering against secretomes from other cell lines of diverse origin, in order to obtain a list of specific proteins from which extrapolate putative biomarkers. Cellular localization was predicted using ab initio web-based software (SignalP 3.0, SecretomeP 2.0 and TMHMM 2.0). Protein lists (secreted, specific and secreted-specific) were analyzed with David 6.7 beta and GeneCoDis 2.0 in order to identify the most relevant associated biological functions and pathways. Finally, protein-protein interaction data from various public databases were integrated through David 6.7 beta and Cytoscape 2.6.3, in order to identify common interactors of secreted proteins. *Results.* The integration of different bioinformatic analysis tools has identified a subset of proteins involved in proteasomal processing and interacting with NF-kB pathway. Data mining from literature has confirmed NF-kB relevance to and proteasome involvement in CLL pathogenesis. It was also noted that most drugs available in clinic and trials for CLL are also inhibitors of NF-kB pathway. Besides, logical filtering coupled with semantic data mining has identified a small subset of proteins, suitable for testing in clinical samples as surrogate biomarkers of disease. *Conclusions.* High-throughput bioinformatic analysis of JVM-13 CLL cell line has enabled the construction of a clinically relevant working hypothesis, to be further tested *in vitro* and *in vivo*.

P052

EQUIVALENT EFFICACY AND LOWER TOXICITY OF SUBCUTANEOUS CLADRIBINE AT REDUCED DOSES (FIVE VERSUS SEVEN CONSECUTIVE DAYS) IN HAIRY CELL LEUKEMIA: AN UPDATE ON 156 PATIENTS OF THE ICGHCL 2004 PROTOCOL BY THE ITALIAN COOPERATIVE GROUP ON HCL

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Background. Hairy cell leukemia (HCL) is a rare B-cell neoplasm generally responsive to Cladribine. Cladribine is generally administered intravenously either as a continuous weekly infusion or as a 2-hour daily or weekly infusion for 7 days. Subcutaneous Cladribine is an alternative route with 100% bioavailability and with efficacy similar to intravenous Cladribine at the dose of 0.7 mg/kg/cycle. In indolent non-Hodgkin lymphomas other than HCL, reduction to 0.5 mg/kg/cycle determined equivalent efficacy and lower toxicity. *Aim.* To investigate efficacy and toxicity of subcutaneous cladribine given at different dose regimens. Methods: In a national multicentre clinical trial (protocol EudraCT code: ICGHCL 2004), we have evaluated efficacy and toxicity of subcutaneous Cladribine given 0.1mg/kg/die for 5 (total dose 0.5 mg/kg, arm A) or for 7 days (total dose 0.7 mg/kg, arm B) as a single course in newly diagnosed HCL requiring treatment. Responses to treatment were assessed on day 60 and day 180 after treatment and defined according to the 1987 Consensus criteria. Complete Remissions (CR) and Partial Remissions (PR) were considered as beneficial responses, while minor Responses (mR) and No Responses (NR) were rated as treatment failures. Toxicity was assessed from day 0 to day 60 after treatment, according to the 2003 NCI/CTCAE v3 criteria. *Results.* Of 156 patients enrolled in 22 italian centers, 9 patients were excluded because diagnosis was not confirmed centrally or due to lack of data. A total of 147 patients were evaluated for toxicity and response to treatment (76 patients in arm A and 71 in arm B). Cladribine was administered at the proposed regimen with no modifications in all but 2 patients of arm B. One-hundred-forty/147 patients (95,2%) had a beneficial response to treatment (101/147 CR, 67,8%; 39/147 PR, 26,5%). The 7/147 treatment failures scored as 5/147 mR (3,4%) and 2/147 (1,4%) NR. Responses were equivalent in the two arms (P=ns), with 72/76 (94,7%) beneficial responses (49/76 CR, 64,5%; 23/76 PR, 30,3%; 4/76 mR, 5,3% and 0 NR) in arm A *versus* 68/71 (95,7%) beneficial responses in arm B (52/71 CR, 73,2%; 16/71 PR, 22,5%; 1/71 mR, 1,4%; 2/71 NR, 2.8%). Overall grade 3-4 toxicity was recorded in 31/147 (21%) patients, of which 23/147 (15,6%) were FUO or documented infections. Grade3-4 toxicity appeared less frequent in arm A (11/76, 13,9%) than in arm B (20/71, 26%) (P=0.04) and was largely represented by FUO/infections (7/76, 9,2% in arm A *vs.* 16/71, 22,5% in arm B, P=0.016), to suggest a higher risk of infection in the 7 day regimen. As a result of toxicity, 9/76 (11,4%) *vs.* 21/71 patients (27,3%, P=0.01) required hospitalization in arm A *vs.* arm B after treatment, respectively. *Conclusions.* The present data indicate that overall activity of subcutaneous Cladribine is similar to the intravenous formulation (Cheson, 1998). Furthermore, the current analysis confirms our previous data that subcutaneous Cladribine given at 25% reduced doses (0.5 mg/kg) has equivalent activity and lower toxicity than subcutaneous Cladribine at standard doses (0.7 mg/kg) and is easy to give in an outpatient setting.

P053

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

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Background. IGHV1-69 gene identifies the most common IGHV gene in chronic lymphocytic leukemia (CLL) and is dominant in unmutated CLL (U-CLL). It is often rearranged to form stereotyped HCDR3 patterns that may suggest antigen selection of the leukemic clones. One of stereotypes is the IGHV1-69/D3-16/J3 subset 6 that may produce antibodies binding non-muscle myosin on apoptotic cells, with potential consequences on clinical behavior (Chu CC, Blood 2010, prepublished). *Aims.* to investigate the prognostic significance of mutational status and of stereotypic B-cell receptors in IGHV1-69$^+$ CLL. *Methods.* Nucleotide sequences of the tumor IGHV1-69/D/J rearrangement, clinical and molecular prognostic parameters at diagnosis and clinical status at follow-up of 271 IGHV1-69$^+$ CLL patients were obtained from 14 different hematological institutes in Italy. CLL B-cell derived IGHV1-69 rearrangements were scanned for HCDR3 stereotypic patterns and assigned to subsets according to the criteria by Murray *et al.* (Blood, 2008 111: 1524-1533). Time to progression requiring first treatment according to NCI criteria (TTFT) was used as the primary endpoint to measure behavior of all or of Rai stage 0 CLL, with mutated (M) or unmutated (U), stereotyped or not stereotyped IGHV1-69 rearrangements. *Results.* Of 271 IGHV1-69$^+$ CLL, 245 (90,4%) were unmutated, 154/257 (59,9%) revealed stereotypic patterns and 15/257 (5,8%) belonged to subset 6. Subset 6 IGHV1-69 rearrangements were unmutated in 14/15 (93,3%) CLL. TTFT was significantly shorter in IGHV1-69$^+$ U-CLL than in IGHV1-69$^+$ M-CLL (29 *vs.* 101 months, P=.001 in all IGHV1-69$^+$ CLL; 52 *vs.* 142 months in Rai 0 IGHV1-69$^+$ CLL, P=.001), while was no different between the CLL assigned or not assigned to subsets (31 *vs.* 37 months in all IGHV1-69$^+$ CLL, 52 *vs.* 78 months in IGHV1-69$^+$ Rai 0 CLL, P=NS). However, specific analysis of the CLL-specific subset 6 revealed TTFT longer than the remaining IGHV1-69$^+$ U-CLL either in all stages (52 months *vs.* 27 months, P=.05) or in stage 0 (no events *vs.* 49 months, P=.016). We expanded the analysis to all IGHV1-69$^+$ and non IGHV1-69$^+$ subsets 1, 3, 8, 9 and 28 (n=50) that also react with autologous non-muscle myosin (Chu CC, Blood, 2010, prepublished). Most remarkably, we found that TTFT of subset 6 was longer than the other anti-myosin subsets (26 months in all stages, P=.021; 44 months in Rai stage 0, P=.02). *Conclusions.* our analysis documents and confirms that unmutated status of IGHV, and not stereotypy, is a relevant prognosticator in CLL. However, the good prognosis of Rai 0 U-CLL assigned to subset 6 suggests a differential clinical behavior irrelevant of unmutated status of this specific subset, particularly if identified in early stage (Rai 0) CLL.

P054

LACK OF ALLELIC EXCLUSION BY SECONDARY REARRANGEMENTS OF TUMOUR B-CELL RECEPTOR LIGHT CHAINS IN HAIRY CELL LEUKEMIA

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Introduction. Analyses of the tumor immunoglobulin (Ig) gene (IG) heavy (H) and light chains show heterogeneity of mutational status, but reveal common features of ongoing IGH isotype-switching with multiple IGH isotype expression and preference of IGL with selective use of IGLJ3. Methods: Immunophenotypic and immunogenetic analyses were performed in a series of 105 HCL patients to estimate prevalence of mul-

tiple IG light chain expression by the tumour cells. Results: We found that 5/105 HCL (4,76%) expressed double tumour-related Ig light chain proteins. Among the 5 HCL, functional mutated double IGKI/IGKII, IGKI/IGLI and IGLI/IGLII transcripts were cloned and sequenced in 3/3 cases investigated. All 3 HCL expressed multiple IGH isotypes with mutated IGHVDJ rearrangements at the time of AID transcript expression. Most interestingly, the 3 cases had reinduced RAG1 transcript. In the double IGL expresser, single-cell analysis documented co-expression of the tumour-related IGLs in 5/6 cells (83%). In the IGK/IGL co-expresser, evidence of surface IgK/IgL isotype proteins confirmed functionality of the tumour-derived transcripts. Conclusion: The evidence of double light chain expression in single HCs and the new observation of RAG re-induction suggest ongoing selective influences on the BCR that may promote or maintain the HCL clone in the periphery.

P055

EFFICACY AND SAFETY OF FLUDARABINE-ALEMTUZUMAB COMBINATION (FLUCAM) AS FIRST LINE TREATMENT OF HIGH RISK CLL: A PILOT ANALYSIS OF ELDERLY CLL PATIENTS WITH TP53 DISFUNCTION

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Background. Concomitant administration of fludarabine and alemtuzumab (FluCam) in patients has been proposed as effective regimen in relapsed or refractory B-cell chronic lymphocytic leukemia (CLL) (T. Elter, J Clin Oncol, 2005) and it is under investigation for efficacy and toxicity in young patients (<60 years) high-risk CLL patients in an Italian multicenter clinical trial. However CLL occurs in elderly patients (median age at diagnosis 72 years) and treatments in high-risk CLL are unsatisfactory. *Aim.* to explore efficacy and toxicity of full dose FluCam regimen CLL elderly (>65 years) patients with TP53 defects. *Methods.* 12 patients with TP53 disfunction (FISH 17p deletion or PCR TP53 mutation), requiring first line (7 patients, 60-84 years, median 65) or >= 2nd line treatment (5 patients, -53-66 years, median 56) according to the 2008 IWCLL/NCI criteria (Hallek, Blood, 2008), received intravenous fludarabine 30 mg/m^2/day and subcutaneous alemtuzumab 30 mg/day for 3 consecutive days (FluCam) every month for a maximum of 4 cycles. CMV profilaxis was given to all patients with oral Valciclovir 2 g t.i.d. Toxicity was assessed during each cycle and response to treatment was defined according to the 2008 IWCLL/NCI criteria (Hallek, Blood, 2008). Dinamic endpoints were treatment-free interval (TFI), event-free survival (EFS) and overall survival (OS) from FluCam. Results of FluCam in first line were also compared to a control cohort of 15 CLL patients (46-81 years, median 57) with TP53 disfuntion having received treatments other than FluCam in first line. *Results.* All patients receiving first line FluCam were >60 years. Treatment was well tolerated in all patients and CMV reactivation was recorded in none of the 7 patients. Six of 7 patients obtained a complete remission (CR) while 1/7 obtained a partial remission (PR). After a follow-up of 6 to 90 months (median 30 months) only the 1 patient with a PR required a new treatment and median EFS was not reached, and none died. Conversely, of 5 patients that received FluCam after first line (3rd-5th line) experienced CMV reactivation in 2 cases during the first cycle, and prolonged cytopenia in 2 cases after the 2nd and the 3rd cycle, respectively, and stopped treatment prior to the 4th cycle in 4/5 cases. Of 5 patients, only 1 obtained a CR, 2 scored as stable disease (SD) and 2 as progressive disease (PD). Median EFS was 5 months and median OS was 17 months. The control cohort of 15 CLL patients with TP53 disfunction receiving nonFluCam regimens in first line obtained 1 CR, 3 PR, 8 SD and 3 PD, had a median EFS of 14 months and a median OS of 34 months. *Conclusions.* this pilot analysis suggests high efficacy and good tolerability of full dose FluCam in elderly patients with high risk CLL (TP53 disfunction) that require treatment for the first time and suggest that alemtuzumab-based regimens may find a preferential location if used as first line treatments.

Lymphomas I

P056

SPECIFIC EFFECTS EXERTED BY B-LYMPHOPROLIFERATIVE DISEASES ON PERIPHERAL T LYMPHOCYTES PROTEIN EXPRESSION

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Background. B-lymphoproliferative diseases represent a wide range of neoplastic syndromes: Hodgkin's lymphoma, aggressive and indolent lymphoma, chronic lymphocytic leukemia, plasma cell dyscrasias. Various studies describe alteration of T-cells in patients affected by such diseases (1-4) arising a great interest about the regulatory function of T-lymphocytes on B-cells pathologies. In our laboratory, we have previously demonstrated that T-lymphocytes and/or their subpopulations from peripheral blood may represent molecular sensors to be used for the evaluation of gene expression modification in physiological and pathological conditions,providing a unique and easily available biological model for integrated studies of gene expression in humans. *Aims.* Here we report on the proteome profile of peripheral T-cells in patients affected by B-lymphoproliferative diseases at the onset. *Methods.* Subjects. Twelve patients (4 females and 8 males, mean age 59±15) with various lymphoproliferative, malignancies were selected for this study by the Haematology division of the Azienda, Ospedaliera Sant'Andrea, Sapienza University of Rome, Italy. Among these, 4 were affected, by diffuse_large-B-cell-lymphoma (DLBCL); 3 by follicular lymphoma (FL); 3 by classical, Hodgkin's lymphoma (CHL); 2 by chronic lymphocytic leukemia (CLL). Informed consent was obtained by all patients. *Samples.* T cells were then isolated by negative selection (mean purity ≥95%) using a magnetic beads system (Pan T Cell Isolation Kit II, human, Miltenyi Biotec, Auburn, CA) and Protein separation was carried out after cell lysing. Two-dimensional (2D) gel electrophoresis and MALDI-ToF mass spectrometry were used for protein separation and identification. Interaction network analysis. Protein-protein interaction analysis was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software version 8.1 (6). The database was queried using only experimental data as prediction method and limiting the search to no more than 100 molecular interactors, with a required minimum confidence score of 0.4 (medium level). *Hierarchical clustering of proteomic data.* Unsupervised analysis of proteomic data was performed by the GeneSpring GX 7.3 expression analysis software (Agilent Technologies). For each analysed patient, the average quantisation of spot values inside its replicate group, reported as the ratio to the average of the control replicate group, was imported as expression data. Analysis included T-cell proteome profiles of: 12 B-lymphoproliferative disorders, 10 polycystic ovary syndrome (PCO), 5 congenital adrenal hyperplasia (CAH), 2 monoclonal gammopathy of undetermined significance (MGUS), healthy subjects (single sample representative of ten pooled control subjects, as described above). Clustering was performed according to an average linkage algorithm using the following parameters: similarity measure by Pearson correlation, similar branches merged with a separation ratio of 1, minimum distance 0,001. Samples were processed following the diagram reported in Figure 1. *Results.* The analysis of the proteome profile of peripheral T-cells of patients affected by B-lymphoproliferative diseases reveals decreased levels of profilin-1 and cofilin-1 and increased levels of coronin1A and prohibitin, compared with healthy controls. The protein-protein interaction network of these proteins was studied, highlighting the actin cytoskeleton regulation as the main biological process involved in peripheral T-cells of such patients. Unsupervised cluster analysis of protein expression data shows that the recorded alteration of T-cell proteome was specifically induced by B-cells pathologies. *Discussion.* Peripheral T-lymphocytes represents easily available living biosensors, and proteins whose expression level changes significantly in specific pathological states might represent new molecular markers for follow-up testing of the disease state. All patients enrolled in this study were at the on-set of the disease, and the identified proteins differentially expressed can be regarded as markers of T-cell activation, according to many recent works reporting alterations of T-cells functionality, number and functional class balance in B-lymphoprolifer-

ative disorders (35-39). We believe it interesting to evaluate the potential prognostic and/or diagnostic value of monitoring the expression level of these factors during the therapy.

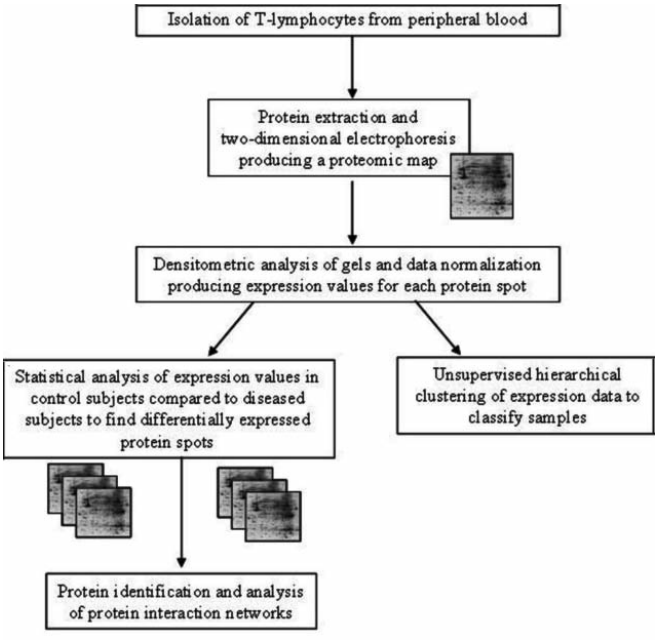

Figure 1.

P057

HODGKIN LYMPHOMA OF THE SKIN: A UNUSUAL ENTITY. REVIEW OF LICTERATURE AND DESCRIPTION OF A CASE

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Introduction. The localization of Hodgkin's disease on the skin is an atypical occurrence and frequently occurs late in the course of Hodgkin's lymphoma. This rare condition is thought to have decreased in incidence in recent decades, likely owing to improved treatment of patients with Hodgkin's disease, who are receiving improved chemotherapy and radiation therapy, and the advent of peripheral blood stem cell transplantation. The most common clinical presentation is of single or multiple dermal or subcutaneous nodules. Direct extension from an underlying nodal focus, hematogenous dissemination, and most often, retrograde lymphatic spread, distal to involved lymph nodes, are the mechanisms usually implicated. We report the case of a patient with Hodgkin's disease who presented skin involvement at diagnosis. *Case report.* An 85 years old woman accused malaise, fatigue and loss of weight; on physical examination the unilateral supraclavicular lymph node was found (diameter 2.0 cm) and ulcerated lesion of the neck of 5 cm of diameter. The remaining of physical finding was normal. Laboratory data were normal. LDH was high: 679 UI/L (v.n.300-600). Total body scan was negative. The biopsy of lymph node was performed and the histology reveled Hodgkin's lymphoma, mixed cellularity subtype. The PET total body after node biopsy showed a single area with excavation at the center in the subcutaneous tissue of the occipital region (SUV max 5.9). The skin biopsy pointed a dermal localization of Hodgkin's lymphoma, $CD30^+$ and $CD15^+$. Histology of bone marrow was normal. The patient was considered in clinical stage II AE. Chemotherapy with VEPEMB protocol was started; the patient received 3 full cycles obtained a complete remission, proved by clinical, biochemistry, and PET/TC imaging techniques. After chemotherapy RT/IF was performed. *Discussion.* Primary cutaneous HD is very unusual and generally represents a rare late manifestation of dissemination of the disease heralding poor prognosis. Specific skin lesions were described in 3.4-7.6 % from all cases of HD and have been categorized as papules, nodules, plaques or infiltration, ulcerative lesions, a combination of these, and erythroderma and often are accompanied by pruritus. The skin of the chest seems to be most frequently involved. More recently, cutaneous HD has been described in patient with human immunodeficiency virus (HIV) infection, advanced clinical stage, and aggressive clinical course, suggesting that alteration in immune system function is important for disease appearance and spread. EBV positively has been previously described in cutaneous HD in only one case. Conclusion: Cutaneous involvement usually represents, or accompanies, Stage IV Hodgkin's disease, and often portends an ominous prognosis. Nevertheless, it also might follow a relatively benign course, and more intensive systemic chemotherapy sometimes is effective in such cases.

P058

IN VITRO COMBINATION OF LOW DOSES OF ENZASTAURIN AND LENALIDOMIDE SHOWS STRONG ANTITUMOR ACTIVITY ON B-LYMPHOMA CELL LINES

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Introduction. Enzastaurin is a potent inhibitor of PKC-β, which has been shown to inhibit cancer cell proliferation and angiogenesis in human cancer cell lines. Lenalidomide, an immunomodulatory drug, has a pleiotropic effect via activity on microenvironment and some direct effects on cell resulting in an anti-angiogenic and antiproliferative action. For its strong activity, lenalidomide is approved for use in combination with dexamethasone to treat patients affected by Multiple Myeloma and in patients with low-or intermediate-1-risk Myelodysplastic Syndromes associated with a deletion 5q cytogenetic. The purpose of the present study was to asses the antitumor activity of the combination of low doses of enzastaurin and lenalidomide on B-lymphoma (B-NHL) cell lines. *Methods.* We utilized WSU-NHL, RL and Karpas-422 cell lines that are carrying the t(14;18). Cell viability was evaluated with Trypan blue exclusion, and IC_{50} values were calculated using the MTT assay. Cell lines were treated with enzastaurin and lenalidomide, alone and in combination. Cell apoptosis was assessed by flow cytometry using Annexin V/FITC Kit. Cell cycle was analyzed by BrdU/PI using flow cytometry. The effect of enzastaurin plus lenalidomide on signaling pathways and caspases activation was evaluated by immunoblotting analysis. The combination effect was examined by isobologram analysis based upon Chou-Talalay method. *Results.* Enzastaurin alone decreased the viability of cell lines with IC_{50} values ranging between 1.5 and 10 μM. Further, enzastaurin induced apoptosis via activation of intrinsic and extrinsic pathways and inhibition of AKT pathways at concentrations of 10 μM. Lenalidomide alone had antiproliferative effect with an IC_{50} between 7 and 15 μM, without inducing apoptosis and inhibition of AKT pathway. Lenalidomide showed an effect on cell cycle by increasing the percentage of cells arrested in G0-G1 phase. In combination studies, we utilized enzastaurin and lenalidomide at concentration of 1 microM that is lower than IC_{50} concentration. Usually at this concentration each drug utilized alone has no effects on cell lines. This combination induced apoptosis by cleavage of caspases 8, 9, 3 and PARP and inhibited AKT phosphorylation as well as downstream GSK-3β, m-TOR, p-70, p-90RSK and MAPK. We demonstrated that low doses combination of enzastaurin and lenalidomide has synergistic effect against B-NHL cell lines, as confirmed by isobologram analysis that showed a combination index minor 1.0. The combination maintained its activity in the co-culture of B-NHL cells with bone marrow stromal cells. Furthermore, enzastaurin plus lenalidomide did not decrease viability of mononuclear cells from healthy donors. *Conclusions.* Our data demonstrate that low doses combination of enzastaurin and lenalidomide shows synergistic activity against B-NHL cell lines with (14;18) and inhibit AKT and MAPK signaling pathways, inducing apoptosis. These results support the use of this combination for treatment of NHL patients in an attempt to overcome the resistance of tumor cells.

P059

DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF BIS-ARYL-UREA DERIVATIVES AS ANAPLASTIC LYMPHOMA KINASE INHIBITORS

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Anaplastic Large Cell Lymphoma (ALCL) is characterized by the chromosomal translocation t(2;5)(p23;q35)that generates the highly oncogenic NMP-ALK fusion gene. NMP-ALK drives the survival and proliferation of ALCL cells. Currently no ALK inhibitor is registered for clinical use. Two known urea inhibitors, Sorafenib and BIRB-796, have been shown to bind to the inactive conformation of tyrosine kinases. Since inhibitors targeted to the inactive conformation are thought to be more specific than the ones binding active conformations, we investigated the possibility of using BIRB-796 and sorafenib to design new urea derivatives as ALK inhibitors. A 3D homology model of the inactive conformation ALK was produced, using the structure of insulin receptor kinase as a template. BIRB-796 and sorafenib were docked into the ALK active site. The results rationalized why BIRB-796 is only weakly, and sorafenib is not active on ALK. Virtual screening of commercially available compounds using the homology model of ALK yielded hits that were tested and confirmed by biochemical assays. Starting from initial hits, new derivatives were rationally designed. Here, the synthesis, biological activity and structure-activity relationship (SAR) of a novel series of urea compounds as potent ALK inhibitors is presented. Some of the inhibitors showed nanomolar activities on the purified enzyme and in NPM/ALK+ cells. One derivative inhibited NPM/AKL-transformed cells growth with an IC50 of 0.5 M and 1-log selectivity over control cells.

P060

BENDAMUSTINE IS HIGHLY ACTIVE IN INDUCING APOPTOSIS ON A T-CELL LYMPHOMA CELL LINE

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Introduction. Bendamustine has relevant clinical activity against a variety of lymphoid malignancies and solid tumors. This drug is a bifunctional agent with structural similarities to alkylating agents and purine analogues, and is particularly effective in patients with B-cell non Hodgkin's lymphomas (NHL). The role of Bendamustine against T-cell NHL, which share an inferior outcome compared to their B-cell counterpart and are frequently resistant to common anti-neoplastic drugs, is unclear. We investigated the pro-apoptotic effect of Bendamustine on an *in vitro* model of T-cell lymphoma (cell line SUP-T1). *Methods.* SUP-T1 cells were incubated with Bendamustine at concentrations ranging between 0 and 250 μg/mL in RPMI medium supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin for 24 or 48 hours. Drug dosages were chosen to calculate the IC25, IC50, IC75 and IC90 defined as the dosage of drug necessary to cause apoptosis of 25%, 50%, 75% and 90% of cells, respectively. Apoptosis was evaluated with the fluorescent DNA-binding agent 7-AAD (7 aminoactinomycin D); the disruption of mitochondrial membrane potential (m) was monitored with the specific fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide (JC-1). This test is based on a lipophilic fluorochrome which exhibits red fluorescence in aggregated form, with high m, and green fluorescence as monomer when m is relatively low. Flow cytometric analysis was used to analyze results. The experiments were repeated a minimum of 4 times with 2 biological repeats. *Results.* Pro-apoptotic effect of Bendamustine was directly proportional to drug concentration, reaching its higher activity at 250 μg/mL, when 81% of the cells were apoptotic by 7AAD test (Figure 1). Similarly, exposure to increasing concentrations of Bendamustine resulted in a decrease of m indicative of altered mitochondrial functions; the percentage of m disruption was significant over 100 μg/mL and increased to 87% at the highest concentration (Fig 1). Interestingly, with this test we observed two distinct apoptotic populations, different in their emission spectrum, suggesting the existence of an early and a late stage of cell damage. For both tests results were similar when cells were incubated for 24 or 48 hours. *Conclusions.* Bendamustine demonstrated a high activity in a T-cell NHL cell line, which was proportional to drug concentration and was not time dependent. A prolonged incubation of the drug did not rely in an increased apoptotic effect, suggesting that Bendamustine in these patients do not require a long exposure. JC1 results indicate that the cytotoxic effect of the drug may not be limited to its alkylating/purine analogue mechanism of action, but may also be mediated by a change in mitochondrial membrane potential, which would then trigger the apoptotic pathway. Based on our preliminary results Bendamustine could represent a future new drug to be used in patients with T-cell NHL.

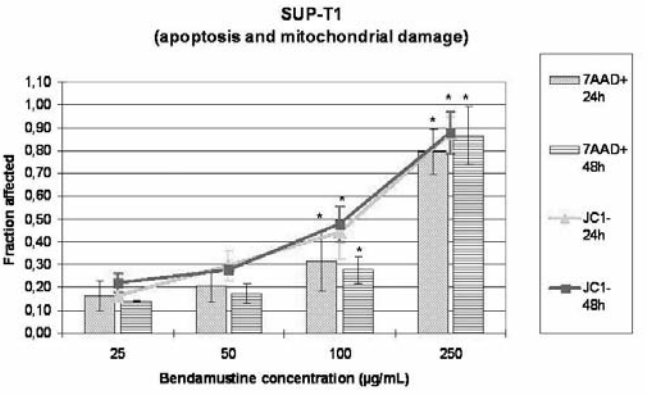

Figure 1. Apoptosis induced by Bendamustine after 24 and 48 hours of incubations on SUP-T1 cells measured with 7-AAD (columns) and disruption of mitochondrial membrane potential evaluated with JC-1 (lines).* P<0.05 compared to control (0 μg/mL).

P061

PATIENTS WITH B-CELL NON HODGKIN LYMPHOMA SHOW INCREASED FREQUENCIES OF REGULATORY T CELLS AND CD8+ T-CELL EXPANSIONS

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Introduction. Although most non NHLs take origin from the B-cell lineage, several studies suggest that any impairment involving the different branches of the immune system may play a role in their pathogenesis. Moreover the cross-talk among lymphoma cells and other cell types, such as for instance T-lymphocytes and antigen presenting cells, within the peritumoral microenvironment seem to deeply influence the onset and evolution of NHL. In order to explore the possible impact that the degree of activation of the T-cell immune system and the balance among different T-cell populations may have on the NHL pathogenesis, we analysed the T-cell receptor (TCR) repertoire and the distribution of different T-cell subsets -including regulatory T-cells (Treg)- in patients with NHL. *Methods.* Our study was based on a flow cytometric analysis performed on the peripheral blood of 8 patients (4 with indolent NHL and 4 with diffuse large B-cell lymphoma, DLBCL) and 15 age-matched controls. We first determined the frequency of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16\text{-}56^+$ T-cells. Treg were then identified by considering the $CD4^+$ cell fraction characterised by a very high (>2 log) expression of CD25 and by a very low (<2 log) expression of CD127, as well as by determining the expression of FoxP3 and CD152. TCR repertoire analysis was based on a panel of 24 beta variable (BV) family-specific antibodies. A BV expansion was defined as any value of BV family expression higher than the mean + 3 standard deviations calculated in normal controls. *Results.* We first showed that patients had reduced frequencies of $CD8^+$ cells (mean 21% *vs.* 33%) and $CD16^+$ $CD56^+$ natural killer cells (11% *vs.* 22%) when compared with normal controls, while $CD3^+$ and $CD4^+$ frequencies were similar. Patients also showed a higher frequency of Treg than controls (mean 2.68% *vs.* 1.14%), although this increase was mainly confined to patients with DLBCL (mean 3.64%) rather than in patients with indolent NHL (mean 1.57%). Finally we determined the frequency of expanded T-cell subpopulations expressing the same TCR BV subfamilies, showing in patients and controls a similar frequency of expansions in $CD4^+$ cells (1% *vs.* 1%), besides an increased frequency of $CD8^+$ expansions in patients (4% *vs.* 2%). When

we looked at the possible influence of several disease-related factors, such as WHO lymphoma subtype, IPI score, presence of constitutional symptoms, bone marrow involvement and stage, only a diagnosis of DLBCL rather than indolent NHL was specifically associated with an increased frequency of $CD8^+$ lymphocyte expansions (5% *vs.* 1%). *Conclusions.* Our preliminary data suggest that the T-cell branch of the immune system in patients with NHL show features which can be distinguished from those observed in normal controls. In particular, NHL patients seem to show an increased degree of activation of the TCR repertoire along with a higher frequency of Treg, which are both even more pronounced in patients with aggressive NHL.

P062

FLOW CYTOMETRIC IMMUNOPHENOTYPIC AND MOLECULAR PROFILES AND FOLLOW-UP IN A CASE OF GAMMA-DELTA HEPATOSPLENIC T-CELL LYMPHOMA

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Hepatosplenic T-cell lymphoma is a rare clinicopathologic entity with poor outcome. It is characterized by malignant T-cell proliferation in the liver, spleen and bone marrow. The diagnosis is based on clinicopathological criteria and can be upgraded with immunophenotypic profile and molecular studies as well. We describe the case of a 51-year old woman affected by HSTCL, who underwent a systematic flow cytometric and molecular follow-up. In May 2006, the patient showed massive hepatosplenomegaly and pancytopenia. She underwent splenectomy, hepatic biopsy and BM analysis, for an abnormal lymphoid cells infiltration a diagnosis of HSTCL was made. The patient was treated with six courses of CEOP14 and complete remission was achieved. The first relapse occurred in September 2008 and the second and last in August 2009. Treatment consisted of DHAP, AutoTMO, GEMOX, Nelarabine. She died in December 2009. At diagnosis BM, spleen and liver biopsy had shown, $CD45^+CD2^+CD3^+CD7^+CD56^+TcR\delta^+CD5^-CD4^-CD8^-$ neoplastic cells at cytofluorimetric analysis. Molecular analysis, performed by denaturing high-performance liquid chromatography (DHPLC), showed a clonal rearrangement of the TcR- chain gene on the same samples and the sequencing analysis of both DNA strands identified the monoclonal rearrangement Vg10-JgP1. At the first relapse BM biopsy was positive for lymphoma infiltration despite the negative cytofluorimetric and morphological analysis, and the liver biopsy was infiltrated by $CD45^+CD2^+CD7^+CD56^+CD3^-CD5^-CD4^-CD8^-TcR\delta^-$ like-NK-cell abnormal lymphocytes as well. Molecular studies showed the Vg10-JgP1 and a new Vg11-JgP1 clonal rearrangements on BM. At the second relapse, the histological study on the liver biopsy failed to detect any neoplastic cells, although the flow cytometric study showed large neoplastic cells. BM biopsy was positive for lymphoma infiltration and the cytofluorimetric analysis identify a like-NK-cell population. This case seems interesting for many aspects, the main being: (a) the immunophenotype change from T-cell $CD45^+CD2^+CD3^+CD7^+CD56^+TcR\delta^+CD5^-CD4^-CD8^-$ to a like-NK-cell immunophenotype $CD45^+CD2^+CD7^+CD56^+CD3^-CD5^-CD4^-CD8^-TcR\delta^-$, (b) the evidence of two different clonal rearrangements of the TcR- - chain gene (Vg10-JgP1 and Vg11-JgP1). We might hypothesise that neoplastic cells rosed from a clonal proliferation of Natural killer T (NKT) cells. NKT cells are characterized by the expression of both TCR and NK receptors on the cell surface; moreover, recent evidence indicates that they are totipotent and can underlie many diseases. Furthermore, this could be a case of neoplastic proliferation of T lymphocytes subpopulation bearing two distinct TCR. This is supported by the evidence that a proportion of circulating T cells co-expresses two distinct TCR chains, although the T cells are ensured by several allelic exclusion processes operating at either the genotypic or phenotypic levels and expressing two distinct or TCR.

P063

PHARMACOGENOMIC MARKERS OF CLINICAL EFFICACY IN A DOSE-DENSE THERAPY REGIMEN (R-CHOP14) IN DIFFUSE LARGE B CELL LYMPHOMA: PRELIMINARY RESULTS

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Background. Diffuse large B cell lymphoma (DLBCL) is one of the most common types of non-Hodgkin's lymphoma. Approximately half of patients (pts) will be cured of their disease by primary therapy, including the R-CHOP regimen (rituximab, doxorubicin, cyclophosphamide, vincristine, desamethasone). The remaining die of the disease, mainly because of the occurrence of tumor drug resistance. Many efforts have been made to explain the biochemical and molecular mechanisms involved in the resistance to the drugs used in the treatment of cancer pts, including those with DLBCL. A dose-intense therapy regimen (e.g. R-CHOP14) may help to improve the treatment outcome of DLBCL pts *Aims.* We have carried out a retrospective study aimed at correlating the mRNA expression levels of genes involved in metabolism, mechanisms of action and resistance to doxorubicin (i.e. MDR1, GSTP1, TOPO-2a, Bcl-2, PKC-β2) that represents the backbone of the R-CHOP regimen with treatment outcome data of 32 pts at various stages of disease. *Methods.* The expression of the 5 above mentioned genes were determined in formalin fixed paraffin-embedded samples from DLBCL using real time RT-PCR. The correlations between gene expression data and clinical/pathological characteristics as well as survival parameters have been evaluated by standard statistical tests. *Results.* The case series included 19 males and 13 females, 6 pts had follicular lymphoma grade IIIb and 26 diffuse large B cell lymphoma, 11 presented symptoms at diagnosis. Eighteen pts showed abnormal LDH values, the IPI was intermediate-high risk or high risk in 8 pts. Twenty-eight pts (87.5%) obtained a complete remission and 4 (12.5%) a partial response. The median overall survival (OS) as well as the median failure free survival (FFS) have not yet been reached after a median follow-up of 31.2 months. The mRNA expression levels of TOPO-2a and GSTP1 were detectable in all samples, that of PKC-β2 in 31 samples, that of MDR1 and bcl-2 in 16 and 12 samples, respectively. The intragenic variation levels (ratio between the maximum and minimum levels of mRNA gene expression) ranged from 799 for bcl-2 to more than 100,000 for GSTP1. No significant differences between FFS and mRNA expression levels of the study genes were observed. An inverse relationship was observed between OS and TOPO-2a mRNA expression (P=0.026): higher TOPO-2a gene expression was associated with shorter OS. Only a trend was observed between OS and PKC-β2 or GSTP1 mRNA expression. No difference was observed between gene expression mRNA levels and clinical/pathological characteristics with the exception of the tumor histology for all genes (p minor of 0.05). This preliminary data and others from related studies may help to identify gene profiles useful for selection pts eligible for intensified or personalized chemotherapy that can achieve greater efficacy than standard therapies.

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P064

PROGNOSTIC VALUE OF POSITRON EMISSION TOMOGRAPHY/COMPUTED TOMOGRAPHY (PET/CT) IN PATIENTS WITH LYMPHOMA TREATED WITH AUTOLOGOUS STEM CELLS TRANSPLANTATION FOR RELAPSED/REFRACTORY OR HIGH RISK DISEASE

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Introduction. PET/CT imaging has increasingly been used for management of lymphoma. Few prospective studies addressed its prognostic value in the setting of high dose therapy with autologous stem cell transplantation (ASCT) so far. *Methods.* We prospectively enrolled 17 patients with HD (9 relapsed, 8 refractory diseases), 43 patients with NHL (15 relapsed, 15 in partial remission after first line treatment, 13 at high-risk while in

complete remission). Mean age was 44 years (18-65). M/F ratio was 34/26. All patients presented a positive PET/CT at diagnosis. We evaluated PET/CT before (PET/CT1) and after ASCT (PET/CT2). Primary end points included: OS and PFS using Kaplan-Meier estimates and Log-rank test. Secondary end points included: predictive value of FDG-PET by Fisher's exact test. *Results.* 56 patients underwent both PET/CT1 and PET/CT2: we observed positive result in 30 subjects (19 NHL, 11 HD). Three PET/CT1 negative subjects became positive at PET/CT2: 2 relapsed. Twelve PET/CT1 positive patients became negative at PET/CT2. We observed 5 relapses in PET/CT1 negative group and 11 relapses in PET/CT1 positive group: in this subgroup 3 patients had a negative PET/CT2 scan. Sixty patients were evaluable for PET/CT2: 21 patients were positive (13 NHL, 8 HD). Respectively 6 subjects relapsed in PET/CT2 negative group and 10 relapsed in PET/CT2 positive group. Mean follow up was 27 months (5-84); mean OS and PFS were respectively 87.1% and 71.8%. On the basis of PET/CT1 we observed statistically significant differences in both OS (100% neg. *vs.* 52.4% pos.; P 0.005) and PFS (82.1% neg. *vs.* 49.4% pos.; P 0.008). On the basis of PET/CT2, OS was 100% neg. *vs.* 62.9% pos. (P 0.002), while PFS was 84.2% neg. *vs.* 49.4% pos. (p 0.003) (Figure 1). All relapses have been documented in PET/CT2 positive group within 16 months from ASCT (median 6 months). Died patients showed same PET/CT status before and after ASCT. In negative group 3 patients died, one of TRM after allogeneic SCT, one of secondary AML after radioimmunotherapy and the last because of progressive disease. Seven positive patients died: 6 for progressive disease and one of TRM after allogeneic SCT. In PET/CT1 cohort we observed 11 positive patients with 19 false positive and 21 negative with 5 false negative: positive and negative predictive value were respectively 0.37 and 0.80. A statistically difference was not seen for PET/CT1 status: relative risk for positive status was 1.91. In PET/CT2 cohort we observed 10 positive patients with 11 false positive and 33 negative with 6 false negative: positive and negative predictive value were respectively 0.48 and 0.85. A statistically difference was seen for PET/CT2 status (p 0.01): relative risk for positive status was 3.1. *Conclusions.* Negative PET/CT status before and after ASCT predicts PFS and OS after ASCT, however the risk of relapse is less clear for positive scan.

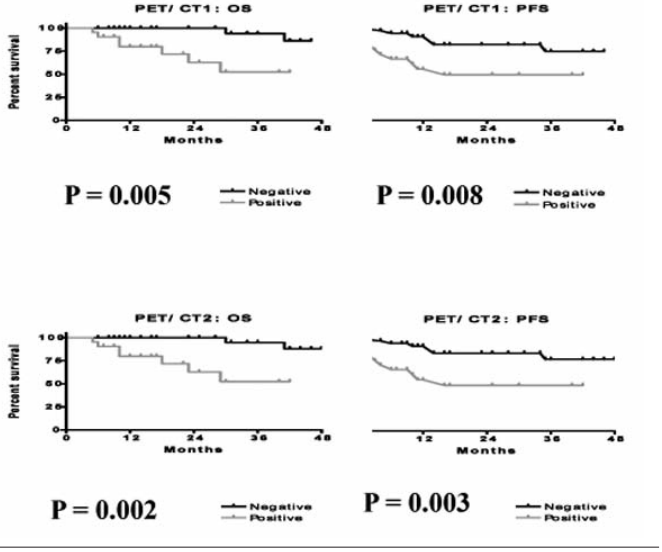

Figure 1.

Lymphomas II

P065

NEW BIOMARKERS OF NEOPLASTIC-RELATED INFLAMMATION BURDEN IN HL

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Background. Hodgkin's Lymphoma (HL) is characterized by the presence of few (1-2%) neoplastic cells called Hodgkin-Reed Stenberg cells (H-RS), often (>90% of cases) derived from germinal-center B in the lymphonode, and a large microenvironment composed by reactive cells originated in the bone marrow. That smouldering inflammation contributes to tumor development through different mechanisms, involving cell cross-talk, epigenetic events, altered gene expression, induction of angiogenesis, immunoediting process, resistance to apoptosis and enhanced proliferation. Myeloid-derived suppressor cells (MDSCs), identified in mice for their immunosuppressive abilities as $CD14^-$, $HLA-DR^-$, $CD15^+$, $CD34^+$, $CD11b^+$, $CD33^+$, and $CD13^+$ cells represent a heterogeneous population of cells including mainly immature macrophages, granulocytes and dendritic cells responsible of progression of solid tumors. Inflammatory monocytes are reported to be a distinct subpopulation of $CD14^+$ monocytes, phenotypically different from classical monocytes, highly "proinflammatory". They are believed to be sensitive markers of the inflammatory milieu or significant contributors to the development and progression of diseases such as atherosclerosis and arthritis. *Methods.* So far, we evaluated 32 patients for circulating levels of MDSCs ($CD11b^+$, $CD13^+$, $CD14^-$, $CD34^+$, $CD45^+$) and inflammatory monocytes ($CD14^+CD16^+$) in peripheral blood by flow cytometry at diagnosis and after chemotherapy. *Results.* At baseline, we found higher levels both of circulating MDSC when compared to matched for sex and age healthy controls (mean 3.66±1.94/mm^3 *vs.* 1,69±0,87/mm^3, P=0.0001) and inflammatory monocytes (mean 93.85±2.49/mm^3 *vs.* 42,69±5.8/mm^3, P=0.04). Among HL patients, a higher number of MDSC was correlated to higher levels of ferritin (P<0.05), but it was independent from ESR, CRP, Hasenclever score values and clinical stage at diagnosis. *Conclusions.* Circulating MDSC and inflammatory monocytes counts in peripheral blood is an easily accessible tool of neoplastic-related inflammation burden in HL.

P066

CD20 EXPRESSION IN HODGKIN AND REED-STERNBERG CELLS OF CLASSICAL HODGKIN'S LYMPHOMAS

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Introduction. Hodgkin and Reed-Sternberg cells (HRS) cells represent the neoplastic population of classical Hodgkin lymphoma (cHL). Recent immunological and molecular studies have shown that HRS cells originate from mature germinal centre B cells. Although cHL is genotypically considered a B-cell lymphoma, the classical B-cell marker CD20 is expressed with a reported frequencies of 5-58%. The prognostic significance of CD20 expression in HRS cells of cHL is still controversial. *Methods.* To further assess the presenting features and the prognostic significance of CD20 expression in cHL, we performed a retrospective single institutional study of 87 cases with a mean clinical follow-up of 12 years. The mean ages were 40.4 years (range, 14-83 years) for the men and 34.3 years (range, 16-77 years) for the women. Sixty-five (74.7%) of the 87 patients (pts) were younger than 45 years of age. The histology were nodular sclerosis in 75, mixed cellularity in 10, lymphocyte rich cHL in 1, and lymphocyte-depleted in 1. Stage III+IV was present in 57 pts (65.5%), bulky disease in 34 pts (39.1%), extranodal disease in 16 (18.4%), 51 pts (58.6%) had IPS score 0-2 (low-risk) and 36 (41.4%) had score 3 (intermediate-high-risk). 57 pts (65.5%) were treated with combined radiochemotherapy, 30 (34.5%) were treated with chemotherapy alone. 24 pts (27.6%) received MOPP/ABVD, 16 (18.4%) received BEACOPP, 47 (54%) received ABVD, chemotherapy regimens. *Results.* HRC expressed CD20 in 27 pts (31% of cases). The negative rate of

CD20 was significantly higher in the patients with B-symptoms (54% *vs.* 32.2%, P=0.046) and with bulky disease (49.4% *vs.* 22.9%, P=0.035). There was no statistically significant difference in CD20 expression between the groups with other different clinical parameters. No statistical differences in terms of response rate (82% *vs.* 85%) was found between CD20 positive and negative cHL. The 5-year progression free survival (PFS) rates were 76.2% in CD20-positive patients and 82.2% in CD20-negative patients (P=n.s.; Figure 1). The 5-year overall survival (OS) rates were 91.4% in CD20-positive patients and 92.5% in CD20-negative patients (P=n.s.). *Conclusions.* CD20 is expressed by HRS cells in 31% of patients with cHL. It is higher in the patients without bulky disease and B symptoms. However, according to our results, the expression of CD20 is not an independent prognostic factor for PFS and OS of naive cHL patients.

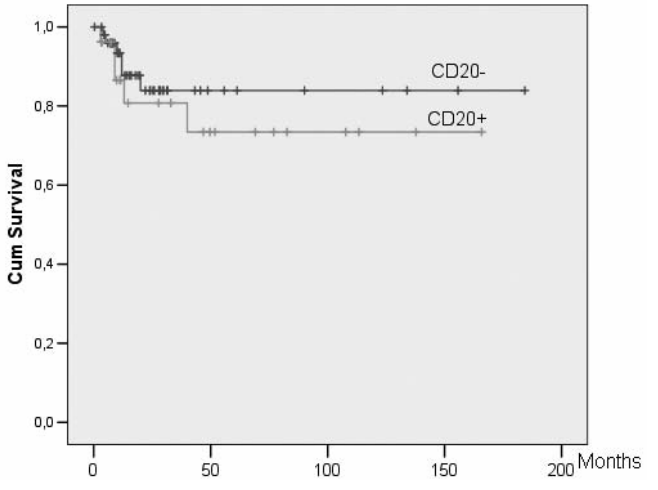

Figure 1. PFS in pts with cHL according to CD20 expression in HRS.

P067

OUTCOME OF PATIENTS AFFECTED BY MANTLE CELL LYMPHOMA IS NOT CONDITIONED BY VASCULAR ENDOTHELIAL GROWTH FACTOR POLYMORPHISMS

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In mantle cell lymphoma, angiogenesis has been shown to be a relevant prognostic factor and a valid target of therapy: the vascular endothelial growth factor (VEGF) expression was identified in 41% of patients, with a significantly shorter overall survival for VEGF-positive patients, in comparison with -negative ones. VEGF is a major angiogenic factor and regulator of endothelial cell proliferation, with a crucial role in vasculogenesis and vascular permeability. The polymorphisms in the promoter region (C-2578A and T-460C, 5'-untranslated C+405G and 3'-untranslated region C+936T) have been associated with different levels of VEGF. In an our previous study we showed that, while there was not a significant difference in allele frequency for VEGF C-460T and C+936T SNPs between MCL and controls, in the case of VEGF C-2578A SNP, the mutant allele (A) was significantly less frequent in the patients. Also in the case of VEGF G+405C, the allele distribution was significantly different between the two groups, the mutant allele (C) frequency being significantly lower in MCL patients. On this basis, we decided to test if VEGF C-2578A and G+405C SNPs would significantly condition response to treatment or survivals in 30 MCL patients treated at our institution according to R-Hyper-CVAD or R-CHOP regimens. The overall response rate for the entire series was 87; complete remissions were achieved by the 60% of patients, without any significant difference between patients receiving R-Hyper-CVAD or R-CHOP (65% *vs.* 50%, P=0.4). The median time to progression of the entire series was 33 months, with 47% of patients alive and free from disease progression at 36 months. No clinical characteristics assessed at diagnosis did significantly influence the achievement of clinical response, nor OS or TTP. OS was significantly influenced only from the quality of response (median 42 months for patients not achieving CR versus not reached for cases in CR, P=0.004). In univariate analysis, TTP was significantly conditioned by 3 variables. 1) The quality of response: TTP was shorter for patients who did not achieve the complete response (36 months-TTP: 25% for cases not achieving CR *vs.* 57% for those in CR, P=0.009); 2) The molecular status at diagnosis. All cases were assayed at diagnosis for either IgH or BCL1/JH rearrangements: in 50% of cases a molecular marker was found. After treatment, 60% of them achieved PCR-negativity. Median TTP was 26 months for patients PCR-positive at diagnosis versus 42 months for PCR-negative ones (P=0.02). On the contrary, the clearance of minimal residual disease (molecular status evaluated at the end of treatment) did not condition TTP; 3) The chosen therapeutic regimen: median TTP was 27 months in the subgroup treated with R-CHOP versus 43 months for those receiving R-Hyper-CVAD (P=0.043). On the contrary, VEGF genotypes or allelic frequencies did not significantly condition the response to treatments, the clearance of minimal residual disease or survivals. In multivariate analysis, the molecular status at diagnosis and the type of treatment retained their statistical significance on TTP. In the present study, we showed that other polymorphisms, those of VEGF, that could be relevant in the pathogenesis of mantle cell lymphoma, are not a discriminating factor in patients candidates to treatment with effective regimens, such as R-Hyper-CVAD and R-CHOP. This observation could be so useful in the clinical practice.

P068

THE ITALIAN MULTICENTER STUDY ON GENE-ENVIRONMENT INTERACTIONS IN LYMPHOMA AETIOLOGY

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The Italian study "Gene-environment interaction in lymphoma aetiology" is part of the international consortium Interlymph, promoted by the US National Cancer Institute -NIH, Bethesda, Maryland (http://epi.grants.cancer.gov/InterLymph/). The main purpose of the Interlymph Consortium is assembling the lymphoma studies conducted world wide to define causal models of lymphoma, using an interdisciplinary approach and state-of-the-art pathology, genomics, molecular biology and epidemiology techniques. Recent publications of the Interlymph Consortium have shown the association between IL-10 and TNF-α gene polymorphisms and risk of diffuse large B-cell lymphoma and follicular lymphoma. The excess risk of various lymphoma subtypes in relation to Hepatitis C infection has also been confirmed, as well as the association of risk with a family history positive for malignancies of the lymphohaemopoietic system, and with previous autoimmune diseases, such as Sjogren sindrome and systemic lupus erythematosus. A protective effect has been observed in relation to infections occurring early in life and exposure to solar radiation. Other studies have shown a positive association with polychlorobyphenil (PCB) serum levels, resulting from environmental and/or dietary exposure, and with occupational exposure to solvents. The "Gene-environment interaction study on lymphoma aetiology" includes the Italian groups that participated thus far individually to the Interlymph Consortium. Main objectives of the study are: 1. to extend the size of the Italian data base, currently including 2800 cases of lymphoma patients and approximately the same number of population and hospital controls; 2. to participate to the international genome-wide association study (GWAS), which could allow to identify conditions of individual hypersusceptibility to be addressed with preventive action, or to optimize the therapeutic approach, by assessing clinical response and long term survival associated with specific gene polymorphisms; 3. to

inquiry with consistent methodology and the required statistical power into aetiological hypothesis of national relevance, about the role of occupational and environmental factors, including military deployment in war areas, living in areas surrounding industrial or military settlements, agricultural use of pesticides, and diet, and their interaction with polymorphisms in genes involved in the metabolism of xenobiotics and in the synthesis of cytokines. Cytokines involved in the Th2 immune response might be of special interest; 4. to increase the specific weight of the Italian group in the International Consortium; and 5. to maximize internally the benefits deriving from such international collaboration, in terms of preventive, diagnostic, and therapeutic success.

P069

A POLYMORPHISM IN THE NFKB1 GENE PROMOTER INFLUENCES IL-6 LEVELS AND PROGNOSIS IN DIFFUSE LARGE B CELL LYMPHOMA

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Introduction. NF-κB signaling plays essential roles in lymphocyte development, activation, proliferation and survival, and aberrant signaling has been linked to lymphomagenesis. Genes activated by the NF-κB pathway include many pro-inflammatory cytokines, such as IL1B and IL6. IL-6 levels are higher in plasma samples of DLBCL patients at diagnosis with respect to healthy individuals, and elevated levels of IL-6 have been correlated to an inferior prognosis. The presence of a deletion/insertion polymorphism at the 94 ATTG promoter site (rs28362491) has been recently reported to modulate the transcriptional activity of the NF-κB1 gene and have been associated to an increased susceptibility to immune diseases and cancer. The aim of this study was to determine whether the NF-κB1 94 insertion/deletion ATTG polymorphism influences the biology and clinical characteristics of diffuse large B cell lymphoma (DLBCL). *Methods.* The 94 insertion/deletion ATTG NF-κB1 polymorphism was genotyped in 112 DLBCL patients by RFLP-PCR (Karban *et al*., Hum Mol Genet, 2004), whereas IL-6 and IL-10 plasma levels were assessed by standard ELISA assay. In particular, IL-6 plasma levels were quantified using an high-sensitive ELISA assay with a minimum detection limit of 0.001 ng/mL. *Results.* NF-κB1 -94 insertion/deletion genotype distribution in the DLBCL patients was in Hardy-Weinberg equilibrium: 47 pts showed the homozygous insertion genotype (wild-type "W"), while 47 and 18 patients were heterozygous or homozygous carriers of the variant deletion ("D") allele, respectively. In 48 of 93 patients, IL-6 levels were detectable in plasma samples at diagnosis. Moreover, carriers of the D allele had higher levels of IL-6 respect to the wild-type individuals (P=0.03). No difference for IL-10 levels according to the NF-κB1 genotype was observed. D allele and advanced stage of disease were independently associated with higher IL-6 levels (both P=0.04). Analyzing for associations with patient characteristics, we found that DLBCL patients with the NF-κB1 D allele had more frequently elevated LDH levels (P=0.02) and an IPI-score >2 (P=0.02). Carriers of the D allele had a significant worse progression-free survival (P=0.04), while there was a trend for worse overall survival (P=0.06). Including IPI as a covariate the NF-κB1 -91 insertion/deletion ATTG polymorphism retained its prognostic significance for progression-free survival (HR 3.18, 95% C.I. 1.19-8.46, P=0.02). *Conclusion.* Our data indicate that the NF-κB1 -94 insertion/deletion ATGG polymorphism impacts on IL-6 production and may modulate prognosis in DLBCL.

P070

REACTIVATION OF AN EPIGENETIC SILENCED TUMOR SUPPRESSOR GENE BIM IN ANAPLASTIC LARGE CELL LYMPHOMA LEADS TO MASSIVE APOPTOSIS

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Introduction. Bim is the major physiological antagonist of the prosurvival Bcl-2 proteins in B and T lymphocytes; it is also essential for the development of T cells, for the induction of apoptosis in activated T cells following an immune response and to prevent autoimmunity. *Methods.* In this study we analyzed the epigenetic status of Bim promoter, by Methylation Specific PCR (MSP) and by Chromatin Immunoprecipitation (ChIP) of acetylated Histone H3 tails, in 7 human neoplastic hematological cell lines, 3 NPM/ALK$^+$ Anaplastic Large Cell Lymophoma (ALCL) cell lines (KARPAS-299, SU-DHL-1, SUP-M2), and 4 NPM/ALK- cell lines (K562, LAMA-84-S, LAMA-84-R and NB-4) origin, lymph nodes from 6 patients affected by NPM/ALK$^+$ ALCLs, lymphocytes from 2 healthy donors. *Results.* Bim expression levels, assessed by Real-Time PCR and Western Blot, are lower in ALCL cell lines compared to NPM/ALK- cell lines (P=0.0054). Bim downregulation is correlated to: the presence of methylation of Bim 5'Untranscribed Region (5'UTR), assessed by clonal sequencing and MSP after bisulfite treatment; the deacetylation of histone tails and chromatin compaction, observed by ChIP. A higher level of methylation is detected in the NPM/ALK$^+$ cell lines lines and jn patients ALCL compared to NPM/ALK- (P<0.0001) and lymphocytes from healthy donors (P<0.0001). The demethylating agent 5-Azacytidine could restore promoter demethylation, promoting Bim expression and apoptosis after exposition. Similarly, treatment with the deacetylase inhibitor (HDACi), Trichostatin, was able to restore the acetylation of the histone tails, to upregulate Bim expression and to induce apoptosis. We assessed by Real-Time PCR that treatment of SUDHL1, with an Npm/Alk inhibitor (1.2 m). After 6 hours, upregulated Bim transcription of 4 fold was induced, causing ALCL cells death (27%). In contrast the inhibitor didn't'promote Bim upregulation and cells death in K562 cells (NPM/ALK$^-$). Similar results were obtained, in SUDHL1 cells through the inducible silencing of NPM/ALK. We analyzed, by ChIP, that the NPM/ALK inhibitor and NPM/ALK downmodulation, didn't modify the acetylation status of Bim promoter. Further experiments on Bim methylation status following NPM/ALK inhibition are ongoing. *Conclusions.*1. Bim epigenetic silencing is an important mechanism by which ALCL cells can escape the potent proapoptotic activity of Bim. 1. Bim 5' UTR methylation is detectable *in vitro* and *in vivo* including lymph nodes from patients affected by NPM/ALK$^+$ ALCLs. 2. Bim expression can be restored using demethylating agents, HDAC inhibitors, NPM/ALK downregulation and specific NPM/ALK inhibitors.

P071

EVALUATION OF INTRALESIONAL ADMINISTRATION OF RITUXIMAB FOR TREATMENT OF ORBITAL LYMPHOMA

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Introduction. Intralesional Rituximab has been developed for application to primary cutaneous marginal zone lymphoma; its employ in the orbit lesion has never been described before in literature. Localized lymphoma of the orbit are generally treated with chemotherapy, immunotherapy or combined chemoimmunoterapy. The intralesional injection of low doses of Rituximab could be effective and reduce the adverse effects. We evaluated the efficacy and toxicity of intralesional administration of rituximab for the treatment of patients affected by orbital B-cell NHL CD20$^+$. *Methods.* We enrolled five patients with orbital lymphomas CD20$^+$, CD5 negative non otherwise specified, detected by incisional biopsy of the lesion, limited to ocular and peri-ocular region. The lesion was localized in lachrymal glands in 2 patients, eyelid in one patients, and medium and posterior orbital space in one patient; all patients had unilateral lesions. They received intralesional injection of Rituximab 5 mg once a week for the first month (4 administrations); in case of partial remission of the intraorbital lesion, higher dosage of Rituximab, until 10 mg, was injected, for a maximum of other two cycles. In patients who obtained complete remission after first cycle, a second cycle at the same dose was planned. Response to therapy was assessed after every cycle. *Results.* Two patients obtained complete regression of the lesion respectively after 2 and 3 cycles; the first, with lachrymal gland localization, still maintains the response after 10 months of follow-up; the second, with eyelid involvement, showed continuous orbital response 7 months after treatment, but he developed abdominal localization of lymphoprolipherative disease and started systemic therapy. In two patients, after 3 cycles of escalating Rituximab dose, was obtained stable disease with regression of subjective symptoms (weeping and

pink eye). One patient, with medium and posterior orbital localization did not showed any response and underwent to systemic treatment. No patients experienced adverse drug reactions. *Conclusions.* Local treatment with Rituximab, in ocular lymphoma CD20 positive, seems to be a safe and useful procedure without the typical adverse effects due to chemo/radiotherapy. Now-a-day these preliminary encouraging results need of confirmation with more patients and a longer follow-up. Further studies would asses the optimal dosage in relation to the localization of lesion in particular in the setting of deep orbital lesions. Moreover the use of intralesional Rituximab does non precludes systemic future approaches.

P072

MANTLE CELL INTERNATIONAL PROGNOSTIC INDEX (MIPI) IS A GOOD SURVIVAL PREDICTOR IN MANTLE CELL LYMPHOMA (MCL) PATIENTS TREATED WITH RITUXIMAB AND CHEMOTHERAPY

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Introduction. The outcome of MCL is unfavourable, with continuous relapses. The MIPI, a clinical score, defined performance status, age, LDH and leucocyte counts as predictors of MCL outcome. Ki-67 as cell proliferation index was evaluated in biological-MIPI (MIPI-b). Aim of the study was to tested MIPI on a retrospective group of MCL patients treated with Rituximab-chemotherapy; secondary endpoints were: to evaluate the feasibility of MIPI-b on a retrospective population and to quantify the predictive discrimination of IPI, MIPI, MIPI-b on the outcome of MCL in the Rituximab era. Methods. Between 1999 and 2009, 136 MCL >18 years at diagnosis consecutively treated in five Italian institutions entered into the study. Histology was centrally reviewed and Ki-67 evaluation was performed. Overall Survival (OS) and failure-free survival (FFS) curves were estimated both overall and stratified by MIPI, MIPI-b and IPI score. Differences between curves were tested using the 2-tailed log-rank test. In order to quantify the predictive discrimination of MIPI, MIPI-b and IPI scores, a Cox's model analysis and univariate logistic models (with death and failure event as binary outcomes) were fitted and the area under the receiver operating characteristic (ROC) curves (c-index) was estimated in a subgroup of 84 patients that fulfilled MIPI, MIPI-b and IPI scores. *Results.* Clinical characteristics were: median age 62 (37-84) years, 78% stage IV, 73% with bone marrow involvement, 15% with blastoid variant; median leucocyte counts at diagnosis was 7.53×10^3 (2.38-175). First-line treatments were: Rituximab with high-dose chemotherapy in 35%, Rituximab-Fludarabine based chemo in 16%, R-CHOP in 37% and other Rituximab containing regimens in 12%. Ki-67 evaluation was performed in 93 patients. Patients at high-risk (HR) were 43 (32%) according to MIPI, 16 (12%) according to MIPI-b and 47 (35%) to IPI. With a median follow-up of 28 months, 2-year Overall Survival OS was 77% (95% CI:68-84%) and 2-year FFS was 60% (95%CI: 50-69%). Two-year OS according to MIPI by risk groups was: LR 95%, IR 88%, HR 51% and according to IPI: LR 88%, IR 91%, HR 60%. In the subgroup of 84 patients that fulfilled MIPI, MIPI-b and IP scores an univariate logistic model and a Cox's model analysis were fitted. The c-index and Cox-index for death event were 73% and 77% for MIPI, 72% and 73% for MIPIb, 69% and 65% for IPI respectively; the c-index and Cox-index for failure event were 66% and 72% for MIPI, 66% and 69% for MIPIb, 66% and 64% for IPI respectively. *Conclusions.* MIPI score was confirmed as a good predictor of death event in MCL retrospective patients treated with Rituximab-chemotherapy regimens. MIPI score should predict outcome better than MIPI-b and IPI in a retrospective analysis. MIPI and MIPI-b score superiority on standard IPI should be demonstrated in prospective trials. New therapeutic strategies are warranted to improve the outcome of MCL namely in MIPI-HR group.

P073

THE ASSOCIATION OF BORTEZOMIB AND RITUXIMAB IS EFFECTIVE AND SAFE IN RELAPSED/REFRACTORY INDOLENT NON-FOLLICULAR AND MANTLE CELL LYMPHOMA: FINAL RESULTS OF PHASE II STUDY BRIL-06 BY INTERGRUPPO ITALIANO LINFOMI

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Introduction. Bortezomib (B) alone or in combination with Rituximab (R) has shown clinical benefit in treatment of Mantle Cell Lymphoma (MCL) and Marginal Zone Lymphoma (MZL). Aim of the study was to evaluate feasibility of R and B combination in relapsed/refractory indolent non-follicular lymphoma (linfocytic lymphoma, LL, or MZL) and MCL not eligible to high-dose chemotherapy. *Patients and methods.* The study was a phase II multicenter trial according to Simon's design. Inclusion criteria were: age 18-75 years, histological proven relapsed or refractory LL, MZL and MCL after 1-4 lines of therapies. Treatment plan was: one course of four weekly intravenous bolus of 1.6 mg/sqm B in combination with four infusion of 375 mg/sqm R followed by two courses of four weekly bolus of 1.6 mg^2 B. Patients with complete (CR), partial remission (PR) and stable disease at the intermediate evaluation were planned to be given three further courses with the same schedule. *Results.* From September 2006 to March 2008, 55 patients entered into the study. Central histology revision was performed. Forty-nine patients fulfilled inclusion criteria and were evaluable. Clinical characteristics were: median age 68 (50-74) years; 16 LL, eight MZL, 25 MCL; 42 stage III/IV; 33 bone marrow involvement; 20 at intermediate-high/high IPI risk. Thirty-eigh patients performed > two prior lines of chemotherapy; 34 were R-pretreated; 21 refractory and 28 relapsed disease. Overall Response Rate (ORR) was 53% (CR 26.5%, PR 26.5%); no response 43% and 4% off therapy for other causes. ORR by histology was: 37% in LL, 50% in MZL and 64% in MCL. ORR was not adversely affected by R pretreatment: R-pretreated 62% and R-naïve 33%. ORR was higher in relapsed patients compared with refractory ones: 64% and 38% (p .06). With a median follow-up of one year, OS was 89% (95%CI: 75-95) and 1-year PFS was 45% (95%CI: 30-58). One-year PFS was 50% for MZL and MCL and 37% for LL (Figure 1). A total of 233 courses were delivered with a median of 4.7 courses/patient. Thirty patients completed the treatment plan; 19 did not because of progression disease in 13, adverse events in five (concomitant gastric neoplasia, neurotoxicity grade II, sepsis, pleural effusion and toxic death due to interstitial pneumonia). Grade 3-4 CTC haematological toxicity was rare: neutropenia in 5% of the courses and thrombocytopenia in <2%. Grade 3-4 CTC cumulative non-hematological toxicity was observed in 4.7% of all courses. The most frequent non-hematological toxicities were: neurotoxicity grade III in four patients, with complete recover or return to grade I in all of them. Infections were observed in eight patient with ten events: viral reactivation in four, pneumonia in three, sepsis in one and micosis in two. *Conclusions.* The combination of R and B in weekly schedule was effective and safe in treatment of relapsed/refractory indolent and MCL. PFS was promising also in R-pretreated patients and mainly in MZL and MCL.

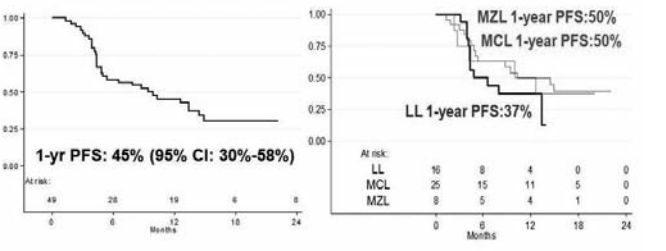

Figure 1.

Multiple Myeloma I

P074

THE DIAGNOSTIC ROLE OF MULTIPARAMETER IMMUNOPHENOTYPING BY FLOW CYTOMETRY IN MULTIPLE MYELOMA: A NEW MODEL

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Background. Multiparameter flow cytometry (FC) represents an attractive approach in the detection of abnormal plasma cells (aPC) in Multiple Myeloma (MM) due to its capacity to combine an examination of both immunophenotype and clonality. Due to the large numbers of cells amenable to analysis by FC, it may be additionally useful in the detection of minimal residual disease (MRD). Problems with such evaluation of PC include those related to the frequent hemo-dilution of bone marrow aspirates (BMA) with peripheral blood (PB) as well as the liability of PC stored outside of the body. The histologic examination of BM remains the gold standard in the diagnosis of MM. We have developed a new statistical diagnostic model that examines what correlation exists between the immunophenotype and clonality detected by FC and histology, defining the diagnostic role of FC in MM. *Methods.* 130 bone marrow samples, from patients and controls, were enrolled in a study for routine diagnostic analysis of MM; a minimum of 100 PC were analyzed for each patient sample. A direct 7-8-color method was applied to study the immunophenotype of PC, utilizing a BD FACSCanto II. Samples were labelled with fluorochrome-conjugated monoclonal antibodies (PacificBlue-FITC-PE-PerCP-APC-PeCy7-PacificBlue-APC-Cy7) to the following antigens: CD138, CD81, CD200, CD221, CD45, CD38, CD28, CD19, CD27, CD117, CD38, CD33, CD20, CD56, CD10, immunoglobulin and light chains. *Results.* CD19 and CD27 expression, when applied to our model, resulted in optimal concordance with histology. *Conclusions.* This statistical model showed a correlation between FC and histology. It represents a new objective and reproducible way to interpret the immunophenotype of PC and correlates this analysis with histological results. Our goal is to use this information to consolidate this model and test its applicability on a larger scale.

P075

ACHIEVEMENT OF COMPLETE REMISSION IS A STRONG PROGNOSTIC FACTOR IN ELDERLY MYELOMA PATIENTS TREATED WITH MELPHALAN-PREDNISONE BASED-REGIMENS: RESULTS OF THE GISMM2001, RV-MM-PI-026 AND GIMEMA-MM-03-05 MULTICENTER ITALIAN TRIALS

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Background. Several studies, mainly in the field of autologous stem cell transplantation, have shown that a better tumor reduction significantly correlates with longer overall survival (OS) or at least progression-free survival (PFS) in multiple myeloma (MM) patients. Complete remission (CR) was a rare event since new drugs have been added to standard melphalan-prednisone (MP). *Aim.* To validate the prognostic value of CR in elderly newly diagnosed MM patients treated with MP based-regimens. *Methods.* 895 patients ≥65 years (or younger but ineligible for high-dose chemotherapy) with newly diagnosed MM, enrolled in the GISMM-2001, RV-MM-PI-026 and MM0305 multicenter Italian trials were included. Patients received MP (n=164), MP plus thalidomide (MPT, n=167), MP plus lenalidomide (MPR, n=53), MP plus bortezomib (VMP, n=257) or MP plus bortezomib and thalidomide (VMPT-VT, n=254). Median cycles number administered was 6 (range 1-9). Best response assessment was available in 847 patients. *Results.* CR was achieved in 195 patients, very good partial response (VGPR) in 171 patients and partial response (PR) in 297 patients. After a median follow-up of 51 months, 4-year PFS was 62% in patients who achieved CR, 28% in those who obtained VGPR, and 28% in patients who achieved partial response (PR) only (P<0.0001). Four-year OS was considerably higher in patients achieving CR compared to VGPR or PR (79% versus 52% *vs.* 54%, P<0.0001). At a landmark analysis performed at 6 months after enrolment (n=644) PFS and OS confirmed significantly better in patients who obtained CR compared to patients with VGPR or PR (P<0.0001). Subgroup analysis according to age, International Staging System (ISS) stage and treatment regimen showed that the benefit of obtaining CR on PFS was confirmed in patients younger than 75 years (P<0.0001) and older (P<0.0001); in patients with ISS stage I (P=0.018), stage II (P<0.0001) and stage III (P<0.0001); and in patients receiving bortezomib-based regimen (VMP, P=0.003) or immunomodulatory agents (MPT and MPR, P=0.002) or bortezomib plus immunomodulatory agents (VMPT-VT, P=0.0004). In addiction, CR achievement was correlated with a significant increase in OS in patients younger (P=0.001) and older than 75 years (P=0.015); in those with ISS stage II (P=0.003) and III (P=0.006); in patients treated with MPT and MPR (P=0.019) and with VMPT-VT (P=0.019). *Conclusions.* In elderly MM patients treated with MP-based regimens, achievement of CR is a strong prognostic factor regardless of age, ISS stage and treatment regimen.

P076

NON CANONICAL WNT SIGNAL PATHWAY (WNT5A/ROR2) ACTIVATION STIMULATES THE OSTEOGENIC DIFFERENTIATION PROCESS OF BONE MARROW MESENCHYMAL CELLS BEING A POTENTIAL TARGET IN MULTIPLE MYELOMA BONE DISEASE

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Canonical Wnt signal pathway is critical in the regulation of bone formation process and its activation in osteoblastic cells improves bone mass in multiple myeloma (MM) mouse models. Together to canonical Wnt signaling, a non-canonical Wnt pathway, independently to β-catenin activation, has been identified. Non-canonical Wnt signaling (Wnt5a, Wnt1/11) is transduced through FZD receptor and Ror2 co-receptor to several cascades as Disheveled pathways involving Rho family small GTPase (ROCK), RAC-JNK pathway and Ca^{++} dependent pathways /PKC involving the nuclear factor of activated T cells (NFAT). Interestingly, recent evidences suggest that non-canonical Wnt pathway activation by Wnt5a or Wnt4, rather than canonical one by Wnt3a, stimulates the osteogenic properties of human mesenchymal cells hMSC through Ror2 activation. In this study the effect of MM cells on the non-canonical Wnt pathway in hMSC and osteoprogenitor cells (PreOB). The effect of MM cells on non-canonical Wnt pathway as well as the role of the activation of this pathway in hMSC on the osteogenic differ-

entiation impairment induced by MM cells are not known and have been investigated in the present study. We performed a series of co-culture between PreOB and MM cells using either the human myeloma cell lines (JJN3, XG-1, XG-6, KMS12, KMS27) or purified $CD138^+$ cells obtained from MM patients finding that MM cells inhibit Ror2 protein expression by PreOB and consistently the activity of NFATc1 at nuclear level. Following activation of non-canonical Wnt signaling pathway either by Wnt5a treatment or by the induction of both Wnt5a and Ror2 overexpression by lentivirus vectors have been performed in primary hMSC obtained from MM patients. The efficiency of Wnt5a and Ror2 trasduction was checked by GFP expression using flow cytometry whereas the efficacy was evaluated by the level of Wnt5a and Ror2 mRNA and protein expression as well as by the intracytoplasmatic increase of Ca^{++} influx, phospho-PKC expression and NFATc1 activity. We found that Wnt5a treatment as well as Wnt5a or Ror2 overexpression significantly increased osteogenic differentiation and the expression of alkaline phosphatase in hMSC. Consistently, in the co-culture system with MM cells, Wnt5a and Ror2 overexpression by hMSC blunted the inhibitory effect of MM cells on alkaline phosphatase expression and osteogenic differentiation. Finally, these observations were further confirmed showing that Wnt5a or Ror2 silencing in PreOB by siRNA or shRNA trasfection, respectively inhibited the expression of osteogenic markers alkaline phosphatase, osteocalcin and collagen I. In conclusion our data indicate that activation of non-canonical Wnt5a/Ror2/Ca^{++} signal pathway in hMSC increases osteogenic differentiation and counterbalance the inhibitory effect of MM cell suggesting that this pathway could represent a potential target in MM microenvironment.

P077

LENALIDOMIDE AS CONSOLIDATION/MAINTENANCE THERAPY IN MULTIPLE MYELOMA PATIENTS

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Introduction. Lenalidomide is a new immunomodulatory drug with a potent biologic activity. It represents an important treatment option for multiple myeloma patient either as first line therapy, either in resistant/refractory disease or consolidation/maintenance therapy. In this way Lenalidomide increases the available treatment options. *Methods.* According to several studies which evaluated lenalidomide in the treatment of multiple myeloma, in our department, lenalidomide was administered in resistant/relapsing myeloma patients and as consolidation/maintenance therapy in elderly myeloma patients with stable disease (partial remission) after induction therapy or more lines of chemotherapy. We treated 20 patients (11M and 9F) with median age of 70 years (range 66-80). Patients completed almost 12 months of therapy with variable doses of Lenalidomide (5-25 mg/die p.o., according to tolerability of each patient, for 21 days every 28 days), in association of very low doses of dexametasone (10 mg/die p.o. days 1,2,3,4) or alone. We used Enoxaparin for prophylaxis of venous thromboembolisms. Clinical restaging was performed after three, six and twelve months, in course of therapy. *Results.* At the present we didn't observe any progression of disease and in 15 cases we observed a good impact on monoclonal component. In all patients the therapy was well tolerated and were not found significant adverse events. *Conclusions.* A role exists for lenalidomide with dexametasone or alone for consolidation/maintenance therapy in previously treated elderly myeloma patients. This therapy seems to lead an improvement in prognosis of these patients, without causing severe complications.

P078

BORTEZOMIB-MELPHALAN-PREDNISONE-THALIDOMIDE FOLLOWED BY CONTINUOUS BORTEZOMIB-THALIDOMIDE FOR INITIAL THERAPY OF ELDERLY MULTIPLE MYELOMA PATIENTS: A PROSPECTIVE RANDOMIZED TRIAL

Bringhen S,[1] Rossi D,[2] Cavalli M,[3] Ria R,[4] Offidani M,[5] Patriarca F,[6] Nozzoli C,[7] Levi A,[8] Guglielmelli T,[9] Benevolo G,[10] Morabito F,[11] Grasso M,[12] Musolino C,[13] Cangialosi C,[14] De Rosa L,[15] Liberati AM,[16] Falcone A,[17] Gottardi D,[18] Crippa C,[19] Montefusco V,[20] Zambello R,[21] Lupo B,[1] Musto P,[22] Petrucci MT,[8] Larocca A,[1] Omedè P,[1] Gaidano G,[2] Boccadoro M,[1] Palumbo A[1]

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Introduction. In newly diagnosed multiple myeloma patients bortezomib-melphalan-prednisone (VMP) and melphalan-prednisone-thalidomide (MPT) are now regarded as the new standards of care. *Methods.* Between May, 2005 and January, 2009, 511 patients aged ≥ 65 years were enrolled in a phase III clincal study and were randomized to receive VMPT-VT (N=254) or VMP (N=257). In the VMPT-VT arm patients received bortezomib 1.3 mg/m^2 days 1,4,8,11,22,25,29,32 in cycles 1-4 and days 1,8,22,29 in cycles 5-9; melphalan 9 mg/m^2 plus prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42 for nine 6-week cycles, followed by VT therapy (bortezomib 1.3 mg/m^2 days 1, 15, thalidomide 50 mg/day). In the VMP arm, patients received the same schedule without maintenance. To evaluate if the treatment regimen could be further optimized by decreasing the toxicity while maintaining efficacy, in March 2007 the protocol was amended and both bortezomib schedules were reduced to once-weekly infusion. *Results.* 503 patients were evaluable: 250 were assigned to receive VMPT-VT and 253 to VMP. Both arms were well balanced for baseline characteristics. Response rates with VMPT-VT and with VMP were: ≥VGPR rate of 59% *vs.* 50% (P=.03) and CR of 38% *vs.* 24% (P<.001), respectively. VT therapy increased response rate in 11% of evaluable patients. After a median follow-up of 23.4 months, the 3-year PFS was 56% and 41% (P=.008), and the 3-year overall survival (OS) was 89% and 87% (P=.77) in the VMPT-VT group and in the VMP group, respectively. VMPT-VT induced a higher incidence of grade 3-4 neutropenia (38% *vs.* 28%, P=.02) and cardiac complications (10% *vs.* 5%, P=.04); the incidence of grade 3-4 peripheral neuropathy (PN) was 8% in VMPT and 5% in VMP (P=.19). During maintenance therapy with VT, the most relevant toxicity was PN (4%), other serious adverse events were less than 1.5%. 133 patients were older than 75 years, response rates and PFS were similar in VMPT-VT (65 patients) and VMP patients (68 patients): ≥VGPR was 49% *vs.* 41% (P=.35) and CR 31% *vs.* 29% (P=.72), respectively; the 2-year PFS was 56% in the VMPT-VT and 53% in the VMP group (P=.49). In patients ≥75 years, the incidence of non-hematologic events (59% *vs.* 31%, P=.001) cardiologic events (18% *vs.* 4%, P=.01), and treatment discontinuation for toxicity (31% *vs.* 16%, P=.04) were higher for VMPT-VT as compared to VMP. *Conclusion.*

These results show that: 1. VMPT-VT was significantly superior to VMP; 2. cardiologic toxicity may reduce the efficacy of the VMPT-VT regimen in elderly frail patients.

Table.

	VMPT-VT (n = 250)	VMP (n = 253)	P (VMPT-VT vs VMP)
CR	38%	24%	<.001
≥ VGPR	59%	50%	.03
3-year PFS	56%	41%	.008
3-year OS	89%	87%	.77

P079

A MINIATURIZED FISH APPROACH (MICROFINDTM) FOR DETECTION OF GENETIC LESIONS FROM SCARCE CELL SAMPLES: APPLICATION TO MULTIPLE MYELOMA

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Introduction. Despite the improvement in sensitivity for the identification of chromosomal aberrations by fluorescence based methods and automated analysis (Ntouroupi *et al*. Br J Cancer 2008; Medintz *et al*. Int J Nanomedicine 2008), a low cell number recovery may make it difficult to perform FISH detection of rearrangements involved in cancer cells. Multiple myeloma (MM), a clonal B-cell malignancy characterized by the accumulation of terminally differentiated bone marrow plasma cells (PC), represents a peculiar example because of the low infiltrate of malignant cells found in a large fraction of patients. Therefore, the manipulation and the analytical processing of PCs still represents a technological challenge and FISH detection requires purification which generally provides a limited number of PCs. *Methods.* To overcome the difficulty, we have used a novel device (microFINDTM) and tested it for FISH analyses by collecting living cells of bone marrow aspirates from MM patients. microFIND is a glass slide coated with a nanomaterial (Carbone *et al*. Biomaterials 2006) that efficiently immobilizes cells (down to 4×103) inside a microchannel: a miniaturized FISH protocol (using one tenth of reagent employed in the standard protocol) is carried out and the slide evaluated by high resolution fluorescence microscopy. PCs were purified from MM bone marrow samples of 15 different patients using CD138 immunomagnetic microbeads (MidiMACS system) as previously described (Fabris *et al*. Genes Chromosomes Cancer 2005). Genetic lesions such as del(13q14), del(17p13) and the t(4;14)(p16;q32) were evaluated using commercially available kits (Abbott Chicago, IL) *Results.* When FISH results by microFINDTM and standard protocols were compared , we found a complete concordance for all tests in all cases studied. Conclusions: mincroFINDTM may represent a promising useful tool for the FISH analysis of genomic alterations in myeloma purified plasma cells even in case of a very limited number of available cells. Furthermore the miniaturization of the assay offers a cost saving approach, suitable for automation and throughput increase in a "chip" configuration.

P080

IN VITRO PRECLINICAL STUDIES OF COMBINATIONS AND SEQUENCES OF OLD AND NEW GENERATION DRUGS USED IN MULTIPLE MYELOMA THERAPY

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Introduction. The treatment for most MM patients today includes chemotherapy with standard drugs in combination with new generation drugs that have anti-angiogenic and immunomodulatory properties (Thalidomide and Lenalidomide) or are able to inhibit the proteasome (Bortezomib). Each of these new drugs has multiple mechanisms of action, targeting both intracellular signalling pathways and the tumour micro-environment. Preliminary studies advanced the hypothesis that the sequence of administration of older drugs and novel drugs is important to improve final therapeutic response. The purpose of this study was to investigate *in vitro* their optimal sequences and combinations in presence or absence of bone marrow stromal cells (BMSC). *Methods.* We utilized RPMI 8226 (IL-6 independent) and U266 (IL-6 dependent) human MM cells lines. BMSC were obtained from healthy donors. Agents tested included Melphalan (1-10 M), Bortezomib (1-10 nM) and Lenalidomide (0.5-2 M). Drugs were administered alone, in simultaneous combination or sequentially. Incubation time was 120 hours, cytotoxicity was assessed by MTT cell viability assay. Statistical significance was examined by 2-way analysis of variance, in all analyses $P<0.05$ was considered statistically significant. *Results.* In RPMI 8226 cells the administration of Lenalidomide followed by Melphalan after 24 hours had a greater inhibitory effect than Melphalan alone, than concomitant administration of both drugs and than reverse combination of Lenalidomide followed by Melphalan ($P<0.05$ in all cases). Similar results were obtained for U266 cells when Melphalan was administrated with Bortezomib. The ordered sequential combination of Melphalan followed 24 hours later by Bortezomib was more inhibitory than Melphalan alone, than simultaneous combination of both drugs and than reverse combination of Bortezomib followed by Melphalan ($P<0.05$ in all cases). In RPMI 8226 cells co-cultured with BMSC, the administration of Melphalan followed by Bortezomib after 24 hours increased inhibition compared to Melphalan alone and to the simultaneous combination of the two drugs ($P<0.05$ in all cases). When U266 were cell co-cultured with BMSC no statistically significant results were obtained between the different combinations of administration. *Conclusions.* Our preclinical *in vitro* findings suggest that the sequential combination of a standard drug followed 24 hours later by a new drug are more favorable in terms of inhibition of tumor cell growth than simultaneous or reverse combination.

P081

DEREGULATION OF MIR-221/222 CLUSTER AFFECTS P27 EXPRESSION IN MULTIPLE MYELOMA

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Introduction. Recent data indicate a deregulation of microRNA (miRNA) in multiple myeloma (MM). In particular, we identified several miRNAs differentially expressed between patients belonging to distinct TC (Translocation/Cyclin) groups and thus potentially contributing to the heterogeneity of the disease. Among these, oncogenic miRs miR-221 and miR-222, encoded in tandem on the X chromosome, are also up-regulated in many types of human neoplasms and thought to facilitate cell proliferation via downregulation of p27 and/or p57 which negatively regulate the cell cycle progression from G1 to S phase. At the moment, the role of the miR-221/222 cluster in MM cells has not been elucidated. *Methods.* Human myeloma cell lines U266 and KMM1 were transfected respectively with 30nM pre- and 50nM anti-miRNAs (Ambion) using the Nucleofector technology (Amaxa Biosystems). Nontargeting pre- and anti-miRNAs were used as negative controls. Cells were collected and processed for quantitative real time PCR (Q-RT-PCR) (TaqMan miRNA assays, Applied Biosystems), immunoblotting (WB) and cell cycle analyses at different time points after nucleofection (24, 48, 72 and 96h). Cell cycle distribution was analyzed by flow cytometry after propidium iodide staining. *Results.* To investigate the role of miR-221/222 cluster in MM, we overexpressed miR-221 and miR-222 in the U266 cell line, that constitutively expresses low levels of these two miRNAs; their expression in transfected cells was enormously higher as compared to negative control at all time points analyzed by Q-RT-PCR. At the same time points no change in cell number was observed in comparison to negative control. Interestingly, cell cycle analysis revealed that transfected U266 cells exhibited an increase in the percentage of cells in S phase, starting from 48h, becoming more marked at 72h and decreasing at 96h. The same increase was observed in sub-G0/G1 populations, suggesting that entry into S phase promoted by high exogenous levels of miR-221 and miR-222 under conditions (such as after electroporation) that are not permissive for normal mitotic division is followed by apoptotic cell death. As miR-221/222 negatively regulate p27 expression in different cell types, we verified if this modulation could occur in our experimental model. WB analysis of whole cell lysate showed a pronounced reduction of p27 protein expression in miR-221 and/or miR-222-transfected cells: based on densitometric analysis p27 levels were more than 90%

lower than controls at 48 and 72h, beginning to raise towards basal levels at 96h. As a complementary approach, we blocked miR-221/222 activity in KMM1 cells, observing a significant up-regulation of p27 expression. *Conclusions.* Our data indicate that the miR-221/222 cluster modulates the expression of p27 protein in MM cells. Such a regulation appears to be crucial for promoting cell cycle entry into S phase.

P082

CHARACTERIZATION OF B CELLS (CD19+), PLASMA CELLS (CD38+) (IGD-CD27-) DOUBLE-NEGATIVE (DN) IN PATIENTS WITH MULTIPLE MYELOMA (MM) AND MONOCLONAL GAMMOPATHIES OF UNDETERMINED SIGNIFICANCE (MGUS) IN THE BONE MARROW

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Introduction. In this paper, we describe the (DN) immunoglobulin IgD-CD27- B cells and plasma cells in bone marrow in patients with MM and MGUS. We have studied the expression of CD27, which is involved in B cell differentiation until antibody-secreting plasma cells. We hypothesize that DN cells are memory cells late developed, or who have exhausted the down-modulated expression of CD27 and filled immunological space in such patients. *Methods.* We studied 15 patients with MM and 5 patients with MGUS. Phenotypic study of membrane and intracytoplasmic Ig was carried out by labeling with specific monoclonal antibodies, the following panel was used in all the case: IgG Fitc / IgD Pe / CD19 PE-Cy5 / CD38 PE-Cy7 / CD45 Pacific Blue / CD27-Apc Cy7. Samples were acquired with Cyan ADP TM (Beckman Coulter), cytometer and analyzed with analysis software Kaluza 1.0TM (Beckman Coulter). We have been able to identify four different populations $CD19^+$ cells (see Table 1). *Results.* From analysis prompt out the reduction of B naïve cells $IgD^+CD27^-CD19^+$ and a significative increase of DN IgD-$CD27^-$ in $CD38^+$ and in $CD19^+$ cells, both. The DN population in $CD19^+$ cells is more expressed in MGUS patients (17%) than in MM patients (12%); in $CD38^+$ cells DN is higher in patients with MM (40%) than patients with MGUS (9%). In MGUS patients intracytoplasmic Ig (cIg) of DN in $CD19^+$ cells is (64%) while in MM patients is (32%); cIg in $CD38^+$ cells is greater in MM patients (34 %) than patients with MGUS (12.85%). *Conclusions.* Studying the DN population, we found overlapping features with those of classical memory B cells (IgD-$CD27^+$), ie the presence of IgG and intracytoplasmic membrane (index case isotypic switch.) The hypothesis is that these cells are B lymphocytes "late memory" who lost their functional capacity, which have down modulated the expression of CD27 and playing a role in the immunological space of patients with MM and MGUS. It is suggested that $CD19^+$ and $CD38^+$ cells DN, so expanded in such patients, could be the result of prolonged stimulation or, alternatively, caused by a dysregulation of the immune system.

Table 1. $CD19^+$ subpopulation.

IgD+ CD27-	virgin B cells
IgD+CD27+	B cell memory unswitched
IgD- CD27+	B cell memory switched
IgD- CD27-	Late memory B cells (switched isotypic)

P083

OSTEOCLAST FORMATION IN MULTIPLE MYELOMA BONE DISEASE PATIENTS: ROLE OF DCR3

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Decoy Receptor 3 (DcR3), a member of the TNF receptor superfamily, overexpressed mainly in tumor cells, is known to be involved in the differentiation of osteoclasts (OCs). In this study we show that human myeloma cell lines (HMCLs), malignant plasma cells and T lymphocytes from multiple myeloma (MM) bone disease patients produce DcR3. We also demonstrate that DcR3 supports the OC formation in peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMNCs) of MM patients. This finding comes from experiments of osteoclastogenesis performed in the presence of increasing concentration of neutralizing anti-DcR3 antibodies (mAb) in both culture systems, in which we found a dose-dependent inhibition of OC formation. In particular, anti-DcR3 mAb totally inhibits OC formation in PBMCs, while in BMMNCs a residual osteoclastogenesis still persists. To demonstrate that the effect of DcR3 on OC formation was mediated by the modulation of osteoclastogenic cytokines, we analyzed the mRNA and protein levels of RANKL and TNF-α expression in PBMCs treated or not with anti-DcR3 mAb. We found that the antibody exerts a dose-dependent inhibitory effect on the expression of both osteoclastogenic cytokines. To better characterize this effect, by Real-time PCR and western blot we showed that DcR3 upregulated the basal levels of TNF-α in both T and $CD14^+$ cells, while RANKL expression was upregulated only in T cells. Moreover, we demonstrated that DcR3 stimulated osteoclastogenesis of $CD14^+$ cells in a dose-dependent manner and, interestingly, this action was abrogated by TNFR1-Fc but not by RANK-Fc. In conclusion, our data provide the evidence of the expression of DcR3 in MM, and the involvement of this molecule in supporting the formation of OCs from MM bone disease patients through the up-regulation of RANKL and TNF-α expression.

P084

ORAL REVLIMID AND PREDNISONE FOLLOWED BY ORAL REVLIMID, MELPHALAN AND PREDNISONE IN NEWLY DIAGNOSED ELDERLY MULTIPLE MYELOMA PATIENTS: A MULTICENTER, OPEN LABEL STUDY

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Introduction. The combination Melphalan-Prednisone-Lenalidomide (MPR) has shown promising acitivity . In the transplant setting, low-dose chemotherapy (induction) precedes high-dose chemotherapy (autologous transplantation consolidation). This approach reduces tumor mass, with few side effects, before achieving the maximum cyto-reduction with autologous transplantation. Elderly patients receive a similar approach, which consists ofinduction with lenalidomide plus corticosteroids and subsequent consolidation with MPR. This study aims to evaluate the safety and efficacy of Lenalidomide-Prednisone (RP) as induction, followed by MPR) as consolidation in elderly myeloma patients. *Methods.* A two-stage phase II trial was planned according to Bryant and Day method. Unfit patients with newly diagnosed symptomatic myeloma older than 65 years were enrolled. No exclusion criteria were included in the protocol to avoid the selection of fit elderly subjects. Patients with low blood count, abnormal performance status, hepatic, renal, cardiac or pulmonary functions were enrolled. The treatment consists of: 4 RP courses (Lenalidomide 25 mg/day for 21 days every 4 weeks, plus Prednisone 50 mg 3 times/week continuously) followed by 6 MPR cycles (Melphalan 2 mg and Prednisone 50 mg 3 times/week continuously, plus Lenalidomide 10-15 mg/day for 21 days every 4 weeks) and maintenance with Lenalidomide (10 mg/day for 21 days every 4 weeks) and Prednisone (50 mg 3 three times/week continuously) until PD . Two different dose-levels of Lenalidomide were tested in combination with MP: 15 mg (dose-level 1) and 10 mg (dose-level 2). Each cohort included 12 patients, with additional 22 patients enrolled at dose-level 2. Patients were evaluated for efficacy and toxicity after completion of at least 2 consolidation cycles. *Results.* Forty-six patients (median age 75, range 65-88) were enrolled. After a median of 7 cycles and a median follow-up of 11.3 months, 24 patients were evaluable. Neutropenia (25%), anemia (20%), thrombocytopenia (8%) were the most common grade 3-4 hematological side effects during RP induction, wheras during MPR consolidation, the most frequent adverse events were neutropenia (42%), and thrombocytopenia (4%). The incidence of neutropenia was increased by the addition of melphalan, while both thrombocytopenia and anemia were reduced. Non-hematological toxicities were more frequent during RP cycles and reduced during MPR cycles (cutaneous rash and infections).

After RP induction, at least partial response (PR) rate was 62.5%, at least very good partial response (VGPR) was 17%. After 2 MPR cycles, PR rate increase to 67% , including 25% of at least VGPR in dose-level 1 and 33% at least VGPR in dose-level 2. *Conclusions.* RP induction followed by consolidation with MPR reduced the risk of haematological toxicity and increased the quality of response in unfit elderly multiple myeloma patients. These data will be updated at the meeting.

Table.

	RP	MPR dose level-1	MPR dose-level 2
Number of patients	24	12	12
Hematological toxicity			
Neutropenia	25 %	42 %	42 %
Thrombocytopenia	8 %	8 %	0 %
Anemia	21 %	0 %	0 %
G-CSF administration	8 %	17 %	17 %
Non-hematological toxicity			
Cutaneous rash	17 %	8 %	0 %
Infections	12.5 %	8 %	0 %
DVT	0 %	0 %	0 %
Response Rate			
> PR	62.5 %	67 %	67 %
> VGPR	17 %	25 %	33 %

Multiple Myeloma II

P085

EVALUATION OF THE EFFICACY AND SAFETY OF WEEKLY INFUSION BORTEZOMIB IN ELDERLY PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA

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Introduction. Peripheral neuropathy (PN), thrombocytopenia and gastrointestinal dysfunction are the most common adverse events with bortezomib-containing therapies. Bortezomib-related PN is a common complication (>G3 22%), especially in elderly patients. it is usually dose-related and reversible with treatment interruption and dose modifications. In this phase III randomized trial of bortezomib-melphalan-prednisone-thalidomide followed by continuous treatment with bortezomib-thalidomide (VMPT-VT) *vs.* bortezomib-melphalan-prednisone (VMP), we evaluate efficacy, safety and the risk factors of bortezomib-related side effects, in particular PN, in elderly newly diagnosed myeloma patients treated with different bortezomib schedules. *Methods.* From May, 2005 to January, 2009, 511 patients >65 years were randomly assigned to VMPT-VT or VMP. VMPT-VT patients received: bortezomib 1.3 mg/m^2 days 1,4,8,11,22,25,29,32 in cycles 1-4 and days 1,8,22,29 in cycles 5-9; melphalan 9 mg/m^2 plus prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42 for nine-6-week cycles, followed by VT (bortezomib 1.3 mg/m^2 days 1, 15, thalidomide 50 mg/day). VMP patients received the same schedule with no maintenance In March 2007 the protocol was amended, bortezomib was given once-weekly in both arms to evaluate if the treatment regimens could be further optimized by decreasing toxicity. *Results.* 503 patients were evaluable for safety and efficacy, 369 in the once-weekly and 134 in the twice-weekly groups. Response rates were similar in the two groups: ≥PR was 85% *vs.* 86% (P=.73), with a VGPR rate of 55% *vs.* 54% (P=.84) and CR of 29% *vs.* 35% (P=.23), respectively. Nodifferences in the 3-y PFS (50% *vs.* 47%, P=.99) and 3-y OS (88% *vs.* 89%, P=.54) were detected between once-weekly and twice-weekly patients. Non-hematologic grade 3-4 adverse events were 36% in the once-weekly group and 51% in the twice-weekly group. The reduction was significant (P=.003) and was mainly related to grade 3-4 sensory PN that considerably decreased from 16% to 3% (P<.001), with no significant difference between VMPT-VT and VMP groups. The only predictive factor, in multivariate analysis,of lower incidence of PN (P<.0001) was the reduction of bortezomib infu-

sion from twice to once weekly. 5% of patients in the once-weekly and 15% in the twice-weekly group (P<.0001) required treatment interruption for PN . 24% and 54% of patients (P<.0001) respectively required bortezomib dose reduction. This improvement in discontinuation and dose reduction rate prolonged the time on therapy: the cumulative delivered bortezomib dose compared with planned dose was 40.0 mg/m^2 (46.8 mg/m^2) in once-weekly and 41.0 mg/m^2 (67.6 mg/m^2) in twice-weekly patients. *Conclusion.* Weekly infusion of bortezomib decreased the rate of severe PN, discontinuation, dose reduction, prolonging the time on therapy. This unprecedented safety improvement came with no substantial efficacy reduction.

P086

MICROARRAY BASED GENOME SCREEN FOR GENES RESPONDING TO METHYLATRANSFERASE AND HISTONE DEACETYLASE INHIBITION IN MULTIPLE MYELOMA CELL LINES

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Introduction. Gene expression profiling is a powerful tool to better understand the biology of multiple myeloma. Several genetic aberrations are demonstrated to be involved in the pathogenesis of MM; moreover, in the last few years there is evidence that epigenetic aberrations play an important role in the development and progression of MM. In our study we used a microarray based genome screen to analyze global changes in gene expression profiles of MM cell lines, responding to demethylating drug 5Aza-2 deoxycytidine (5Aza-dC) and histone deacetylation inhibiting drug trichostatin A (TSA). *Methods.* The human MM cell lines U266, and H929 were treated either with 0.5 micromol/L 5Aza-dC for 7 days or with 100 ng/mL TSA for 24 hours or with the combination of 0.5 micromol/L 5Aza-dC for 7 days and 100 ng/mL TSA for additional 24hours. Control cells received no drug treatment. Total RNA was extracted from the cells and the quality of RNA was determined using the Agilent Bioanalyser. 1 μg of RNA was converted into cDNA and the Applied Biosystems microarray platform ABI 1300 was used for carrying out microarray profiling and analysis. To classify up-regulated genes into functional categories the PANTHER Classification System was used (http://www.pantherdb.org). *Results.* After treatment with 5Aza-dC 698 genes resulted up regulated in H929 cell line and 258 genes in U266 cell line. After treatment with TSA 719 genes resulted up regulated in H929 cell line and 742 genes in U266 cell line. The exposure to the combination of 5Aza-dC/TSA resulted in up-regulation of 921 genes in H929 cell line and 615 genes in U266 cell line. We evaluated overlaps of 5Aza-dC and TSA up regulated genes between MM cell lines and we found that there is only a limited overlap of 5Aza-dC or TSA-induced genes in the two MM cell lines: after 5Aza-dC treatment, 5 genes were found up regulated in both MM cell lines and 24 genes after TSA treatment. When we analyzed genes whose expression was up-regulated as response to a particular drug in each cell line individually we found that 34 gene were commonly up-regulated after 5Aza-dC and TSA treatment in U266 and 72 genes in H929 cell line, indicating that 5Aza-dC and TSA can affect same groups of genes and pathways unlike what has been proven by other studies. To classify up-regulated genes into functional categories and thus identify biological processes possibly involved in myeloma disease the panther classification system was used.We identified several 5Aza-dC or TSA up-regulated genes that are involved in important cancer-related pathways as cell cycle, apoptosis, cell adhesion, oncogenesis and cell metabolism, including DNA repair and nucleosome assembly (Table 1). We particularly found interesting the expression changes in members of cancer testis antigen (CTA) family like TSPY1, TSPYL3 and TSPYL4. In our study TSPY1 resulted up regulated after treatment with 5-azacitidine in cell line H929 (level of induced expression was 101,73 fold); TSPYL3 and TSPYL4 resulted up regulated after treatment with Trichostatin A in cell line H929 with a level of induced expression of 4,82 and 10,71 fold respectively. Moreover was remarkable the gene AKAP12, a putative suppressor of tumorigenesis and metastasis, that resulted up regulated after treatment with 5-azacitidine in cell line H929 and after treatment with Trichostatin A in cell line U266 (level of induced expression was 18,5 and 84,7 fold respectively). AKAP 12 expression after exposure to the combination of 5AzadC/TSA, resulted also up regulated in cell line H 929 (10,67 fold). Significant was also the expression changes of IGFBP-3, a gene involved in regulation of cell growth and death, that resulted up regulated after treatment with 5-azacitidine in cell line U266 (level of induced expression was 5,16). *Conclusions.* Identification of epigenetically inactivated cancer-related genes in MM is extremely important to better understand initiation and/or progression of the disease. In our study we found that expression of a large number of genes that are involved in cancer-related pathways is affected by 5Aza-dC and TSA in myeloma cell lines. Our next goal will be to validate data through microarray in MM cell lines and patients' bone marrow samples and investigate possible correlation with clinical features.

Table 1. Gene Ontology functions of genes whose expression is up-regulated >4-fold in MM cell lines after treatment with Aza-dC or TSA.

(742)	*5AZAdC vs untreated H929 (698)*	*5AZAdC vs untreated U266 (258)*	*TSA vs untreated H929 (719)*	*TSA vs untreated U266*
Immune and defense	43	23	44	46
Response to stress	8	6	8	9
Immunity response	35	17	36	37
Cell proliferation	21	11	28	29
Signal trasduction	89	42	101	133
Cell cycle	19	6	6	19
Cell metabolism and biogenesis	124	52	116	171
Protein	65	21	49	72
Carbohydrate	12	2	11	14
Nucleic acid	38	19	45	60
Lipid	14	7	10	21
Other	2	5	3	11
Apoptosis	14	6	9	13
Cell Motility and structure	31	10	30	48
Transport	35	24	35	49
Neuronal activities	30	10	30	29
Development	53	17	63	92
Cell adhesion	17	9	20	28
Oncogenesis	14	5	8	16
Muscle contraction	7	2	8	6
Unclassified	154	63	178	189

P087

THE INVESTIGATION OF GENOMIC PROFILES IN PRIMARY PLASMA CELL LEUKEMIAS BY MEANS OF AN INTEGRATIVE MICROARRAY APPROACH

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Introduction. Multiple myeloma (MM) is a clonal proliferation of malignant plasma cells (PCs) characterized by a marked genomic instability. Primary Plasma Cell Leukemia (pPCL) is an aggressive, rare variant of myeloma characterized by poor prognosis. Global genomics studies in pPCl are still limited. *Methods.* Highly purified PCs from 38 newly diagnosed MM and 8 previously untreated pPCL patients were characterized for the main chromosomal aberrations by FISH. pPCL cases were recruited in a multicenter GIMEMA clinical trial testing the lenalidomide/low dose dexamethasone combination. Gene expression profiling was generated by means of Gene 1.0 ST array (Affymetrix). Global miRNA expression (33 MM and 5 PCL) was obtained by means of the miRNA Microarray V2 (Agilent). Genome-wide DNA profiles (6 pPCLs) were generated using the 250K Nsp SNP array (Affymetrix); copy number (CN) values were inferred through the FISH-based normalization procedure. *Results.* Unsupervised analysis resulted in a dendogram in which the 8 pPCLs were grouped all together. Supervised analysis evidenced 85 differentially expressed genes, all upregulated in pPCLs, with a relevant localization on 22q12-q13 (11%) and 1q (7%). Several modulated tran-

scripts were associated to cytoskeleton cellular component (24%), in particular to actin cytoskeleton pathway and to the myosin complex, as well as the LYN and RHOA proto-oncogenes known to play a role in MM cell adhesion and chemotaxis. Regulation of intracellular transport and some metabolic processes such as RNA processing, glycolysis and cholesterol biosynthesis resulted also modulated. As regards miRNA profiling, an unsupervised clustering of 33MM and 5 pPCL samples grouped all the PCLs along with 4 MMs in a distinct sub-branch. Supervised analysis identified 24 differentially expressed miRNAs, all upregulated in pPCLs. Some of them may have a particular importance such as miR-142-5p and miR-142-3p translocated in human B-cell malignancies; members of the miR-17-92 and miR-106b-25 clusters and the miR-21 known to be over-expressed in MM with respect to normal PCs; miR-155 implicated in many B lymphoproliferative disorders; and miR-146a, a NF-KB-dependent miRNA involved in immune and inflammatory responses and cancer. SNP-array genome-wide profiling confirmed the main CN alterations in accordance to FISH. In addition, gains of several odd-chromosomes were evidenced in one case. Extracopies of the entire 1q chromosome were found in 3 PCLs, while 2 samples showed a partial 1q gain. Complete or partial loss of chromosome 13 was evidenced in all cases, chromosomes 14 and 1p were totally or partially deleted in up to 5 cases. Chromosome 17 was deleted in 4 PCLs, involving the TP53 locus in 3 cases; 8p and 16q regions were deleted in 3 cases. *Conclusions.* The integrative genomic approach in pPCLs compared to MM may help to identify genetic lesions and altered molecular pathways potentially involved in this aggressive form of PC dyscrasia.

P088

CD200 INTENSITY EXPRESSION CORRELATES WITH CD56 INTENSITY EXPRESSION: INSIGHT INTO THE ANTIGENIC PROFILE OF MYELOMA PLASMA CELL

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Introduction. Currently, morphological assessment of plasma cells (PC) percentage in the bone marrow (BM) represents a major criteria for Multiple Myeloma (MM) diagnosis and for evaluation to response to therapy, however, numerous evidence have suggested PC immunophenotype as a useful tool for MM diagnosis, identification of prognostic markers and evaluation of minimal residual disease as well. CD200 molecule, has been reported in MM PC, however the relationship with the other well known MM PC immunophenotipic markers and its possible prognostic significance have not been fully elucidated yet. The aim of this study was to analyse the antigenic pattern of MM PC to identify a possible antigenic profile correlated with CD200 expression. *Methods.* The study included 54 MM patients who were all in stage II-IIIA except for two patients in stage IA (Durie-Salmon). Immunophenotype was performed on BM aspirate samples using direct immunofluorescence tecnique. PC, identified with CD38/CD138 staining, were tested for the antigens CD200, CD19, CD27, CD45, CD56, CD20 and CD117 (BD Biosciences). The samples were analysed in a FACSCalibur flow cytometer using the CellQuest Program (BD Biosciences). Clinical characteristics were evaluated and related to immunophenotypic features. *Results.* 46/54 cases were $CD200^+$ with a median percentage value of 88% and a median MFI value of 66. 49/54 cases were $CD56^+$ (percentage 96%, MFI 330), 40/54 cases were $CD27^+$ (percentage 52%, MFI 79) and 27/54 cases were $CD45^+$ (percentage 68%). 5 cases were $CD19^+$ (percentage 32%), 10 were $CD20^+$ (percentage 86,5%). Expression of CD117 was evaluated in 31/54 cases and it was positive in 17/31 (percentage 58%). On the basis of CD200 expression three groups of patients were identified: $CD200^-$, CD200low(MFI 66) and CD200high (MFI>66). Incidence and percentage of expression of CD27, CD56, CD20 and CD117 was similar among the three groups, except for CD45 that was expressed in all the $CD200^-$ cases but only in 43% CD200low cases and 39% $CD200^{high}$ cases and the percentage expression of CD19 antigen that decreased among $CD200^-$ (74%), CD200low (29,5%) and CD200high (19%). Interestingly, CD56 MFI directly correlates with CD200 MFI, being 106, 295,5 and 634 in $CD200^-$, CD200low and CD200high groups respectively (CD200- *vs.* CD200low P=0,09; CD200- *vs.* $CD200^{high}$ P=0,057). With respect to the clinical features, patients CD200- showed a time to treatment of 23,12 months (mean value) *vs.* 8,7 months of $CD200^+$ patients however it was irrespectively of the presence or intensity of CD56 antigen. No other clinical difference were associated with the antigenic pattern observed. *Conclusion.* Our results showed that CD200 MFI significatively correlates with CD56 MFI but it did not seem to identify a specific group of patients with common clinical features. Further studies are ongoing to better clarify the clinical-biological impact of this antigenic association.

P089

LONG-TERM RESPONSE TO LENALIDOMIDE IN PATIENTS WITH MULTIPLE MYELOMA RELAPSING AFTER MULTIPLE CHEMOTHERAPY LINES

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Introduction. Lenalidomide is effective for the treatment of relapsed multiple myeloma (MM), but evidences of long-term response are lacking. This retrospective multicenter study aimed to evaluate whether long-term responders (LTRs) to lenalidomide exist and which patients' characteristics may predict the long-term response. *Methods.* All MM patients relapsed after ≥2 chemotherapy lines receiving lenalidomide were enrolled. Response was defined as per International Uniform Response Criteria, long-term response was defined as >=PR lasting ≥12 months. Patients with a follow-up ≥12 months or progressed at any time from the treatment start were evaluable for long-term response analysis. 104 patients were enrolled in 13 Italian hematology centers, 61% had IgG, 27% IgA, 12% light chain MM. Median age at lenalidomide treatment start was 65 (40-89). Cytogenetics showed del(13) in 25%, t(4;14) in 3%, t(11;14) in 10%, del (17) in 4%, was normal in 25% and was not available in 56% of patients. Median number of previous lines was 3 (range 2-7), 64% of patients had failed an autoSCT and 13% an alloSCT. All patients received lenalidomide for 21 every 28 days per cycle at a daily dose of 25 mg (84%) or at reduced dose due to hematologic toxicity (16%). Dexamethasone was administered with lenalidomide in 98% of patients, either at low (≤160 mg/cycle, 88%) or at high-dose (12%). The median number of courses per patient was 6 (range 2-72). *Results.* Median follow-up was 375 days (84-2005). The responses were: 4% sCR, 8% CR, 10% VGPR, 50% PR, 16% SD and 12% PD. Response was achieved after a median time of 94 days (range 25-637), and lasted a median of 431 days (range 74-2005). Median time to progression was 272 days (range 74-540); 87 patients (84%) were evaluable for long-term response analysis. LTRs to Lenalidomide were 41 (47%). LTRs had significantly better OS than no-LTRs (2-year OS of 97% of LTRs *vs.* 26% of no-LTRs, P<0.001). LTRs and no-LTRs did not significantly differ by age, stage, previous lines, autoSCT and dexamethasone dose. No-LTRs had more frequently light chain MM than LTRs (22% *vs.* 2.5%, P=0.03). LTRs had a higher incidence of t(11;14) and a lower incidence of del(13) and del(17) as compared to no-LTRs (12%, 10% and 2.5% *vs.* 0%, 26% and 7%, respectively, P=0.05). Strikingly, 7 of 8 alloSCT patients (87%) were LTRs (P=0.04). LTRs had 98% response >=PR as compared to 48% of no-LTRs (P<0.001) and 76% of LTRs are still responding at last follow-up. The multivariate analysis showed that previous alloSCT and best response to Lenalidomide independently predicted long-term response (P=0.04 and P<0.001, respectively), whereas isotype, previous lines and autoSCT were not significant. *Conclusions.* In conclusion, 47% of MM patients relapsed after ≥2 lines who start a

treatment with lenalidomide are long-term responders, and alloSCT and best response to lenalidomide are the best independent predictors of long-term response.

P090

LIVER INVOLVEMENT IN MULTIPLE MYELOMA: REPORT OF A CASE

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Introduction. Multiple Myeloma (MM) is a plasma cell malignancy, mostly affecting bone marrow and the bones. Extraskeletal involvement is relatively frequent at autopsy, especially in organ rich in reticuloendothelial elements but is often clinically silent. Liver involvement in MM is detected in about 40% of autopsies, but rarely reported in living patients. Clinical or laboratory hepatic manifestations are rare at diagnosis, more frequent at relapse or in terminal phase We report a patient experiencing liver involvement during the late stage of MM. Methods and results: A Caucasian 47-year-old man was diagnosed MM IgG k stage IIIA (DS), ISS 1. He was treated with 4 cycles VAD (vincristine, adriamycin, dexamethasone), HD cyclophosphamide, two autologous SCT (melphalan 200 mg/sqm) achieving complete remission (CR). After 5 years, a relapse was successfully treated with 3 cycles EDAP (etoposide, dexamethasone, cytarabine, and carboplatin) followed by one year thalidomide. Unfortunately after discontinuing maintenance he presented a lumbar paravertebral extramedullary relapse and new bone lesions. He underwent radiotherapy and allogeneic BMT with poor clinical benefit. After 9 months for progressive bone disease 7 VTD cycles (bortezomib, dexamethasone, thalidomide) were administered, and medullary CR was reached. Nevertheless a PET/TC revealed an abnormal 18FDG hepatic uptake. An abdomen MRI confirmed 3 nodular lesions in the right lobe with a diameter of 47, 71 and 34 mm respectively. The patient complained only mild dyspepsia and all liver function and viral tests were normal, except increased gammaGT and LDH. The patient refused to perform liver biopsy, thus MM liver involvement was clinically diagnosed. He was treated with 4 RCD cycles (lenalidomide, cyclophosphamide, dexamethasone). After initial response at MRI he experienced progression of the hepatic lesions (100 mm) and the appearance of multiple nodular $CD56^-$, $MIB1^{+++}$, plasmablastic skin lesions. New unsuccessfully chemotherapeutic regimens with bendamustine-bortezomib and with HD etoposide were administered; the patient finally died for septic complications, 14 months after hepatic relapse and 9 years after initial diagnosis. *Conclusion.* Liver involvement probably derived from selection of plasmacellular clones after HD chemotherapy. The histological patterns of MM liver involvement include amyloidosis, light chain deposition, extramedullary plasmacytomas or diffuse infiltrative process. Massive hepatic infiltration generally is distinguished in two types: tumor forming plasmacytomas and diffuse infiltrative process. The diffuse pattern consists of sinusoidal flooding by plasma cells of varying degrees of differentiation with poor propensity to destroy parenchyma, form tumors or aggregate in portal area. Our patient belongs to the nodular forming type (plasmacytoma), which is less common and can be detected radiographically as space-occupying lesion. Treatment of MM with hepatic involvement requires systemic therapy, eventually with novel agents. However even with aggressive treatment responses are short lasting and the prognosis is extremely poor, presumably because of the long disease course and the aggressive plasma cells behaviour.

P091

IMMUNOLOGICAL IMPAIRMENT AND INCREASED NUMBER OF CIRCULATING MDSC IN MM BUT NOT IN MGUS

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The immune function in MM is impaired consequently to an immunologically hostile microenvironment and cellular defects, differently from MGUS. Treg cells maintain immunological self-tolerance, control autoimmunity and modulate an immune response against infections and tumors. CD200, a membrane glycoprotein belonging to the immunoglobulin superfamily, play an important role in regulating immune system. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells of myeloid origin identified in mice as $CD11b^+Gr\text{-}1^+$. Human MDSC have an immature phenotype, including lineage negative (Lin-), $CD14^-$, HLA-DR^-, $CD15^+$, $CD34^+$, $CD11b^+$, $CD33^+$, and $CD13^+$ cells. Their accumulation has been described in the peripheral blood of patients affected by solid tumours, chronic infections, inflammatory diseases, and traumatic stress. *Aims.* Flow cytometry evaluation in patients with multiple myeloma and MGUS : 1) the expression of CD200 on B and T- lymphocytes, 2) T-reg cells $CD4^+$ $CD25^+$ $Foxp3^+$, 3) MDSC. *Methods.* We collected peripheral blood in EDTA from 54 MM at diagnosis, 41 MGUS patients and 25 healthy donors (HC). 1×10^6 cells were stained of the following monoclonal antibodies for 20 minutes at room temperature: 1) CD3, CD19, CD200, CD45 for CD200 detection on both B and T lymphocytes; 2) CD13, CD34, CD11b, CD14, CD45 for MDSC. T-reg cells were identified as $CD4^+/CD25^+/FoxP3^+$ T according to manufacturer's instructions and expressed as a percentage of the $CD4^+$T-cell population. *Results.* We observed a significant decrease in expression of CD200 on T and B- lymphocytes of patients with MM (2.8±1.22%, and 52.2±15.1%) compared to MGUS (4.56±1.94%, and 63±17%) (P=0.004 and P=0.005 respectively) and HC (5.95±2.96% and 65.67±11.64%) (P<0.0001). No statistical difference was observed in expression of CD200 on T and B- lymphocytes of patients with MGUS compared to HC. MM patients had a significant decrease in T-reg cells $CD4^+$ $CD25^+Foxp3^+$ (5.5±1.9%) compared to MGUS (7.1±1.7%) and healthy controls (8.03±1.6%) (P=0.01 and P=0.002 respectively), while this difference was not observed in patients with MGUS *vs.* HC. Finally, MDSC were higher in MM compared to MGUS, but no difference was appreciable between MGUS and HC. MDSC levels in MM were related to clinical stage and disease activity. Interestingly, patients with complete remission in follow up had similar levels of MDSC compared to MGUS/HC. *Conclusion.* Taken together, our findings suggest a role for immunological impairment in driving the evolution of MGUS in MM through mechanisms immunosuppressive and proangiogenetic. Increased levels of circulating MDSC, and the concomitant downregulation of tolerogenic $CD200^+$ lymphocytes and Treg contribute to immune dysfunction in patients with MM.

P092

BORTEZOMIB, PEGYLATED LIPOSOMAL DOXORUBICIN AND DEXAMETASONE (PAD) IN RELAPSED/REFRACTORY MULTIPLE MYELOMA. A SINGLE – CENTER EXPERIENCE

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Introduction. Bortezomib has been reported to affect myeloma cell growth by NF-kB blockade, downregulation of adhesion molecules, inhibition of angiogenesis and by inhibiting DNA repair, all of which results in a proapoptotic effect on myeloma. This drug produces significant responses in about one-third of patients with relapsed/refractory disease. Pegylated liposomal doxorubicin (PegLD) was used because it was anticipated that many patients would have had prior anthracycline-based therapy, and there was evidence that PegLD may have less cardiac toxicity. Also, the prolonged half-life (t1/2) of doxorubicin in the liposomal preparation allowed maximal overlap between the 2 agents with a convenient dosing schedule. Finally, PegLD was the only anthracycline for which there was *in vivo* data showing enhanced antitumor efficacy in combination with bortezomib and dexametasone. *Methods.* Between April 2007 and September 2009, twelve patients affected by relapsed/refractory multiple myeloma were treated for a median of six 28-day cycles (4-8). Bortezomib was given at 1.3 mg/m^2 (days 1, 4, 8, 11); Dexamethasone at 40 mg (days 1-4) and Pegylated Liposomal Doxorubicin (Caelix) at 30 mg/m^2 (day 4). The characteristic of the patients were the following: median age = 74 years (68-82), M/F = 8/4, median number of prior regimen 2 (1-3), IgG/IgA = 9/3 κ/λ = 7/5, Stage II/III = 8/4, Median beta 2 microglobulin 3.9 mg/L (1.9-7), Median C reactive protein 2.45 mg/L (1.14-39), Median lactate dehydrogenase 520 U/L (430-1150), Median BMPC 50% (40-70), Median PLT 140.000 mm^3 (88.000-215.000), Median Hb 8.4 g/dL (7.2-11.5). *Results.* After 3 (1-6) median course to response, 4 patients archieved a complete response (CR); very good partial response (VGPR) in 6, partial response (PR) in 2. Median time to progression was 10.5 months (7.4-21.3). Adverse events included thrombocytopenia in 3 patients (Grade 2/3), leukopenia in 1 (Grade 3/4), peripheral neuropathy in 2 (Grade 3/4, required a dose reduction), herpes zoster in 1. All of these adverse reactions could be controlled with routine supportive treatment. *Conclusion.* In conclusion, PAD regi-

men should be considered as an appropriate treatment for relapsed or refractory multiple myeloma. The side effects are usually manageable but the duration of response is short.

P093

IMPACT OF THALIDOMIDE THERAPY ON LENALIDOMIDE RESPONSE AND OUTCOME IN RELAPSE/REFRACTORY MULTIPLE MYELOMA PATIENTS

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Introduction. Lenalidomide (len) is a thalidomide analog, designed to have improved efficacy and tolerability over the parent drug. The aim of this retrospective study is to analyse the impact of thalidomide (thal) therapy on len response and outcome in relapse-refractory MM patients. *Methods.* A total of 153 MM patients received len treatment and were categorized as follows: 1) patients with no previous thal exposure (T0) (n=47); 2) thal sensitive patients (TS) (thal stop in PR, n=25); 3) thal resistant patients (TR) (progressive disease while on thal, n=81). Twenty-three patients in T0 group received MPR at diagnosis while all other patients received len-dex as salvage therapy. Mean prior lines of therapy in T0, TS and TR groups was 1 (range 0-3), 2 (range 1-5) and 3 (range 1-6), respectively. *Results.* No statistical difference was found in the overall response rate (more than PR) between T0 and TS patients (78.7% *vs.* 64%, P=0.17) and between TS and TR patients (64% *vs.* 55.5%, P=0.45). Treatment with len-dex led to a significant improved overall response when compared T0 *vs.* TR patients (78.7% *vs.* 55.5%, P=0.008). Len treatment was significantly more effective in prolonging PFS in T0 with respect to TR group (median, 19,5 *vs.* 10 months, P=0.007) but not in prolonging PFS in T0 *vs.* TS patients (median, 19,5 *vs.* 12 months, P=0.16) and in TS *vs.* TR patients (median, 12 *vs.* 10 months P=0.27). Median OS was not reached in T0 group (P=0.001) and was similar in TS and TR groups (median, 15 *vs.* 17 months, P=0.75). No difference was found in term of PFS on len between TS group (thal discontinuation in PR, n=25) and TR thal responsive patients (thal continuation until progression, n=44) with a median PFS of 12 and 13 months, respectively (P=0.67). Of the 75 patients who obtained at least a PR while on thal, 48 (64%) and 27 (36%) were responsive and refractory to len, respectively (P=0.036). Viceversa of the 31 patients thal resistant, 18 (60%) and 13 (40) were respectively len refractory and sensitive. *Conclusions.* Len is highly effective in thal pre-treated MM patients. Discontinuation of thal in PR may not confer a positive impact on len outcome. Response to thal may be predictor of len activity and response.

P094

ROLE OF INFLAMMATION SUSTAINED BY MONOCYTIC-MACROPHAGYC SYSTEM IN PROGRESSION FROM MGUS TO MM

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Background. The immune function in MM is impaired consequently to an immunologically hostile microenvironment and cellular defects, differently from MGUS. Activin-A is a transforming growth factor-beta (TGF-β) superfamily member that plays a pivotal role in many developmental and reproductive processes. Activin A secretion is potently upregulated in monocytes as well as stromal fibroblasts by cognate interaction with activated T cells in the bone marrow milieu and plays a functional role in the suppression of inflammation. Recently, it has been activin-A has potent autocrine effects on the capacity of human monocyte-derived dendritic cells (DCs) to stimulate immune responses. Antagonizing this signaling resulted in significantly enhanced expansion of antigen-specific effector $CD8^+$ T cells. Inflammatory $CD14^+/CD16^+$ monocytes have a wide range of chemokine pathways for recruitment into tumour microenvironment, in which they differentiate into macrophages or alternatively in dendritic cells and as shown exclusively in mouse model they might enhance oxidative stress and endothelial dysfunction. *Methods.* So far, circulating levels of Activin A have been detected by a commercially available ELISA kit in sera of 15 MM, 3 smouldering MM and 12 MGUS and compared to 10 healthy subjects matched for age and sex. In the same cohort, we evaluated the absolute count monocytes and all subset of inflammatory monocytes, identified by flow cytometry as $CD14^+CD16^+$. *Results.* We observed a significant increase of circulating Activin A in sera of patients affected of MM (260.8±29.24 ng/mL) compared to MGUS/smouldering MM (189.5±32.43 ng/mL) and healthy subjects (mean 62.18±8.83 ng/mL) (P<0.0001, one way ANOVA). Among MM patients, Activin A was significantly increased in presence of osteolytic lesions (280.4±26.82 *vs.* 173.5±24.15, P=0.014, unpaired T test). Even though absolute count of monocytes was similar between healthy subjects *vs.* MGUS *vs.* MM, inflammatory monocytes in MM were higher than in healthy subjects (mean 48.32±7.82/mm^3 *vs.* mean 37.75±3.45/mm^3, P=0.027, unpaired T test), but similar to those circulating in MGUS/smouldering MM (mean 51.39±4.47/mm^3, P=0.33), so suggesting that chronic inflammation is activated at the same manner in all gammopathies, but it is characterised by different kind of soluble mediators. *Conclusion.* Taken together, our findings suggest a role for Activin A in driving the evolution of MGUS in MM through mechanisms immunosuppressive, and contributing to sustain osteolysis. Activin A could be used as surrogate of macrophages activity in bone marrow, with higher fidelity than absolute count of inflammatory monocytes circulating in peripheral blood.

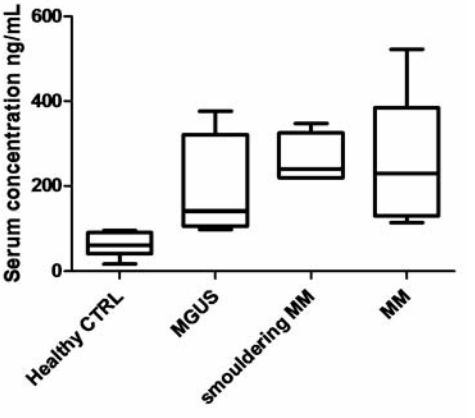

Figure. Activin levels.

Thrombosis and Transfusion Medicine

P095

CIRCULATING ENDOTHELIAL CELLS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA: RESULTS FROM CD146+ IMMUNOMAGNETIC ENRICHMENT FOLLOWED BY MULTIPARAMETER FLOW CYTOMETRY

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Introduction. circulating endothelial cells (CECs) have recently emerged as a marker of endothelial damage in cardiovascular disorders and, concurrently, as a marker of angiogenetic activity. In addition to its well-established role in solid tumours, angiogenesis plays an important role in the pathogenesis of haematological diseases, modifying bone marrow microvascular density and displaying an increase of endothelial progenitor cells (EPCs). Although CECs and EPCs research studies may have important clinical implications, they're often impeded by methodological issues and a lack of consensus on phenotypic identification and distinction of these cells and particles. In our study we determined CECs in patients (pts) with Essential Thrombocythemia (ET), in order to investigate their possible role in the pathogenesis of this disease. *Methods.* CECs are defined in our study as $CD146^+/CD45^-$ nucleated cells, determined in peripheral blood from 21 healthy volunteers (median age 47 yrs) and from 32 ET pts (median age 54 yrs). CECs measurement was performed using a combination of preenrichment of $CD146^+$ circulating cells and multiparametric flow cytometry measurement (FCM). $CD146^+$ cells were isolated using CD146-coated magnetic nanoparticles and labeled using CD45-fluorescein isothiocyanate and CD146-PE or isotype control antibody and propidium iodide before FCM. ASO-PCR for JAK2V617F mutational status was performed on genomic DNA from bone marrow cells or peripheral blood granulocytes. Differences in CECs levels among ET pts and controls were evaluated by Mann-Whitney test; linear regression was performed in order to evaluate correlations between CECs and clinical variables (age, duration of disease, thrombosis, WBC, PLT and Ht values). Data were processed using the Graph Pad PRISM 5 Software.

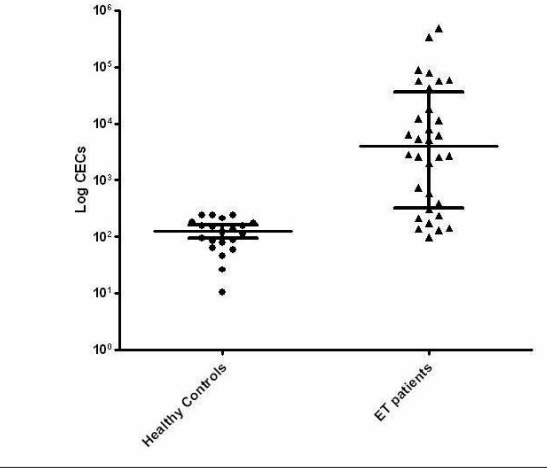

Figure. Increased CECs levels in ET patients (Mann-Whitney test).

Results. CECs levels in ET pts were significantly higher respect to healthy controls (median 4014 CECs/mL *vs.* 121.3 CECs/mL, P<0.001), as shown in Figure 1. No significantly differences were found in CECs between JAK2V617 positive (18) and negative (14) pts or in terms of correlations with gender, age, duration of disease, history of thrombosis and hematology values at the time of detection. Apparently hydroxyurea treatment seems not influence CECs levels in cytoreducted pts (26), respect to pts receiving only disaggregation (6). *Conclusions.* Our data suggest that endothelium in ET is activated; it would be interesting to confirm this status by assessing other estabilished markers of endothelial activation, such as p-selectin and von Willebrand factor. We purpose to extend our case study in order to better investigate a possible endothelial role in thrombosis in ET and to better define a possible linkage between endothelial activation and JAK2V617F mutation. Moreover, the increase in CECs levels observed in ET pts, respect to controls, may indicate a significant role of angiogenesis in the pathophysiology of chronic myeloproliferative disorders.

P096

FREQUENCY, TYPES AND MANAGEMENT OF BLEEDING EPISODES IN ACQUIRED VON WILLEBRAND SYNDROME: A FIVE YEARS PROSPECTIVE STUDY IN A COHORT OF 18 PATIENTS WITH CHRONIC LYMPHO-MYELOPROLIFERATIVE DISORDERS (LPD/MPD)

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Background. Acquired von Willebrand Syndrome (AVWS) is a rare bleeding disorder with laboratory findings similar to those of inherited VWD. Actual prevalence, frequency and types of bleedings of patients with AVWS are unknown because large prospective studies are not available. AVWS is frequently associated with monoclonal gammopathy of uncertain significance (MGUS) or Essential Thombocythemia (ET). *Aims.* To determine frequency and types of bleeding episodes which might need therapeutic interventions we have prospectively followed-up for five years our cohort of AVWS. *Patients and Methods.* 18 patients were enrolled in the study on January 2005 with diagnosis of AVWS performed according to the criteria of ISTH-SSC. A bleeding severity score (BSS) was calculated after exposing patients at enrollment to a detailed questionnaire. Bleeding time (BT) and VWF activities were measured in all cases while platelet nucleotides only in ET. Bleeding-free survival was computed with the Kaplan-Meier method. Types and number of treatments needed to manage bleeding were also recorded. *Results.* AVWS associated with MGUS (n=10) showed higher mean values of BSS than those (n=8) with ET (15 versus 6) in agreement with lower mean levels of VWF:RCo (8 versus 35 U/dL) and FVIII (12 versus 65 U/dL). Among the 8 cases with ET, BSS was relatively higher when low VWF:RCo levels were associated with lower platelet nucleotides. BT was moderately prolonged, with values of 16 (MGUS) and 12 (ET) min. The bleeding-free survival at five year calculated according to MGUS and ET was 20% and 50%, respectively. Mucosal (n=36) and non-mucosal (n=23) bleeds in MGUS (case n=8) or ET (case n=4) were treated with DDAVP (n=38), VWF concentrates (n=43), IVIg (n=26), rFVIIa (n=12). *Conclusions.* Patients with AVWS associated with MGUS/ET can show severe bleeding requiring intensive treatment. An early correct diagnosis should improve morbidity and mortality of these patients.

P097

ACQUIRED VON WILLEBRAND SYNDROME TYPE 2A ASSOCIATED WITH JAK2 POSITIVE MYELOPROLIFERATIVE DISORDER IN ONE AFFECTED MEMBER OF A LARGE FAMILY WITH AUTOSOMAL DOMINANT A1716P MUTATION OF THE VWF GENE

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Introduction. Acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder occurring in subjects with no personal or family history of bleeding and is similar to inherited VWD in terms of laboratory findings and clinical symptoms. A 2A-like AVWS has been found to be associated with myeloproliferative disorders (MPD), especially in patients with very high platelet count, and the VWF defect can be corrected with reappearance in plasma of High Molecular Weight (HMW) VWF multimers when platelet count is normalized upon cytostatic treatment. *Methods.* We have followed-up a 41 year-old male with increased platelet count (6.5×10^8/mL) and a JAK2 positive MPD who met WHO criteria for Essential Thrombocythemia (ET). At diagnosis, PFA-100 C/Epi closure time was prolonged and the ristocetin-induced platelet aggregation (RIPA) reduced. VWF activities were also reduced with low VWF:RCo/VWF:Ag (0.34), VWF:CB/VWF:Ag (0.48) and loss of the HMW multimers, suggesting VWD2A. Considering the absence of oth-

er cardiovascular risk factors and the VWF defect, the patient was left untreated until an episode of acute myocardial infarction occurred; he was then immediately started on anti-platelet and cytostatic therapy (aspirin 100mg/die, hydroxyurea 500 mg/die). *Results.* During his follow-up, sixteen family members were studied and 6/16 showed reduced levels of VWF activity with all the HMW multimers in plasma suggesting VWD1. Sequencing analysis of the VWF gene revealed a nucleotide transversion in exon 29 (c.5146G>C) leading to an alanine to proline substitution (p.A1716P) in the collagen binding domain (A3) of VWF in affected members, including the JAK2-positive patient. Following treatment the patient had the platelet count normalized (3.5×10^8/mL) and the acquired 2A-like defect turned into VWD1. *Conclusions.* We can conclude that bleeding defects can be present in the families of patients with MPD and should be always searched for to exclude inherited defects underlying acquired platelet disorders.

P098

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

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Background. Cancer-related disseminated intravascular coagulation (DIC) is a rare but life-treating condition. Acute management is mainly based on administration of fresh-frozen plasma (FFP) and Antithrombin (AT) with the aim of restoring coagulation factors deficiency. The Protein C (PC) pathway is a modulator of the coagulation as well as the inflammatory system; PC deficiency leads to increased activation of the coagulation system, resulting in thrombin generation. Some clinical trials have demonstrated the efficacy of administering activated recombinant (a-r) PC in adult patients with sepsis-related coagulopathy, but increasing the risk of major bleeding. Plasma-derived PC has a self-limiting process in determining anticoagulation thus it seems more suitable than a-rPC in patients at high risk for bleeding, such as cancer patients. Objective. To describe the efficacy and safety of PC concentrate to restore physiological values in adult cancer patients with overt DIC. Study Design. Not controlled clinical trial (NCCT).

Table 1. Changes in laboratory findings abtained from all patients during the study period (mean ± SD).

	Baseline	24 h	48 h	72 h	7th day	14th day
PC (%)	27.3 ± 7.1	71 ± 15.6*	85.9 ± 12.5*	91.2 ± 11.6 *	92.2 ± 13.4 *	99.1 ± 13.5 *
WBC ($\times10^9$/L)	8.2 ± 3.1	7.8 ± 2.2	6.5 ± 1.9	6.7 ± 1.5	7.3 ± 1.5	8.1 ± 0.6
Platelet ($\times10^9$/L)	49.3 ± 20.4	51.2 ± 19.4	71.2 ± 33.4	91.7 ± 41.1	113.4 ± 65. 1	154.8 ± 10 9.2*
d-Dimer (µg/L)	2.133.6 ± 1.643	2.366 ± 1.56 1	1.230 ± 1.045 *	800.2 ± 686 *	350 ± 225*	541 ± 246*
Fibrinogen (g/L)	2.1 ± 1.4	2.8 ± 1.1	3.6 ± 1.5	4.4 ± 1.4*	4.5 ± 1.2*	4.2 ± 1.3
PT (%)	46.4 ± 11.5	46.2 ± 12.1	51.8 ± 13.8	63.3 ± 15.2	65.4 ± 0.9*	69.7 ± 14.3 *
aPTT (s)	40.1 ± 13.4	34.8 ± 7.6	35.4 ± 6.1	33.4 ± 6.1	32.9 ± 7.5	31.2 ± 3.6*
AT (%)	54.2 ± 12.2	61.6 ± 23.3	73.4 ± 21.4	77.7 ± 22.2 *	80.6 ± 16.5 *	87.1 ± 18.5 *
DIC score	6.26 ± 1.12	5.38 ± 1.42	4.26 ± 0.96	3.16 ± 0.98 *	2.97 ± 0.87 *	2.21 ± 1.43 *

*$P < 0.05$ versus baseline

Materials and Methods. Adult cancer patients affected by DIC, having PC plasma concentration less than 50%, were treated with PC concentrate (Ceprotin®, Baxter) as an adjusted bolus of 30 to 50 UI/Kg/die to restore normal PC values (70-120%). Clinical outcomes (bleeding, thrombosis and mortality) were recorded up to a follow-up of 28 days from the initial diagnosis of DIC. PC activity, WBC, platelets, D-dimer, fibrinogen, PT, aPTT, AT and DIC score were measured after 12, 24, 48, 7 and 10 days. *Results.* Twenty-two patients were included over a period of 3 years; among them 16 had solid cancer and 6 had haematological cancer. All patients had advanced/metastatic neoplasm. PC concentrate normalized PC activity in all patients within 48h and remained upper the lower normal value for the following days. Baseline PC levels were lower in non-survivors than in survivors although this difference was non-significant. During the study period, there was a significant increase of platelets, fibrinogen, PT, AT, and a significant decrease of D-dimer, aPTT and DIC score (Table 1). No bleeding or thrombosis were observed; mortality at 28 days was 35%. *Conclusions.* Our investigation shows that PC concentrate is safe and normalizes laboratory variables in cancer patients with overt DIC.

P099

RESIDUAL VEIN THROMBOSIS FOR ASSESSING THE OPTIMAL MANAGEMENT OF DEEP VEIN THROMBOSIS IN CANCER PATIENTS: AN INTERIM ANALYSIS OF THE CANCER DACUS STUDY

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Type and duration of anticoagulation is still matter of debate in cancer patients with acute Deep Vein Thrombosis (DVT) of the lower limbs. 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). *Results.* Over a period of 18 months, 134 patients were evaluated across 12 centers in Italy. 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).

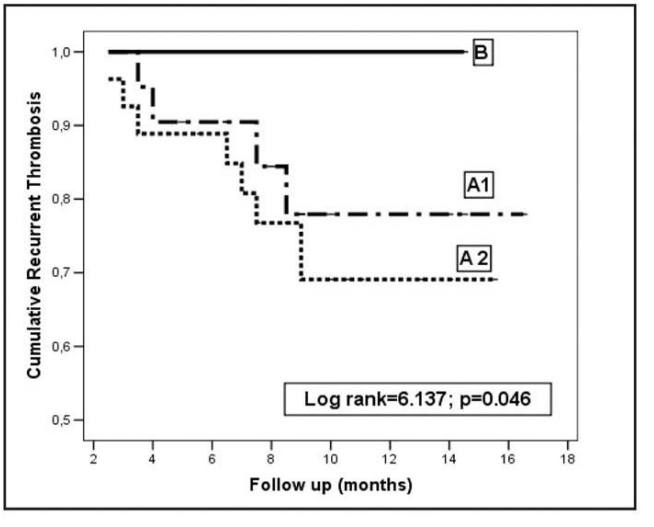

Figure 1.

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=.145). Of the 42 (31.3%) patients without RVT, one had a recurrence (2.3%). The adjusted HR (B *vs.* A1) was 4.54 (CI 2.3-6.66; P=.028). One major bleeding event occurred in each group of patients who stopped (Group A2 and B) and 2 in those who continued anticoagulation (Table 2). 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.

P100

CANCER-RELATED VENOUS THROMBOSIS: RESIDUAL VEIN THROMBOSIS IMPROVES SCREENING FOR OCCULT CANCER

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Introduction. Clinical advantage of extensive screening for occult cancer in patients with idiopathic Deep Vein Thrombosis (DVT) is unknown. We have demonstrated that a Residual Vein Thrombosis (RVT)-based screening for occult cancer improves early detection as well as cancer-related mortality (Siragusa S *et al*. Blood 2007;110(699):OC). *Aim.* We conducted a prospective study evaluating whether a RVT-based screening for cancer is sensitive and influences cancer-related mortality. Study design. Prospective with two cohorts of DVT patients: the first cohort was monitored for clinical overt cancer only (Group A), while the second (Group B) received complete screening for occult neoplasm and subsequent surveillance. *Materials and methods.* Consecutive patients with a first episode of DVT who presented RVT after 3 month of anticoagulation and without signs and/or symptoms for overt cancer. Screening for occult cancer was based on: ultrasound and/or CT scan of the abdomen and pelvis, gastroscopy, colonoscopy or sigmoidoscopy, hemoccult, sputum cytology and tumor markers. These tests were extended with mammography and Pap smear for women and ultrasound of the prostate and total specific prostatic antigen (PSA) for men. *Results.* Over a period of 8 years, 537 patients were included in the analysis: first cohort included 346 patients (Group A), second cohort 191 (Group B). Clinical characteristics between groups were homogenous. During the follow-up, 8.3% of patients developed overt cancer in group A; in group B, 7.8% of patients had diagnosed cancer at the moment of extensive screening while one new case (0.7%) occurred during the follow-up (Table). The sensitivity of this approach was 92.1% (95% confidence intervals 75.2-104.2). Cancer-related mortality was 7.5% in group A and 3.6% in group B (P<0.001) (Figure). *Conclusions.* At the long-term follow-up n 537 patients, the RVT-based screening for occult cancer shows high sensitivity for improving early detection as well as cancer-related mortalit.

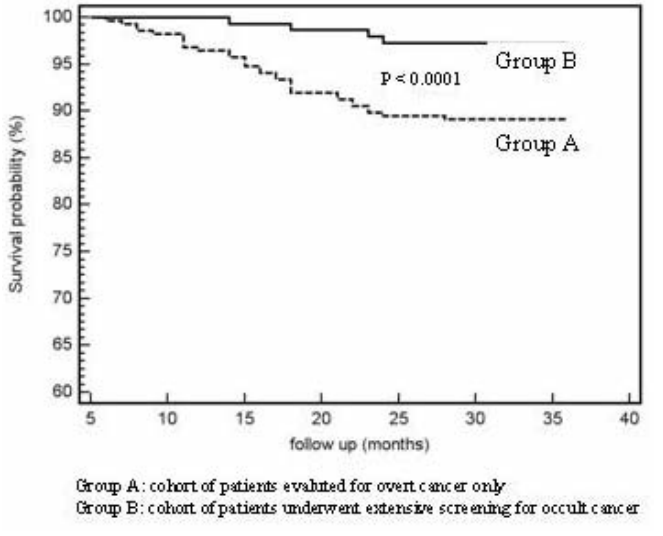

Figure. Cancer related mortality.

P101

ABSENCE OF RESIDUAL VEIN THROMBOSIS AFTER AN EPISODE OF IDIOPATHIC DEEP VEIN THROMBOSIS: SHORT-TERM ANTICOAGULATION IS SAFE. THE EXTENDED DACUS STUDY

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Background. The optimal duration of Oral Anticoagulant Therapy (OAT) for Deep Vein Thrombosis (DVT) can be tailored by Residual Vein Thrombosis (RVT) (Siragusa S *et al*. Blood 2003;102(11):OC183), a marker able to assess the individual risk for recurrent thrombosis. However, in patients with idiopathic DVT the safety of early interruption of OAT, because of absence of RVT, is still debated. Objective of the study. In the present study, we evaluated the safety of withholding OAT, in patients with idiopathic DVT and without RVT, three months after the index thrombotic episode. Study design. Prospective controlled study with two groups: patients without RVT stopped OAT after 3 months while those with RVT continued for additional 3 months. *Materials an methods.* Consecutive patients with a first episode of idiopathic DVT of the lower limbs; patients with cancer or known thrombophilia were excluded. At the third months of OAT, RVT was assessed as previously described; briefly, RVT was considered absent when a clot occupying lessthan 40% of the vein lumen was detected by compression ultrasonography. Events, classified as recurrent DVT and/or Pulmonary Embolism (PE) and/or major and minor bleeding were evaluated; all patients were followed-up for at least 12 months after OAT discontinuation. *Results.* During the period 1999-2006, 518 patients were included in the study. In 206 (39.7%) RVT was considered absent (RVT negative group) and they stopped OAT; the remaining 312 patients continued anticoagulants for additional 3 months (RVT positive group). Total duration of followup (FU) was 184.7 years for RVT negative group (with a mean FU of 3.0+0.83 years) and 191.3 years for RVT positive group (with a mean FU of 3.1+0.89 years). The rate and type of events during FU is reported in Figure 1. *Conclusions.* This investigation shows that in patients without RVT, three months of OAT are safe even after an episode of idiopathic DVT. This hold for at least 30% of the entire DVT population and has an important clinical impact; in fact, it is possible to select a group of patients with a very low risk for recurrences over a period of 3 years. This approach carries also a negligible risk for bleeding.

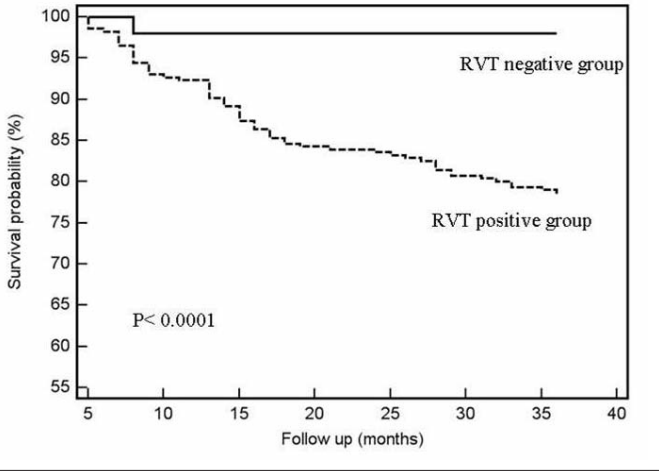

Figure 1. Recurrent thrombotic events.

P102

EFFECTIVENESS OF DESMOPRESSIN (DDAVP) IN A CLINICAL CASE OF VON WILLEBRAND ACQUIRED DISEASE (AVWS) IN ESSENTIAL THROMBOCYTHEMIA (E.T.)

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Introduction. AVWS associated with E.T. is characterized by normal factor VIII and von Willebrand factor antigen (VWF:Ag) levels and a selective deficiency of functional ristocetin co-factor activity (VWF:RCo) and colla-

gen-binding activity (VWF:CBA). We report a case of a 29-year-old white woman with AVWS in Essential Thrombocythaemia, Ehlers-Danlos Syndrome(EDS) typical form (previous classified as EDS I-II) referred in Surgical Department of San Raffaele Giglio Hospital for a video laparoscopic cholecystectomy (VLC). *Methods.* We monitored the response of the AVWS to intravenous desmopressin (DDAVP) dosing exclusively activated partial thromboplastin time (APTT) and factor VIII coagulant activity (FVIIIC). The baseline level of FVIIIc was 60% (previous assay tested was 88%), therefore, the day before VLC we decided to start a prophylaxis with DDAVP at dose 0.3 μg/Kg/die plus tranexamic acid; the therapy was stopped three days after the VLC. *Results.* During the DDAVP prophylaxis the FVIIIc level was always higher 90%. We monitored our patient with serial abdomen ultrasound, and we observed a gradual reabsorption of endopelvic effusion post VLC. *Conclusions.* DDAVP intravenous is a safe and effectiveness drug as like as factor VIIIc/VWF concentrate. In our patient the platelets count remained lower than 550.000/μL avoiding both the anticoagulant prophylaxis and the cytoreductive therapy.

P103

ELEVATED LEVELS OF FACTOR VIIA-ANTITHROMBIN (FVIIA-AT) COMPLEX IN PATIENTS WITH ACUTE PROMYELOCYTYC LEUKEMIA (APL)

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An excessive activation of blood coagulation characterizes the onset of APL which clinically translates into both severe haemorrhage and thrombosis. In this setting, a key role is played by promyelocytic blast procoagulants, including Tissue Factor (TF). All-trans retinoic acid (ATRA) produces complete remission in >90% of patients and rapidly resolves the associated coagulopathy. We evaluated for the first time, in APL patients, plasma levels of FVIIa-AT complexes which may reflect the degree of intravascular TF exposure and inhibition. TFPI, TAT complex and D-dimer were also evaluated. We enrolled 54 consecutive patients (25F/29M) in a prospective registry of major haemorrhage and thrombosis in APL patients receiving ATRA+Idarubicin (AIDA 2000, GIMEMA protocol): 8 had early major haemorrhages (3 fatal); 3 presented with thrombosis (1 fatal Budd-Chiari syndrome). Two patients developed thrombosis during the induction therapy period. Blood samples were obtained in 26 patients, before ATRA (D0), and on days 7 (D7), 15 (D15) and 25 (D25) of ATRA therapy. At D0 FVIIa-AT levels were significantly higher in APL compared to healthy controls and remained persistently elevated at D7 and D15, but significantly dropped at D25 ($P<0.05$ *vs.* D0). A persistent elevation of TFPI until D15 was also observed. Differently, TAT and D-dimer levels, significantly elevated at D0, rapidly decreased starting from D7 ($P<0.05$ *vs.* D0) and remained downregulated thereafter. Elevated FVIIa/AT levels in APL may reflect an increase in TF exposure by leukemic cells which persists over time. However, the parallel decrease of hypercoagulation markers, combined with high TFPI levels, demonstrates that the clotting activation at the cellular site is efficiently controlled by FVIIa-TF inhibition by indirect (AT) and direct (TFPI) inhibitors. The study suggests that monitoring the levels of FVIIa-AT complex in APL is important and the elucidation of its role as a marker of the coagulopathy is warranted.

P104

INCREASED IMMATURE PLATELETS CORRELATES TO INCREASED THROMBIN GENERATION IN PLATELET RICH PLASMA OF PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA

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Introduction. Platelet abnormalities have been variously involved in the pathogenesis of thrombofilia of patients with Essential Thrombocythemia (ET) and Polycythemia Vera (PV). In this study we aim to characterize the haemostatic properties of platelets from ET and PV patients. Methods: In a group of 46 ET and 38 PV patients we performed the thrombin generation (TG) assay in platelet rich plasma (PRP) and the PFA-100 assay to characterize the platelet procoagulant and adhesive properties, respectively. In addition, in the same experimental conditions, we quantified the number of peripheral blood immature platelets (IPF) using the Sysmex XE-2100 system. The correlation analysis was performed to evaluate the possible association of increased IPF parameters with platelet adhesive and procoagulant properties. *Results.* Our results show significantly increased levels of IPF, as both absolute count and percentage, in whole blood as well as in PRP samples from PV patients compared to controls, and an increase in IPF, as absolute count, in whole blood from ET patients. The ET and PV patients carrying the JAK2V617F mutation had significantly increased IPF compared to controls. The correlation analysis showed that higher IPF count ($R=-0.5$, $P<0.001$) in ET patients and higher IPF count ($R=-0.5$, $P<0.01$), IPF% ($R=-0.4$, $P<0.05$) and H-IPF% ($R=-0.4$, $P<0.05$) in PV patients predispose to shorter CADP and CEPI closure time. PRP from both ET and PV patients generated significantly higher TG compared to control subjects. Linear regression analysis showed that a higher IPF count determined higher TG activity ($B=0.52$; $P<0.05$) in PV patients. *Conclusions.* This study provides new insight into the characteristics of platelets from PV and ET patients. The analysis of IPF might be helpful in identifying the patients at high risk for thrombosis. New perspective studies are warranted to evaluate the usefulness of IPF as a treatment target in these patients.

P105

RAPID CYTOFLUORIMETRIC EVALUATION OF REFRACTORINESS TO PLATELET CONCENTRATES IN PATIENTS WITH GLANZMANN'S THROMBASTHENIA

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Background. Glanzmann's Thromboastenia (GT) is a rare autosomal recessive bleeding disorder characterized by quantitative or qualitative defects of the glycoprotein IIb-IIIa (GPIIb-IIIa) receptor on the platelet surface, prolonged bleeding time with normal platelet count, no response to agonists (ADP, collagen, arachidonic acid) in platelet aggregation. Flow cytometry is a sensitive and specific technique to distinguish platelets with normal or reduced levels of GPIIb-IIIa receptors. Standard therapy for GT is platelet concentrates (PC) transfusion and/or administration of recombinant activated factor VII (rFVIIa) in refractory patients. *Aim of the study and methods.* The rapid evaluation of PC refractoriness in GT patients exposed to PC as tested by cytofluorimetric assays with mAbCD41 (clone p2). *Patients.* two patients with type I GT (GPIIb-IIIa<5%) were followed-up at the Division of Hematology and Transfusion Medicine, L. Sacco University Hospital. *Case 1.* 44 years old woman with severe gastrointestinal bleeding was treated with 42 units of RBC (median Hb value 7.2 g/dL during hospitalization) and 19 PC (apheresis). *Case 2.* 47 years old woman with upper urinary tract hematuria and hydronephrosis who arrived to our observation after being previously treated with 6 RBC units and 6 PC units. During hospitalization she received 8 RBC units (median Hb value 9.6 g/dL) and two PC units (apheresis) with a poor clinical response: therefore rFVIIa 90 mg/kg three times daily for 4 days was given. *Results.* $CD41^+$ platelets were consistently shown after PC in case 1 who recovered from bleeding. In case 2, the absence of $CD41^+$ platelets was proven immediately after two PC units consistently with the poor clinical response. This cytofluorimetric analysis could rapidly identify refractoriness to PC in this patient who could be immediately shifted to an intensive therapeutic regimen of rFVIIa. *Conclusion.* Therefore, this rapid method should be strongly recommended during transfusion with PC in GT patients.

P106

BILATERAL SEROUS RETINAL DETACHMENTS IN A YOUNG PATIENT WITH THROMBOTIC THROMBOCYTOPENIC PURPURA: ONE TREATMENT FOR TWO DISEASES

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Introduction. Thrombotic thrombocytopenic purpura (TTP) is charac-

terized by microangiopathic hemolytic anemia, thrombocytopenia and constitution of microthrombi. The ocular involvement in TTP is far from uncommon and can occur in up to 10% of patients; the most findings include palpebral purpura, scleral jaundice, chemosis, subconjuntival and retinal bleeding, hypertensive retinopathy, often suggesting a systemic damage. Other specific ocular lesions are retinal detachment, choroidal bledding, homonimus hemianopsia, diplopia, anisocoria, papilledema. Serous retinal detachments are rare; only 11 cases have been described, predominantly associated to hypertension. *Methods.* We report a rare bilateral serous retinal detachment in a 24-year old woman with acute TTP (Hb 4.5 gr/dL, PLT 21.0×10^6/mL, LDH 1983 U/L, Coombs' test negative, schistocytes 20%, ADAMTS13 activity dosage <5%, ADAMTS13 autoantibodies titer high). According to the SidEM protocol, TPE with fresh frozen plasma inactivated by photochemical treatment system was performed daily. The patient also received oral prednisone (1 mg/kg). On the 7th day a complete haematological response (CHR) was reached and alternate days maintenance TPE procedures were started; 2 days later the patient had a TTP exacerbation (PLT 27.0×10^6/mL, LDH 1138 U/L). Furthermore she developed a serious visual reduction with distortion of images at the right eye. The funduscopic examination showed multiple bilateral chorioretinal cystic formation at the posterior pole, identified as hypofluorescent areas by the retinal fluorescein angiography (Figure 1 a, b); at the delayed enhancement sequences, an impressive chorioretinal exudation was demonstrated at the limit of these areas (Figure 1 c, d). The optical coherence tomography (OCT) demonstrated bilateral serous retinal detachments of the neuroepithelium, while the ocular sonography confirming the exudative chorioretinopathy and the retinal detachments. *Results.* A reinduction phase of TPE was started, (6 daily procedures, 4 on alternate days). The patient showed a progressive improvement of the right visual field defect, confirmed by retinal fluorescein angiography. A CHR was obtained after the 6th TPE procedure. A month later the patient's visual acuity improved (from 5/10 OD to 11/10 OD) and the OCT exam showed the resolution of the retinal detachment. *Conclusions.* Ocular involvement in TTP is not a common event but it may be possible; it is frequently due to thrombocytopenia, hypertension and thrombotic microangiopathy. Moreover the retinal detachment, which is an event rarely reported in TTP, may be a symptom of an underlined TTP autoimmune disease. A clinical suspicion may be difficult if the retinal damage is located far from the fovea, as it does not cause particular visual problems. Many TTP-linked ocular manifestations may be resolved with adeguate plasmapheresis associated to oral corticosteroid therapy. We suggest a complete ophthalmologic examination at every TTP diagnosis.

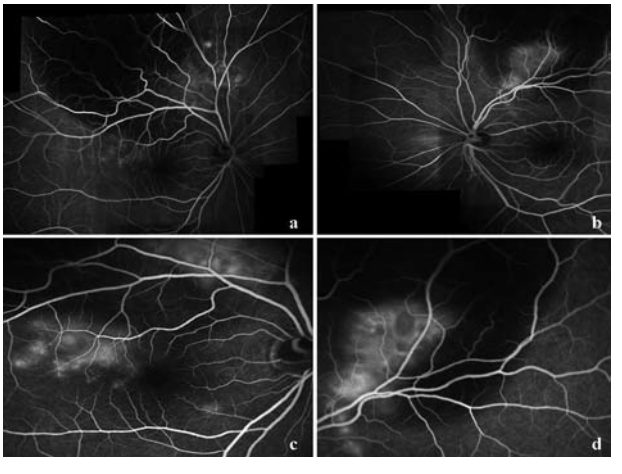

Figure 1. The retinal fluorescein angiography of right (a, c) and left (b, d) eye. In a and b large hypofluorescent areas due to serous retinal detachments of the neuroepithelium; in c and d at the delayed enhancement sequences, an impressive exudative chorioretinopathy was demonstrated as intense hyperfluorescent areas at the limit of the above mentioned hypofluorescent areas.

P107

PROMPT THERAPEUTIC INTERVENTIONS IN RECURRENT THROMBOTIC THROMBOCYTOPENIC PURPURA (TTP) IMPROVE CLINICAL OUTCOMES WITH REDUCED NUMBER OF PLASMA EXCHANGE (PEX)

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Background. Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening disorder characterized by microangiopathic haemolytic anemia, consumptive thrombocytopenia leading to microvascular thrombosis and variable signs and symptoms of organ ischemia and damage. Congenital and acquired TTP are due to inherited and acquired defects of the VWF protease ADAMTS13. TTP can be triggered by pregnancy, infections, sepsis, autoimmune disorders, malignancies, major surgery. TTP is more frequent in females. The onset of TTP is often dramatic and life-threatening, requiring immediate treatment with plasma-exchange (PEX) and/or fresh frozen plasma (FFP) infusion. If left untreated, the mortality rate of TTP is as high as 85% but correct PEX has reduced the mortality to 15-30%. No recommendations are available so far on the management of recurrent TTP. *Aim of the study.* to evaluate the efficacy of a prompt and correct PEX without further FFP infusion in the clinical outcomes of 3 pts previously diagnosed as inherited or acquired TTP during relapse. *Patients and Methods.* case 1: a 43 years old woman with acquired TTP associated with neurological symptoms; case 2: a 41 years old woman with acquired TTP with urinary tract infection; case 3: a 59 years old woman with inherited defects of ADAMTS13 with TTP relapse postcolecistectomy. Baseline haemoglobin at onset were 10.6, 9.8, 10.2 g/dL while platelet count were 15, 4, 12×10^9/L in cases 1, 2 and 3 respectively. ADAMTS13 values were always <10 % (normal range: 50-130%) while VWF measured as collagen binding assay (VWF:CB)/VWF antigen (VWF:Ag) were 270/225, 320/295 and 380/320 U/dL (normal range: 54-156U/dL). At the first onset, a mean of 9 PEX procedures plus 3.5 mean U of FFP were used to induced their remission. At TTP relapse, patients underwent PEX at early first clinical symptoms and initial reduction in platelet count and followed up with lab tests during their recurrent TTP episodes. *Results.* All the 3 patients had their recurrent TTP episodes between 2005 and 2009 and immediate diagnosis of relapse was based on rapid reduction of platelet count down to 112, 86 and 95×10^9/L associated with fever, infection or surgery. At relapse, a mean of 5 PEX procedures were sufficient to induce remission without any other FFP infusion in all 3 cases and all patients responded with a rapid platelet increase (375, 170, 220×10^9/L) and clinical improvement. ADAMTS-13 activity was increased (35, 40, 45%) and VWF activities reduced with VWF:CB/VWF:Ag values of 115/170, 130/158, 110/170 U/dL. *Conclusions.* Our clinical data obtained in such a small cohort of cases confirm that prompt and correct PEX procedures are essential for improving the outcomes of patients with relapsed TTP. A 24-48 hours delay in initiating PEX treatment might worsen their prognoses or at least might require an increased number of PEX and/or plasma products.

Hematopoietic Stem Cells I

P108

IDENTIFICATION OF MOLECULAR BASIS OF ANTITHROMBIN DEFICIENCY: CLINICAL FEATURES OF 24 INVESTIGATED PROBANDS AND REPORT OF EIGHT NOVEL MUTATIONS

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Introduction. More than 200 mutations of antithrombin (AT) gene cause type I (quantitative) or type II (qualitative) deficiency (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1). Type II is subclassified according to dysfunction of the reactive site (RS) or heparin binding site (HBS) or pleiotropic effects. Type II HBS is associated with a low thrombotic risk. *Aims.* To investigate the gene mutations causing AT deficiency. *Patients and methods.* DNA of 17 patients with AT deficiency and venous thromboembolism (VTE) was sequenced according to Picard *et al*. (Thromb Haemost 2005;93:57). Seven asymptomatic women with AT deficiency identified by screening before pregnancy or oral contraceptive intake were also analyzed. *Results.* Fifteen different mutations were identified in 17 patients with VTE. Two novel (W307X, Y260_P352del in two cases) and five known (C-4X, A94V, R129X in two cases, R132X, R425QfsX8) mutations were found in nine heterozygous patients with type I deficiency. One novel (E265K) and one known (M251I) mutations were found in two heterozygous patients with type II RS deficiency; three known mutations (L270P, A404T, L409P in two cases) were found in four heterozygous patients with pleiotropic (quantitative-qualitative) AT deficiency. For one novel heterozygous mutation (E205K) the phenotype was not characterized. Finally, one patient with type I deficiency was double heterozygous for two novel mutations, L210PfsX43 and G2R; familial analysis showed type I deficiency in the father heterozygous for L210PfsX43 and normal AT level and activity in the mother heterozygous for G2R. Among the seven asymptomatic women, three had type I deficiency due to two novel (S250IfsX16 and V303CfsX13) and one known (C-4X)heterozygous mutations. Four had type II HBS deficiency due to three known heterozygous mutations (R47H, R47C, L99F in two cases). In four pregnant women (one with type I and three with type II HBS deficiency) antithrombotic prohylaxis was tailored according to phenotype. Conclusions: The molecular bases of AT deficiency are heterogeneous; their identification can provide data to understand AT structure-function and to give advice for antithrombotic prophylaxis tailored according to different AT deficiency subtypes.

P109

THE IMID CC-4047 (POMALIDOMIDE) INHIBITS MYELOMA-INDUCED OSTEOCLAST FORMATION TARGETING RANKL IN THE BONE MARROW MICROENVIRONMENT

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Multiple myeloma (MM) is a plasma cell malignancy characterized by high incidence of osteolytic bone lesions due to an increase of osteoclast formation and activation that occur into the bone marrow (BM). The alteration of the RANKL/OPG ratio in BM stromal cells (BMSC) and osteoprogenitor cells in favor of the critical osteoclastogenic factor RANKL is induced by MM cells through the cell contact and it is mainly involved in MM-induced osteoclast formation. MM cells also up-regulate RANKL expression and secretion by activated T cells that contribute to the high RANKL level observed in the MM BM microenvironment. Soluble factors such as CCL3/MIP-1, IL-3 and IL-7 produced by MM cells contribute to the increase of osteoclast formation both directly and indirectly trough RANKL stimulation. Recent data suggest that thalidomide and the IMiD® immunomodulatory drug, lenalidomide, may inhibit osteoclast formation directly through blocking osteoclast maturation. In this study we have investigated the potential *in vitro* effect of another IMiD immunomodulatory compound, CC-4047 (pomalidomide) on MM-induced osteoclast formation. First we show that pomalidomide reduced osteoclast formation from the $CD14^+$ progenitor cells in the presence of RANKL and M-CSF. Secondly we found that pomalidomide significantly inhibits RANKL expression and secretion by primary BMSC and osteoprogenitor cells obtained from MM patients but not from activated T lymphocytes at concentration ranging from 2 to 100 M. In addition, pomalidomide blunted RANKL up-regulation in BMSC/osteoprogenitor cells induced by MM cells in a cell-to-cell contact co-culture system decreasing the RANKL/OPG ratio level. Consistently the pro-osteoclastogenic property of the conditioned medium of MM cells co-cultured with BMSC/osteoprogenitor cells was reduced in the presence of pomalidomide. To go further inside to the capacity of pomalidomide to blunt MM-induced RANKL/OPG imbalance in co-culture, we investigated the effect of pomalidomide on cell adhesion molecules. We show that pomalidomide significantly reduced the expression of CD49d (VLA-4), a molecule critically involved in RANKL up-regulation, by MM cells co-cultured with or without BMSC/osteoprogenitor cells. On the other hand, we did not find a significant inhibitory effect of pomalidomide on the production of soluble pro-osteoclastogenic factors as CCL3/MIP-1 , IL-3 and IL-7 by MM cells. In conclusion our data suggest that pomalidomide inhibits MM-induced osteoclast formation both directly and indirectly targeting the microenvironment through the block of RANKL overexpression induced by MM cells.

P110

EFFECTS OF VWF ON ERYTHROID DIFFERENTIATION

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Introduction. Von Willebrand Factor (VWF) is a large multimeric adhesive glycoprotein that circulates in plasma and is also found in platelets, megakaryocytes (MK), endothelial cells, and the subendothelial matrix. VWF is a well-known mediator of platelet adhesion to the vessel wall and of platelet-platelet interactions under high shear-stress conditions. VWF has also been identified as a sensitive and distinct marker for early MK and exposure of human MK to VWF at high shear rates was reported to accelerate platelet production. We recently demonstrated that VWF accelerates platelet production in a dose-dependent way, suggesting a novel and relevant regulatory role of VWF-GPIb interaction in megakaryocytopoiesis. The complexity of hematopoiesis consists in the requirement of a highly regulated progression through different steps of proliferation and maturation, that span from the self-renewal of stem cells (HSC), their commitment, the arrest of proliferation and terminal differentiation of mature, functional elements with a defined lifespan. The downstream progeny of HSC has been characterized, and lineage restricted oligopotent megakaryocyte-erithrocyte progenitors (MEP) have been identified. Cytokine and growth factors (i.e. erythropoietin – EPO – and thrombopoietin – TPO –) are essential for these functions, and act by binding to their cell-surface receptors, triggering complex cascades of intracellular signaling. We studied here the effects of VWF on erythroid differentiation. *Methods.* Primary $CD34^+$ cells were isolated from 150-200 mL of peripheral blood of healthy donors (HD) by immunomagnetic positive selection. Purified human $CD34^+$ cells were cultured up to 14 days in serum free *ex-vivo* medium (BioWhittaker, Walkersville, MD) supplemented every 3 days with 3 ng/mL of recombinant human interleukin-3 (IL-3), 40 ng/mL of recombinant human stem cell factor (SCF) and 100 ng/mL of recombinant human thrombopoietin (TPO) or 5U/mL of recombinant human erythropoietin (EPO), in the presence or absence (controls) of 10 µg/mL of purified VWF. The erythroid and megakaryocytic differentiation was evaluated both by histochemistry and flow cytometry. *Results.* VWF boosted the TPO-induced differentiation of human CD34-derived MK, as expected. The opposite effect of VWF was observed on EPO-treated CD34 cell cultures, where it blocked erythroid maturation. *Conclusion.* Our data demonstrate that VWF has opposite roles in the control of both erythropoiesis and megakaryocytopoiesis, and we hypothesize that VWF could be involved in the MEP commitment. Further experiments are needed to elucidate the effects of VWF on the common precursor.

P111

HEMATOPOIETIC STEM CELLS IN COCULTURE WITH BM STROMAL CELLS: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE HEMATOPOIETIC STEM CELL NICHE

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Introduction. Hematopoietic stem cells (HSCs) are located in the bone marrow (BM) in a specific microenvironment referred as the hematopoietic stem cell niche, which plays a pivotal role in regulating their survival, self-renewal and differentiation. In this specific microenvironment, HSCs interact with a variety of stromal cells including fibroblasts, endothelial cells, reticular cells, osteoblasts and adipocytes. According to several recent studies, osteoblasts are able to support HSCs maintenance *in vitro* through the secretion of soluble factors and cell-cell contact, whereas adipocytes have been shown to act as negative regulators of the hematopoietic microenvironment. Though several components of the stem cell niche have been identified, the regulatory mechanisms through which such components regulate the stem cell fate are still unknown. In order to address this issue, we investigated how osteoblasts and adipocytes can affect the molecular and functional phenotype of HSCs and vice versa in a coculture system. *Methods.* We set up 2 different coculture systems: $CD34^+$ cells purified from the Cord Blood (CB) in coculture with human osteoblasts isolated from the trabecular bone or with adipocytes differentiated from human $CD146^+$ bone marrow stromal cells. After coculture, $CD34^+$ cells and the hematopoietic cell fraction were separated from stromal cells and analyzed by gene expression profiling and clonogenic assay to assess how coculture with osteoblasts or adipocytes could affect the self-renewal and differentiation capacity of HSCs. On the other hand, we also assessed how the contact with $CD34^+$ cells influence the molecular and functional phenotype of the stromal cell compartment. *Results.* Our preliminary results show that osteoblasts induce a strong increase in the clonogenic capacity of $CD34^+$ cells after coculture. Moreover, clonogenic assay results show an increase of the macrophage colonies and a decrease of the erythorid ones in $CD34^+$ cells after coculture with osteoblasts. Gene expression profiling analysis will allow us to understand which signalling pathways are activated in the hematopoietic cell fraction and in the stromal compartment, therefore enabling us to identify the molecular mechanisms responsible for such biological effects. *Conclusions.* Our preliminary data suggest that osteoblasts induce an increase in the clonogenic ability of HSCs and are able to affect the differentiation capacity of $CD34^+$ cells by favouring macrophage commitment at the expense of the erythroid lineage. Gene expression profiling and analysis of the cell culture medium by means of ELISA are necessary to understand the complex mechanisms underlying the effect exerted by stromal cells on HSCs and vice versa.

P112

HDAC INHIBITOR VALPROIC ACID ALTERS MYELOID DIFFERENTIATION OF HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS

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Introduction. Modifications of chromatin such as histone acetylation and DNA methylation are important for gene expression regulation. Compounds like as histone deacetylases (HDAC) inhibitors are used in AML and MDS therapy because modify chromatin conformation by mean the deacetylation of core histone proteins, modulating gene expression patterns involved in cell growth, differentiation, and/or apoptotic cell death. Recently, the HDAC inhibitors have been shown to maintain the self renewal ability on hematopoietic stem/progenitor cells (HSPCs). However, the effects of HDAC inhibitors in changing the differentiation behaviour of normal human myeloid progenitors are poorly described. The aim of this study is clarify the interference of HDAC inhibitors on myeloid commitment and investigate molecular basis underlying biological effects on HSPCs following HDAC inhibitors treatment. *Methods.* HSPCs were treated with HDAC inhibitor valproic acid (VPA), then proliferation and differentiation capacities of VPA treated cells was studied. Moreover, to investigate changes in gene expression induced by VPA treatment on HSPCs, we performed microarray analysis of mRNA expression profile in VPA treated and untreated (CTR) $CD34^+$ cells. *Results.* First, we demonstrated VPA treatment induced H4 histone acetylation on $CD34^+$ cells. VPA treated $CD34^+$ cells showed increased number of cells in G0-G1 phase of cell cycle compared CTR cells. Immunophenotype of CD34 cells at 6 day of treatment showed a physiological decrease of CD34 expression in CTR cells; on the contrary, in the VPA treated cells CD34 expression is maintained for a longer time. Moreover, VPA $CD34^+$ cells showed higher expression of erythroid marker GPA, as well as a significant increase in the megakaryocyte marker CD41; on the contrary, granulocyte (CD66b, CD15, and MPO) and monocyte-macrophage (CD14, and CD163) markers resulted decrease in VPA $CD34^+$ cells compared with CTR cells. According to this data, the clonogenic assay showed a significant increase in the percentage of BFU-E and CFU-E in VPA $CD34^+$ cells, coupled to a decrease in CFU-G. Furthermore, a collagen-based culture system that supports megakaryopoietic progenitor growth *in vitro*, demonstrated VPA treatment induced a remarkable increase in CFU-MK. Gene expression profile analysis showed that among transcripts increased in VPA $CD34^+$ cells, there are cell cycle and self renewal related genes. Moreover, VPA treatment upregulated important genes involved in erythrocyte and megakaryocyte differentiation; on the contrary down-regulated monocyte and granulocyte marker genes. *Conclusions.* These results indicate that the VPA treatment enhances erythrocyte and megakaryocyte differentiation at the expense of granulocyte and mono-macrophage differentiation. Moreover, microarray data provide for the first time a detailed molecular support for the biological effect promoted by VPA treatment on the proliferation, self-renewal and differentiation capacities of HSPCs.

P113

CLEARANCE OF HEPATITIS B VIRUS INFECTION IN A PATIENT RECEIVING ALLOGENEIC BONE MARROW TRANSPLANTATION FROM AN HBV NAIVE DONOR

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Introduction. HBV infection is a serious complication in allogeneic BMT, especially in the presence of HBsAg positive recipients and HBsAb negative donors. We describe a case of an acute HBV infection during allo-BMT from an HBV naïve donor. Thanks to combination of antiviral therapy and donor's immunologic reconstitution, clearance of HBsAg and seroconversion have been documented and no signs of hepatitis has ever been detected. *Methods.* Humoral anti-HBV response was weekly monitored until +60, then monthly. HBVDNA was biweekly monitored until disappearance, then monthly. Lymphocyte immunophenotype ($CD3^+$ T cells, $CD3^+CD4^+$ T helper, $CD3^+CD8^+$ T cytotoxic and $CD19^+$ B cells) was performed on peripheral blood by flow cytometry every 3 months. *Results.* A 46 year-old woman underwent BMT from HLA-identical sibling in December 2008 for AML. Donor's HBV serologic and molecular analysis were negative. A months before BMT recipient's HBV serology showed HBsAb positivity and HBV DNA was negative. Previous HBV infection was supposed because the patient has never received vaccine. Myeloablative conditioning regimen included Busulfan and Cyclophosphamide. GVHD prophylaxis consisted with Methotrexate and Cyclosporine A which was tapered on day +90 and discontinued on day +120. No signs of GVHD have ever been detected. During conditioning regimen, HBV serology monitoring revealed HBsAg positive, HBsAb negative and HBVDNA was also positive. Sexually and transfusion transmitted HBV infections were ruled out and a reactivation of an occult HBV infection was suggested. Entecavir (0,5 mg daily) was promptly started. 2.1×10^6 per kg $CD34^+$ and 4.38×10^7 per kg $CD3^+$ cells were infused and neutrophil engraftment was documented on day +24. Early donor complete chimerism was documented. On day +60 clearance of HBsAg was documented, an increase of HbsAb titer was showed, HBcAb total has been positive and HBVDNA was negative. HBcAb IgM achieved the maximum level on day + 90 and then progressively reduced until disappearance on day +365. Prevalent T cell population has ever been $CD3^+CD8^+$ T cells and B cells progressively increased. At the last follow up (day +365), Entecavir was continued and a sustained humoral

response has been confirmed. No signs of hepatitis have ever been detected. *Conclusions.* Liver disease has never developed probably because of promptly start of pre-emptive therapy and the degree of immune competence during both immunosuppression and immunological reconstitution. Immunosuppression may attenuate immune mediated cytolisis of the virus laden liver cells and, in the absence of adoptive transfer immune, the effective donor immune response could play a role in the clearance of HBsAg and seroconversion. Even if HBV infection is resolved, the risk of reactivation remains high. Entecavir will be discontinued at least 12 months after the withdrawal of immunosuppression and viral serologic markers and liver tests will be strictly monitored. *Acknowledgments.* This work was supported by Progetto Regione Lombardia.

Table 1. Serological test results for Hepatitis B Virus (HBV), HBV DNA levels, lymphocyte subsets (CD3⁺ T cells and CD3⁺CD8⁺ T cells), aspartate transaminase (AST), alanine transaminase (ALT) levels and antiviral therapy during clinical course of HBV infection in an allogeneic bone marrow transplantation recipient.

Time	*Days before transplantation*		*Days after transplantation*			
	- 30	- 7	+ 60	+90	+180	+365
Serological test results HBsAg	-	+	-	-	-	-
HBsAb (mUI/mL)	+ (16)	-	+ (17)	+ (460)	+ (> 1000)	+ (> 1000)
HBcAb total	-	-	+	+	+	+
HBcAb IgM	-	-	+ (2,5)	+ (7,7)	+ (3,9)	-
HBeAg	-	-	NA	NA	NA	NA
HBeAb	-	-	-	+	+	+
HBV DNA level (copies/mL)	< 2000	880.067	< 2000	< 2000	< 2000	< 2000
AST level, mU/mL*	27	45	31	22	31	100
ALT level mU/mL*42	78	41	26	51	45	
CD3⁺ T cells/mL	NA	NA	740	830	1049	1260
CD3⁺CD8⁺ T cells/mL	NA	NA	430	560	660	810
Antiviral therapy (Entecavir 0.5 mg daily)	No	No	Yes	Yes	Yes	Yes

*Note: HBsAg hepatitis B surface antigen ; HBsAb antibody to hepatitis B surface antigen ; HBcAb hepatitis B core antibody; HBeAg hepatitis B envelope antigen; HBeAb antibody to hepatitis B envelope antigen; NA not available; +, positive; -, negative; * normal value 5-50 mU/mL.*

P114

LABORATORY AND CLINICAL PREDICTIVE FACTORS OF ACUTE GRAFT-VERSUS-HOST DISEASE AFTER REDUCED INTENSITY ALLOGENEIC TRANSPLANTATION OF T-CELL REPLETED HLA IDENTICAL PERIPHERAL STEM CELLS

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Introduction. Reduced intensity conditioning (RIC) has decreased the transplant-related mortality (TRM) in patients affected by relapsed/refractory haematological malignancies undergoing an allogeneic stem cell transplantation (alloSCT). Nevertheless, after RIC alloSCT from a sibling donor TRM still remains above 12-15%. A further decrease of the TRM rate is hampered by the development of acute graft-versus-host disease (GVHD) and eventually progressive chronic GVHD. *Methods.* In order to investigate the predictive factors of acute GVHD in the setting of RIC alloSCT we performed a retrospective analysis in 40 patients with the following characteristics: i) having an HLA identical sibling donor; ii) receiving T-cell repleted allogeneic peripheral stem cells; iii) taking the scheduled immune suppressive therapy without an earlier withdrawal due to progressive disease or other causes. The median age was 51 (range, 18-68). Diagnoses were the following: multiple myeloma n=12, acute myeloid leukaemia n=6, non–Hodgkin's lymphoma n=17, Hodgkin's lymphoma n=4, sarcoma n=1. The median number of previous chemotherapy was 2 (range, 0-6). The conditioning regimens and GVHD prophylaxes were: thiotepa/fludarabine/cyclophosphamide with short course methotrexate/cyclosporine, n=28; low-dose total body irradiation (TBI) +/- fludarabine with mycophenolate mofetil/cyclosporine, n=10; fludarabine/busulfan with short course methotrexate/cyclosporine, n=2. All the patients were evaluated until day +100 and acute GVHD was assessed using the international standard criteria. *Results.* The rate of acute GVHD on day +100 was 42.5%. In the analysis we included both clinical and laboratory parameters: female donor/male recipient, CMV-positive patient/CMV-negative donor, the conditioning regimen (TBI+/-Fludarabine versus others), IgG value <600 mg/dl before transplant, infused $CD34^+$ cells/kg > 8×10^6, infused $CD3^+$ T-cell/kg > 5×10^8, serum level of cyclosporine <200 ng/mL on day 0 and on day +10, an abnormal value of C-reactive protein on day +10, fever > 38°C lasting > 2 days before the engraftment. In univariate analysis the significant predictive factors of acute GVHD were IgG level <600 mg/dL (P<0.001) and the conditioning regimen other than TBI+/-Fludarabine (P=0.0088). The multivariate analysis confirmed as predictive factors of acute GVHD the IgG value <600 mg/dL (P<0.001), the conditioning regimen other than TBI+/-Fludarabine (P=0.023), but also fever >38°C before engraftment (P=0.033) and the pair female donor/male recipient (P=0.001). *Conclusions.* In conclusion, in the setting of HLA-identical T-cell repleted RIC alloSCT the intensity of the conditioning regimen, fever before engraftment, the female donor with a male recipient and hypogammaglobulinemia may be risk factors of acute GVHD. These very preliminary results need further investigation with a larger sample and in a prospective manner.

P115

A PROSPECTIVE, RANDOMIZED STUDY OF MELPHALAN, PREDNISONE, LENALIDOMIDE (MPR) VERSUS MELPHALAN (200 MG/M2) AND AUTOLOGOUS TRANSPLANTATION (MEL200) IN NEWLY DIAGNOSED MYELOMA PATIENTS: AN INTERIM ANALYSIS

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Introduction. in multiple myeloma (MM) patients, high-dose chemotherapy with haemopoietic stem-cell support improves quality of response and survival. The study questions the role of high-dose chemotherapy, when new drugs are incorporated in the conventional treatment or in the autologous transplant setting. Patients with newly diagnosed MM, who received induction with lenalidomide-dexamethasone, were randomly assigned to receive consolidation with lenalidomide-melphalan-prednisone (MPR) or tandem melphalan (200 mg/m^2) (MEL200). Progression free survival (PFS) was the primary endpoint. *Methods.* during the induction phase, all patients (N=402) received: four 28-day cycles of lenalidomide (25 mg d 1-21) and low-dose dexamethasone (40 mg d 1,8,15,22) (Rd). Cyclophosphamide plus G-CSF was used to mobilize stem cells. In the consolidation phase, patients (N=202) were randomly assigned to receive MPR [six 28-day cycles of melphalan (0.18 mg/k g d 1-4), prednisone (2 mg/kg d 1-4) and lenalidomide (10 mg d 1-21))] or MEL200 (N=200)[tandem melphalan 200 mg/m^2 with stem-cell support]. *Results.* in both groups the median age was 58 years and patient characteristics were similar. After Rd induction, at least partial response (PR) was 83%, at least very good partial response (VGPR) was 34%, including 6% complete responses (CR). The median yields of $CD34^+$ cells harvested was 8.85×10^6 $CD34^+$ cells/Kg; 94% of patients collected

the minimum dose of 2×10^6/kg CD34$^+$ cells. After 3 cycles of MPR, at least VGPR rate was 56% and CR 14%. After the first MEL200, at least VGPR rate was 52% and CR 25%. In both groups, after a median follow-up of 12 months, response rates, PFS and overall survival (OS) were similar. In a sub-study, patients were also randomized to receive aspirin or low molecular weigth heparin (LMWH) as thrombo-prophylaxis, incidence of DVT was 2% and 1%, respectively (P=.68). Induction with Rd was well tolerated: grade 3-4 adverse events were <10% and early deaths were 2% (see Table). *Conclusions.* in this study we confirm the efficacy and safety of Rd induction regimen. The quality of response achieved by Rd induction improved in both groups (MPR and MEL200), while PFS and OS were similar in the two groups.

Table. Clinical outcome after MPR or MEL200 consolidation.

	MPR	*MEL200*	*P value*
CR	14%	25%	0.19
≥VGPR	56%	62%	0.24
1-year PFS	91%	91%	0.77
1-year OS	97%	98%	0.27
G3-4 hematologic toxicity			
Neutropenia	45%	86%	0.001
Thrombocytopenia	8%	87%	0.001
G3-4 Extra-hematologic toxicity			
Infections	0%	15%	0.001
Gastrointestinal	0%	23%	0.001

P116

A LINKED DONOR RECIPIENT STUDY TO EVALUATE B AND C HEPATITIS VIRUS TRANSMISSION AND LIVER DYSFUNCTION AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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In pairs patient/donor (pt/don) with HBV$^+$ or HCV$^+$, allogeneic transplant is at high risk of developing severe HBV and HCV reactivation. The risk would be even higher if unrelated donor is used. The reduced-intensity is similar, because they are equally immunoablative. No studies have systematically determined the impact of HBV$^+$ or HCV$^+$ of don or pt. Between March 1993 and March 2010, a total of 178 pairs donor/recipient were included in the study, 151 with related donor, 27 with unrelated donor. HCV RNA and HBV DNA PCR and serologic markers, were tested for pt and don. 161 pairs (90.4%) were negative for all markers, 17 pairs (9.6%) were positive, 2 (1.1%) both don and pt, 7 (3.9%), only don was positive, 8 (4.5%) the pt was positive. About don, 5 were positive for HBV markers, 4 for HbcAb, 1 was inactive carrier, 4 were positive for HCV markers (2 HCV Ab$^+$, 2 HCV RNA$^+$); about pt, 7 were HBV positive (5 HbcAb$^+$, 1 was active carrier, 1 was inactive carrier), 1 HCV Ab$^+$, 2 had coinfection. The outcome of Hepatitis C was: in two pairs with don positive and pt negative, 1 pt is alive with infection, 1 pt early died for acute GVHD, in two pairs with don negative and pt with coinfection HBV$^+$HCV$^+$, both pts are alive in treatment with Lamuvidine or Adenofovir. In conclusion, 3 pts of 4 are alive at +11, +12, +15 years after allo-SCT, with not evidence of clinical flare of HCV infection. About Hepatitis B, in 1 pairs with HBV DNA$^+$, both pt and don, the pt was in preemptive treatment with Tenofovir, is alive with Hbsag$^+$ HBV$^-$, in 2 pairs with pts Hbsag$^+$ and don seronegative, in post transplant the pts are Hbsag$^+$ HBVDNA$^-$ and alive in treatment with Lamuvidine. In 4 pairs with don HbcAg$^+$ 2 pts, 1 in therapy with Entecavir and 1 in therapy with Lamuvidine, are not evaluable because developed aGVHD and Graft failure respectively, 2 pts in therapy with Lamuvidine are alive and the postransplant status is HbcAb$^+$, HbsAb$^-$,HbvDNA$^-$, in 4 pairs with don HbcAb$^+$ HbsAb$^+$ and the pts negative, 1 pt in treatment with Lamuvidine died early for acute liver necrosis, 3 are alive, 1 seronegative, 2 with Hbcab$^+$ and HbsAb$^+$ in preemptive treatment with Lamuvidine (2 pts) or Entecavir (1 pt). In conclusion, HBV infection and HCV infection do not seem increase the rate of aGVHD, cGVHD, VOD. HBV and HCV infection do not prohibit BMT, but hepatitis virus infection can cause liver dysfunction and active prophylaxis of hepatitis virus infection remains necessary in accordance with guidelines.

P117

EXPRESSION OF TOLL-LIKE RECEPTORS ON PERIPHERAL BLOOD CELLS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: ONGOING RESULTS OF A PROSPECTIVE STUDY

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Background and aim of the study. Emerging trends emphasize the importance of both innate and adaptive immune system in the response against infections, in the pathogenesis of autoimmune and graft-versus-host (GVHD) diseases. In the cross-talk between innate and adaptive immune system, pattern recognition receptors such as Toll-like receptors (TLRs) play a key role. TLRs recognize common protein, carbohydrate or DNA/RNA pattern motifs leading to signaling for cytokine production and T cell and dendritic cell maturation, and may act as tuners of inflammatory and immunologic reactions. Very little is known about the expression and the function *in vivo* of TLRs in patients who underwent allogeneic stem cell transplantation (SCT). The aim of this study is to evaluate the expression of TLRs on lymphocytes and monocytes in relation to infections (especially CMV and HHV-6) and the onset of GVHD. *Patients and methods.* The expression of TLRs on T cells and monocytes was analyzed by flow cytometry at day +30,+90,+180 after SCT and at the onset of GVHD. The levels of receptors for lipid-based pathogen-associated molecular patterns (PAMPs: TLR 1,2,4 and 6 surface receptors), receptors for nucleic acid based PAMPs (TLR 3,7,8 and 9 located in cytoplasmic compartments), TLR5 and, TLR10 (surface receptors) were expressed as mean fluorescence intensity (MFI). Since the beginning of the study, we have analyzed data of 12 healthy donors and 12 patients. Median age was 46 years (range, 25-64); 6 patients were male. *Results.* Acute GVHD developed in 6 patients. Patients without acute GVHD after SCT and healthy donors showed different MFI of TLR3 on T cells (5.8±1.4 *vs.* 4.2±1,05 P=0.02), of TLR4 on monocytes (26.1±1.01 *vs.* 15.8±4,9 P=0.004), and of TLR6 on T-lymphocytes (7.3±3.2 *vs.* 4,6±1.1 P=0.02) and monocytes (27±12.1 *vs.* 14.9±4.6 P=0.01). TLR3 expression was significantly lower on T-lymphocytes and monocytes in patients with acute GVHD than in those without GVHD (4.06±0.8 *vs.* 5.8±1.4 P=0,02; 9,3±7,2 *vs.* 38,02±30 P=0,04). The levels of TLR5 on T cells and monocytes were significantly higher in patients with acute GVHD than in healthy donors (8.4±2.1 *vs.* 6.4±1.6 P=0.04; 54.2±20.2 *vs.* 33.2±16.5 P=0.04). The rate of infections (especially CMV reactivation), clinical and transplant characteristic were not significantly different between patients with and without GVHD. *Conclusion.* We found a different expression profile of TLRs in healthy donors, in patients after SCT without acute GVHD and in those with GVHD, suggesting that the innate immune response via TLRs activation could be involved in the development of GVHD. The assessment of a larger number of patients and the still ongoing functional analysis of TLRs will be useful to understand the complex interplay between pathogens, self or non-self DNA and RNA, and the immune system after SCT. *Acknowledgements.* This work was supported by Project Regione Lombardia and COFIN 2007.

Hematopoietic Stem Cells II

P118

EVALUATION OF RANKL AND OSTEOPROTEGERIN SERUM LEVELS IN PATIENTS WITH MULTIPLE MYELOMA TREATED WITH CY-BOR

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Introduction. The alterations of bone metabolism in multiple myeloma are well defined. Effects of RANKL and Osteoprotegerin (OPG) on osteoclasts and osteoblasts activity and on the regulation of bone resorption in patients with advanced disease are well defined too. RANKL and OPG represent osteoblast-derived paracrine cytokines and they work mainly in the microenvironment marrow. In several studies, however, it is show to be the feasible to measure their serum concentrations at various stages of disease. We have previously evaluated serum levels of Osteocalcin and Parathormone in patients with multiple myeloma treated with Cy-Bor (Bortezomib, Cyclophosphamide and Dexamethasone). We observed a reduction of Osteocalcin and an increase of PTH levels early in the days following the beginning of therapy. The increase of PTH is more marked in patients receiving Zoledronate too. *Methods.* In this study we evaluated RANKL and OPG levels in the same setting of patients. We studied 13 patients at first or second course of therapy. None of them received Zoledronic Acid. *Results.* Table 1 shows the values of RANKL (expressed as absorbance) and of OPG (expressed as picomole per liter), graph 1 shows percentages of the changes relative to initial values. As reported in other studies we found a low concentration of RANKL (often near the minimum value of detection) in all samples. These data confirm that RANKL plays its role mainly in the bone marrow microenvironment and that the assays currently used have perhaps a low sensivity. Serum levels of OPG are always well evaluable, with repeatable results (all determinations are made in duplicate). The main concentration of OPG in baseline samples is 5.8 pmol/L, the mean value at second determination (day 4 of therapy) is 7.42 pmol (130% of initial value). This increase is present in about ’80% of cases. OPG levels come back to baseline in all patients at the subsequent measurements. *Conclusions.* Due to the limited number of patients involved in this study, we need to study a larger number of patients to perform a meaningful statistical evaluation. If we confirm the early increase of OPG, we could assume a early positive effect of therapy on bone metabolism with subsequent adjustment of regulatory mechanisms. Therefore we will try to identify the drug of Cy-Bor combination that acts more on the levels of OPG, and what can be the influence of association with zoledronate.

Table 1.

	Day 1	Day 4	Day 8	Day 11	Day 15
Rankl (absorbance)	183	184,7	161,8	173,6	201,3
OPG (pmol/L)	5,8	7,42	5,1	6	5,6

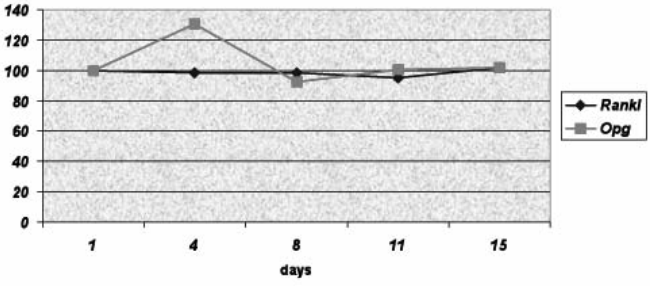

Figure. % of RANKL and OPG serum levels.

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LONG TERM EXTRACORPOREAL PHOTOCHEMOTHERAPY AFTER REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION TO PREVENT GRAFT *VS.* HOST DISEASE: PRELIMINARY DATA OF A PHASE II PROSPECTIVE STUDY

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Introduction. Extracorporeal photopheresis (ECP) is well recognized to be effective in the treatment of steroid refractory/dependent graft versus host disease (GVHD). So far, only few data exist about the role of ECP in preventing acute and/or chronic GVHD. Aim of our study is to prospectively evaluate whether a long term ECP schedule given prophylatically after reduced intensity conditioning (RIC) allogeneic stem cell transplantation (SCT) can reduce (i) GVHD incidence/severity and (ii) the need of steroid treatment during the first year after transplant. *Methods.* According to the study protocol, 28 consecutive patients submitted to RIC allogeneic SCT will be enrolled, soon after the falling of cyclosporine blood level below 100 mg/dL after dosage tapering. Patients need to be free from disease recurrence and active GVHD to be enrolled. ECP schedule is as follows: 8 weekly procedures, followed by 4 procedures every other week, followed by 8 monthly procedures, for a total of 12 months. *Results.* As yet, 4 patients have been enrolled in the study (male: n=3; female: n=1; diagnosis: lymphoblastic lymphoma: n=1; acute myeloid leukemia: n=1; myelofibrosis: n=2) after RIC allogeneic SCT (from a sibling donor: n=2; from matched unrelated donor: n=2). RIC SCT had been performed at a median of 175 days before enrollment (range: 104-182 days). A median of 8 procedures (range: 6-48) per patient have been safely administered; one patient has completed the treatment course, while another patient has been dropped out due to graft failure occurring during the first phase of treatment. Each patient is alive, and 3 out of 4 patients are in complete remission of their disease. After a median of 228 days (range: 159-533 days) from transplant, patients are free from clinically relevant acute or chronic GVHD. *Conclusions.* These preliminary data encourage the use of long-term ECP in the prevention of GVHD after RIC allogeneic SCT.

P120

CMV-DNA DETECTION BY QUANTITATIVE POLYMERASE CHAIN REACTION IN CEREBROSPINAL FLUID (CSF) IN TWO CASES OF LATE-ONSET CYTOMEGALOVIRUS ENCEPHALITIS AFTER REDUCED-INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION

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Cytomegalovirus (CMV) encephalitis is a problematic infectious complication, often considered to be rare in patients undergoing allogeneic hematopoietic stem cell transplantation (SCT). Indeed only few cases have been reported in SCT recipients. In the last 2 months, at our Center, we diagnosed 2 cases of CMV encephalitis (Patient 1 and Patient 2) that occurred in patients undergoing reduced-intensity conditioning transplantation (RIC-SCT). The underlying disease and encephalitis clinical findings are shown in Table 1. Both cases were HIV negative but their CD4 counts were lower than 100 cells/mm^3. The diagnosis of CMV encephalitis was made by detection of CMV-DNA in cerebrospinal fluid (CSF) using a quantitative polymerase chain reaction (PCR) method. To confirm the diagnosis the analysis of CSF was performed, for both cases, in 2 different laboratories. After diagnosis the value of viral load in CSF was monitored every 2 weeks during therapy (Table 1). None of two cases had a retinitis but both patients, before the onset of encephalitis, experienced repeated infectious reactivation of CMV and Pts 1 also experienced a CMV pneumonia one month after RIC-SCT. Magnetic Resonance imaging (MRI) demonstrated, in both cases, a diffuse increase of signal flair, without other brain abnormalities. In these two patients the course of this neurologic complication was unfavourable, despite a prolonged combination therapy with ganciclovir and foscavir associated with specific anti-CMV immunoglobulins. The clinical outcome was related with viral load in CSF only in Patient 1. Instead, in Patient 2, despite a decrease in CSF viral load, there was a progressive neurological deterioration. We think that this severe neurological infectious com-

plication could be more common than previously suspected and would be important, when SCT patients show persistent and/or progressive neurological symptoms to consider this disease and perform a targeted CSF study by quantitative CMV-DNA specific PCR assay. In conclusion: a)We confirm the late-onset of this infectious complication after SCT and its unfavourable outcome, probably due to a partial penetration of antiviral drugs (ganciclovir and foscavir) into CSF. b)Our patients had severe and long-lasting immune deficiency with CD4 lymphocytes count lower than 100 cells/mm^3 and received several lines of immunosuppressive therapy. Therefore, in the absence of immunological recovery, the cure of this complication is difficult, despite appropriate anti CMV specific therapy. Our data, along with those recently reported by Reddy *et al.*, strongly suggest that the diagnosis of CMV encephalitis should be considered and excluded in all SCT patients with severe immunosupression (CD4 count less than 100 cells/mm^3) who develop rapid and progressive brain dysfunction.

Table 1. Underling haematological disease and Cytomegalovirus (CMV) Encephalitis clinical findings.

	Patient 1	*Patient 2*
Patient characteristics	Male, 56 years	Male, 44 years
Underlying disease	Diffuse large B-cell Lymphoma (third complete remission)	Hodgkin Lymphoma (refractory)
Transplant characteristics	Allo SCT MUD RIC (thiotepa, Edx, Fluda)	Allo SCT MUD RIC (thiotepa, Edx, Fluda + ATG)
Status CMV, Donor/Recipient	NEG/POS	POS/POS
Onset of CMV encephalitis	7 months from SCT	8 months from SCT
Clinical manifestations	Cognitive deterioration, short-term memory deficit, lethargy, confusion	Cognitive deterioration, cranial nerve palsies, left lower extremity weakness
CSF findings at Diagnosis	CMV-DNA: 141.341 copies/mL Leukocytes: low Proteins: high Glucose: low	CMV-DNA: 2.225.735 copies/mL Leukocytes: low Proteins: high Glucose: low
Therapy	Ganciclovir plus Foscavir plus specific Immunoglobulins	Ganciclovir plus Foscavir plus Immunoglobulins
CMV-DNA copies in CSF during therapy	13.541 copies/mL (after 2 weeks) 54.021 copies/mL (after 4 weeks) 255.000 copies/mL (after 8 weeks)	1.700.000 copies/mL (after 2 weeks) 1.295.760 copies/mL (after 4 weeks) 256.000 copies/mL (after 8 weeks) 45.000 copies/mL (after 12 weeks)
Outcome	Death, 2 months from diagnosis	Alive, 3 months from diagnosis with progressive neurologic deterioration

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CONDITIONING WITH HIGH DOSE MELPHALAN (HDMEL)PLUS BORTEZOMIB IS SAFE AND ACTIVE IN MULTIPLE MYELOMA ELDERLY PATIENTS AND CAN INDUCE NEGATIVIZATION OF THE MINIMAL RESIDUAL DISEASE

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Introduction. Autologous Stem Cell Transplantation (ASCT) is recommended as part of the initial treatment strategy or at time of relapse in patients (pts) with newly diagnosed MM eligible to this procedure. Novel agents, in combination with conventional strategy, in the setting of High Dose Therapy (HDT), have improved response rates (RRs) especially in younger patients; currently, in intent-to treat (ITT) analyses, 20% to 30% of pts achieve CR with new induction regimens followed by HDT. High-dose melphalan (HDM: 200 mg/m^2) is the recommended conditioning before ASCT but, recently, synergistic effects have been reported (both *in vitro* and *in vivo*), by adding Bortezomib (BOR) to HDM. We started a phase 2 trial in MM elderly patients, fit for ASCT, combining BOR, CY and dexamethasone (DEX) as induction and mobilizing therapy (CY-BOR), followed by ASCT with supplemented BOR-HDMEL. We report preliminary results on the feasibility (in terms of safety and RRs) of this approach on an intention to treat basis. *Patients and methods.* We enrolled 30 pts (17F/13M, median age: 65 yrs, range 52-77). Pts achieving at least PR after induction (4 CY-BOR courses) were mobilized after BOR and DEX standard dose (days 1,4,8,11) with CY 3g/m^2 (day 8). Patients collecting at least 2.5×10^{6}CD34$^+$/kg underwent ASCT with HD-MEL (day -1) and BOR (1.0 mg/m^2 on -6, -3, +1, +4), followed by thalidomide consolidation until Relapse/Progression or unacceptable toxicity. *Results.* Of 25 pts evaluable for response before ASCT, 20 (80%) achieved at least PR and 18 (72%) were mobilized: 17 (68%) of them were able to mobilize ≥2.5×10^{6}CD34$^+$/kg and underwent ASCT. Median time for PMN engraftment was 11 days (range 10-13) and 14 (range 12-20) for PLT≥20.000/mcl. We did not observe any grade 3/4 infections; 2 patients experienced a grade 4 neurologic toxicity. With a minimum follow up of 197 days (median 478, range 197-814) 23 pts are alive; two died of Disease Progression. Six of the 8 patients who did not undergo ASCT are alive: 1 in CR after mobilization failure, 3 are refractory and received salvage treatment, 2 pts withdrawing consent are in VGPR and CR; all the 17 transplanted pts are alive. At day +90 after ASCT the RR was 100%: 3 CR, 9 nCR and 5 VGPR (68% on the ITT basis); 7/17 patients improved their response after ASCT. Flow cytometry evaluation of clonal plasmacells (cPC) in the harvest from 9 patients, showed that the cPCs were <0.1% in all and <0.01% in 8; 3/12 patients achieved MRD clearance at day +90 (cPC <0.01%). *Conclusions.* ASCT conditioning with HDM with BOR is feasible also in older patients, allowing to achieve a very high RR without major toxicities. We need a longer follow up and a larger number of pts to assess if these results will translate in a benefit in terms of outcome. Preliminary data on MRD should be correlated with Real time PCR analysis in negative patients (ongoing).

P122

PERCUTANEOUS LIMB BONES OSTEOPLASTY, ILEOPLASTY AND SACROPLASTY IN THE TREATMENT OF BONE LYTIC LESIONS IN ADVANCED MULTIPLE MYELOMA

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Introduction. The aim of this report is to evaluate feasibility, safety and efficacy of transcutaneous limb bones osteoplasty, ileoplasty and sacroplasty under fluoroscopic and computerized tomography (CT) guidance in the treatment of mielomatous osteolytic lesions with refractory pain and high risk of pathological fracture. *Methods.* Since November 2004 through Avril 2010 seventeen consecutive patients (nine men and eight women, mean age 68 years) with advanced multiple myeloma (stage III Durie and Salmon, ISS 3) who had developed bone lytic lesions in the pelvis and limb bones, have been treated with percutaneous bone-cement injection with an solution of polymethylmethacrylate (PMMA) into the the supracetabular (seven cases), sacral (four cases), femoral (four cases, one with bilateral intervention), humerus (one case) and tibial bone cavity (one case). Before the osteoplasty in all patients was present pain in side of lesions, valued with visual analogue scale score (VAS) system (mean value 6) and immobilization, valued with functional mobility score system in four grade (mean value 4), and was needed analgesic therapy with oppiods and FANS. The lesion approach was performed using a 10 gauge bone biopsy needle under CT and fluoroscopic guidance. Subsequently, under fluoroscopic guidance was injected 8 to 22 mL of PMMA. In a patient with a large osteolytic defect in the head and neck of right femor the tip of the needle is inserted with percutaneous transtrochanteric approach. In four cases, before the PMMA injection, a termoablation with radiofrequency of the tumor was associated. The procedures was performed using local anaesthesia and conscious sedation in all patients, under antibiotic profilaxis with ceftriaxone 2 gr i.v. The aim of osteoplasty was analgesic and of stabilization of the bone with prevention of the patological fractures, you attend in these types of patients. *Results.* Technical success was achivied in all patients. The immediate post-procedural control, with CT as not demonstrate complicance. Complete pain relief occurred whitin 1 month in 15/17 patients and a significant decrease in the visual analogue scale score (VAS score 7.5 to 1.5) occurred in 2/17. Only three patients ever need minimal oppioid therapy. During the mean follow-up of months 32 (4-60 months) the improvement of the deambulation in as been had in all patients with complete abandonment of orthopedic tutor. The mean functional mobility score scale was o after osteoplasty. *Conclusion.* Percutaneous osteoplasty of the pelvis and limb bones is feasible and safe in patients affected by myelomatous localizzations. The methymetacry-

late injection is a minimally invasive procedure that provide immediate pain relief and it contributes to reinforce the bone structure with prevention of the pathological fractures and improvement of the ambulation. This intervention contribute to treatment of the multiple myeloma in association with conventional therapy.

P123

IMMUNOHISTOCHEMESTRY ANALYSIS OF BONE MARROW BIOPSIES IN MULTIPLE SCLEROSIS PATIENTS UNDERGOING AUTOLOGOUS BONE MARROW TRANSPLANTATION

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Introduction. Multiple Sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS) determining demyelization and progressive neurodegeneration. *Methods.* Fourteen bone marrow biopsies of SM patients screened for autologous haematopoietic stem cells transplantation (HSCT) were evaluated to assess cellularity, morphology, immunological profile and microenvironmental bone marrow. The expression of the following molecules: CD3, CD4, CD8, CD20, CD45LCA, and MMP-9 was evaluated through immunohistochemestry analysis. Antibodies expression was evaluated as percentage of positive cells in a total of eight consecutive areas at 400x magnification. *Results.* We detected a normal cellularity according to their age (range 22-55 years) only in 6 patients. The percentage of B lymphocytes in MS patients was the half of the controls, whereas the CD4/CD8 ratio was 2/1 in both groups. In all MS cases the expression of MMP-9 was slightly higher than controls, with a median rate of 8.3% in patients *vs.* 5.2% in controls. We demonstrated that patients with MS showed an age-related reduction of bone marrow cellularity, probably due to previous therapies. Bone marrow T immunological profile was similar in patients and controls, whereas CD20 and CD45LCA expression was double in controls. Finally, in our study we observed an increased expression of MMP-9 in bone marrow of MS patients. *Conclusions.* In conclusion these data confirm that MS pathogenesis is not a district event limited to SNC, but a systemic process that involve all the immunological compartment. In the future, will be very interesting to study MMP9/TIMP-1 ratio in MS and changes of bone marrow micro environmental after AHSCT.

P124

REPLACEMENT OF G-CSF WITH THE DPP-IV INHIBITOR SITAGLIPTIN INDUCES BETTER LONG-TERM IMMUNE RECONSTITUTION AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA (EUDRACT 2008-005042-23)

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Introduzione. Il G-CSF, usato routinariamente per la mobilizzazione delle cellule staminali ematopoietiche dalle nicchie midollari al sangue periferico, viene anche usato dopo autotrapianto per accelerare l'attecchimento delle staminali reinfuse. Perplessità rimangono sull'effettivo effetto midollare piuttosto che di replicazione dei neutrofili periferici, e sui potenziali effetti immunosoppressivi del G-CSF. Nella ricerca di alternative, le gliptine, inibitori della dipeptidil-peptidasi IV (DPP-IV) in grado di diminuire il catabolismo della chemochina CXCL12 che guida la staminale nella nicchia ematopoietica midollare, rappresentano farmaci già approvati per l'uso nei pazienti con diabete mellito di tipo II. Alla luce di dati preliminari che mostrano come la sitagliptina (JanuviaTM, Merck & Co) sia in grado di modulare i livelli sierici di SDF-1α, è stato disegnato un trial clinico di fase I/II (spontaneo e monocentrico) con sitagliptina al posto di G-CSF per valutarne gli effetti sull'attecchimento e sulla ricostituzione immunitaria (EudraCT 2008-005042-23). *Metodi.* 10 pazienti consecutivi con mieloma multiplo da sottoporre ad autotrapianto secondo condizionamento melfalan 100 mg/m^2 (Mel100) sono stati trattati sostituendo il consueto G-CSF del giorno +1 con sitagliptina 100 mg b.i.d dal giorno -1 al giorno +10. I pazienti sono stati monitorati per necessità trasfusionali, eventi ipoglicemici (mediante stick preprandiali), livelli sierici di citochine (IL-2, IL-2R, IL-4, IL-6, IL-7, IL-10, IL-12, IL-15, IL-17, IFN-gamma, SDF-1alfa, e TNF-alfa mediante saggi ELISA), citofluorimetria su sangue periferico per sottopopolazioni linfocitarie (CD4$^+$, CD8$^+$, CD4$^+$25$^+$, CD16$^+$56$^+$, CD8$^+$57$^+$, TcRgammadelta$^+$) e per il numero di copie del virus orfano TTV mediante real-time PCR. *Risultati.* Confrontati con una coorte storica di 30 Mel100 supportati con G-CSF (simile età mediana e dose di CD34$^+$ reinfuse) praticati presso la nostra Divisione, sebbene i tempi per l'ottenimento della conta neutrofila assoluta superiore a 500/microlitro fossero più lunghi (13 *vs.* 10 giorni, rispettivamente) e non vi fosse alcuna differenza nelle cinetiche di ricostituzione piastrinica e nelle necessità trasfusionali, a partire dal giorno +70 i livelli assoluti e percentuali di linfociti CD8$^+$57$^+$ ed il numero di copie di TTV risultavano significativamente inferiori, entrambi ad indicare una miglior immunocompetenza funzionale. Nessun episodio di ipoglicemia veniva descritto durante il trattamento con sitagliptina. *Conclusioni.* Sitagliptina si è rivelata un farmaco sicuro e con potenziali effetti benefici sulla ricostituzione immunitaria a lungo termine, con possibili ricadute in termini di infezioni opportunistiche e recidive di malattia. Studi prospettici randomizzati su un maggior numero di pazienti dovranno determinare la sua effettiva superiorità sul G-CSF.

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BORTEZOMIB (BOR) AND CYCLOPHOSFAMIDE (CY) COMBINATION, FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) AND THALIDOMIDE (THAL) CONSOLIDATION IS SAFE AND FEASIBLE IN THE MAJORITY OF OLDER PATIENTS WITH MULTIPLE MYELOMA (MM)

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Introduction. BOR and CY have been associated in young MM patients (pts) with high response rate, good safety profile, without affecting PBSC collection. Combined BOR and High Dose MEL (HD-MEL) do not affect engraftment and morbidity after ASCT. Thal is active and well tolerated as consolidation after chemotherapy or ASCT. On this background we started a phase 2 study in MM elderly patients, fit for ASCT, combining BOR, CY and dexamethasone (DEX) as induction and mobilizing therapy (CY-BOR), followed by ASCT with HD-MEL+ BOR. Main end point was percentage of CR after whole program; secondary end points were clearance of Minimal Residual Disease (MRD) assessed by flow cytometry (FC), in the harvest and *in vivo*, safety, PFS and OS. *Patients and methods.* 30 pts (17F/13M, median age: 65 yrs, range 52-77) were enrolled; they received four 3 weeks courses of BOR1.3 mg/m^2+DEX 40mg/day i.v. (days 1,4,8,11)+CY i.v. 300 mg/m^2 (days 1,8,15). Pts achieving at least PR, were mobilized after BOR and DEX standard dose (days 1,4,8,11) with CY3g/m^2(day8) and G-CSF from day9. Pts collecting >2.5×10^{6}CD34$^+$/Kg underwent ASCT with HD-MEL (200 mg/m^2 day-1) and BOR (1.0 mg/m^2 days -6,-3,+1,+4). 90 days after ASCT, pts received Thal 100mg/day until relapse/progression or toxicity. Not responding pts received Lenalidomide (Lena) 25 mg/day +Dex (3 courses); those achieving at least PR were subsequently mobilized, treated as scheduled for patients responding to induction and evaluated according to ITT. *Results.* 25 pts are evaluable for response after induction: 20 responded (ORR=80%): 2PR, 11VGPR, 5nCR+2CR (28%). 5(20%) pts did not respond. Notably, 1 patient has t(4;14), 1 t(11;14) and 3 a hyperdiploid kariotype; none of them was rescued with Lena: 2 received double ASCT, obtaining VGPR and nCR and 3 received a third line therapy without success. Among 20 pts responding to CY-BOR, 2 refused ASCT and received 3 more CY-BOR. 18 were mobilized: 17 collected PBSC (median 3; range 2.5-11.2×10^6 CD34$^+$/kg) and were transplanted (68% according to ITT). All 17 are alive; 7 improved quality of response: 5VGPR and 3CR+9nCR (48%). We did not observe thromboembolic events during induction/transplant; 2 pts had grade 4 neurotoxicity,requiring reduction of BOR dose. Among 11 pts starting Thal consolidation/maintenance 4 improved the response; 5 stopped Thal due to: relapse(2),deep venous thrombosis(1), grade III neurologic toxicity(1), allergic reaction(1). No treatment related-deaths were observed. With a median follow up of 16 months(range 7-27), 23/25 (92%) are alive; 2 died of Disease Progression. EFS at 16 months is 80%. *Conclusions.* CY-BOR followed by ASCT is feasible in more than two thirds of fit elderly patients, allowing to achieve a rapid and deep response without major toxicities. A larger number of pts and a longer follow up are needed to verify both if failure of Cy-BOR is predictive of resistance to salvage with Lena and to evaluate ASCT outcome after Thal.

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FEASIBILITY, SAFETY AND THERAPEUTIC POTENTIAL OF SYSTEMIC PRE-OPERATIVE BONE MARROW-DERIVED CELL MOBILIZATION BY G-CSF TO ENHANCE OSSEOINTEGRATION OF BONE SUBSTITUTE IN HIGH TIBIAL OSTEOTOMY

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Introduction. Bone substitutes are widely used to improve bone repair in orthopaedic surgical procedures. Osseointegration is a slow process that takes place both at bone-implant interface and inside the tridimensional structure. The process might benefit from the addition of bone marrow-derived cells (BMC). In order to exploit this possible effects, a study protocol has been designed including preoperative BMC mobilization induced by granulocyte-colony stimulating factor (G-CSF). Aim of the study was to verify feasibility, safety and efficacy of BMC-mobilization in patients undergoing high tibial valgus osteotomy (HTVO). *Methods.* Twenty-four patients undergoing medial open wedge HTVO to treat genu varum were enrolled in a prospective, randomized study. The osteotomy gap was filled by hydroxyapatite and tricalciumphosphate bone graft substitute. Preoperatively, patients were randomly assigned either to receive (GROUP A) or not receive (GROUP B) a daily dose of 10 μg/kg of G-CSF for 3 consecutive days and half the dose 4 hours before surgery. BMC mobilization was monitored by flow cytometry analysis of circulating $CD34^+$ cells; WBCs were monitored as well. All patients underwent a clinical (Lysholm Score and SF-36) and X-ray evaluation preoperatively, and at 1-3-6 months since surgery. Anteroposterior standard radiographs were analyzed to compare bone structure of the osteotomy areas. The percentage of integration was estimated by 2 blinded observers. A computed tomography (CT) evaluation of the interface between host bone and bone substitute was performed at 2 months. *Results.* All patients of both groups completed the study. The most common adverse events among patients assigned to G-CSF were mild to moderate bone pain and muscle discomfort, well controlled by oral analgesics. There were no severe adverse events in both Group A and B. Mobilization of $CD34^+$ cells occurred in all patients receiving G-CSF: mean preoperative WBC and $CD34^+$ values were 39.09×10^3/μL (21,57-51,11) and 131,58/μL (29.1-404) in Group A, and 6,77 (2,8-12-06) and 7.67/μL (5,4-12) in Group B, respectively. Clinical evaluation at 1-3-6 and 12 months did not show significant differences between groups. Patients of Group A experienced pain and a slight impairment in overall performance at 1 month, while they displayed a slight increase in overall performance at 3 an 6 months compared to Group B, although the differences did not reach statistical significance. Semiquantitative radiographical evaluation revealed a higher rate of osseointegration in Groups A than in Group B at 1, 3 and 6 months; CT evaluation at 2 months showed a greater osseointegration at interface between host bone and bone graft substitute in Group A. *Conclusions.* Repeated G-CSF administrations to mobilize $CD34^+$ cells is feasible and safe in patients undergoing orthopaedic surgery. The careful radiographical evaluation suggests that mobilized BMCs may hasten bone graft substitute integration.

Chronic Myeloid Leukemia I

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MONITORING OF COMPLETE CYTOGENETIC RESPONSE (CCGR) BY INTERPHASE FLUORESCENCE IN SITU HYBRIDIZATION (I-FISH) ON PERIPHERAL BLOOD IN ELDERLY PH^+ CML PATIENTS WITH STABLE CCGR TREATED WITH INTERMITTENT IMATINIB (IM)EUDRACT NUMBER 2007-005102-42, CLINICALTRIALS.GOV NCT 00858806

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Objectives. Complete cytogenetic response (CCgR) determined by chromosome banding analysis (CBA) of marrow cell metaphases is the most solid surrogate marker of progression free survival and overall survival in the CML patients treated with IM. However, CBA requires marrow cells, which cannot be always sampled and an adequate number of banded metaphases, which cannot be always obtained. These limitations are more frequent and severe in the patients treated with IM for a long period of time. Fore these reasons I-FISH is increasingly used as a substitute of CBA once all metaphases are Ph^-, that means once the CCgR is defined by CBA. Recently, a study of GIMEMA CML WP demonstrated a significant correlation (98.4%) between the CCgR by CBA (NO Ph^+ metaphases) and CCgR by I-FISH performed on marrow cells (less than 1% of BCR-ABL + nuclei). Furthermore, I-FSH was better correlated than CBA with molecular response. *Matherial and methods.* In our study we monitored the CgR status by I-FISH on peripheral blood every 3 months. I-FISH was performed according to the technique previously described and by the use of Dual-Colour Dual Fusion or extra-signal probes (DCDF; Vysis-Abbott). The number of nuclei analyzed by I-FISH had to be more than 200. *Results.* From baseline to the 12 th month, 95% to 87 % of patients showed a I-FISH negative (0% Ph^+ cells); 1% to 6% a I-FISH ≤ 1% Ph^+ cells; and 4% to 11% a I-FISH >1% Ph^+ cells. As concern as molecular response, 99% of the patients had a major molecular response (MMR=<0.001-0.1 BCR-ABL/ABLISX 100) at the baseline. One patient had NO Ph^+ metaphases and >1% BCR-ABL + nuclei at baseline, 3, 6, 9 and 12 month and the BCR-ABL transcript level were, 0.01, 0.05, 1.31 and 0.12 respectively. Thirteen out of 50 (26%) patients who completed 12 months of InterIM (trial time), showed I-FISH variations of the percentage of BCR-ABL + nuclei (see Table 1). One patients at the baseline and the 6th month and 4 patients at the 9th and 12th months showed an I-FISH > 1% BCR-ABL cells but all of them had NO Ph^+ metaphases by CBA. In only one case (n°12) we observed a persistent increase of I-FISH >1% from the 6th month thereafter. There was other 9 cases who shoved a spot positivity of I-FIH ≤ 1%. Interestingly, we found a significant correlation between the % of BCR-ABL + nuclei and the BCR-ABL transcript levels in the 50 patients who completed the trial time (12 mo) (r=0.22; P=0.001). *Conclusions.* These data strongly suggest the I-FISH from peripheral blood could substitute CBA for monitoring CCgR in patients treated with IM. Furthermore, I-FISH from

peripheral blood significantly correlates with the molecular response. *Acknowledgments.* This work was supported in part by CML-Leukemia Net and Progetto Regione Lombardia.

Table 1. 13/50 (26%) PTS who completed 12 months showed FISH variations.

pts	Baseline	3°rd month	6° month	9° month	12° month
1	0.8	0	0	0	0
2	0	0	0	0	2
3	0	0	0	1	5
4	0	0	0	9.5	0
5	0	0	/	3	0
6	0	0	0	1.3	0.5
7	0	0	0	0.5	0
8	0	0	1	0	0
9	0	0	1	0	0
10	0	1	0	0	0
11	0	1	1	0	0
12	0	0	2.5	2.5	4
13	15	1	0	0	5

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ASSOCIATION BETWEEN ESSENTIAL THROMBOCYTHEMIA AND NON-HODGKIN'S LYMPHOMA: A RARE ENTITY. CASE REPORT AND REVIEW OF LICTERATURE

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Introduction. The coexistence of essential thrombocythemia (ET) and non-Hodgkin's lymphoma (NHL) is an extremely rare event, with only two such cases having been reported in the medical literature. We describe a 58 year old woman affected by essential thrombocytemia and high-grade B-cell NHL with only bone marrow involvement. Given the relative frequency of ET and the fact that the present case represents only the fourth reported instance of NHL developing in such patients, the coincidental ocurrence of both diseases is a possibility that cannot be excluded. *Case report.* A woman of 58 years came to our observation for an increase of platelets with normal white blood cells and hemoglobin. No evidence of disease association. Bone marrow biopsy reveled significant lymphocyte infiltration and a great number of megakaryocytes, most of which showed atypical features. Immunohistochemical analysis demonstrated that lymphocytes were CD20 and CD19 positive. The JAK2 mutation was present. A total body CT scan which proved normal, without evidence of enlarged lymph nodes or splenomegaly. On the basis of these findings, and after exclusion of possible causes of secondary thrombocytosis, the simultaneous occurrence of essential thrombocythemia and non-Hodgkin's lymphoma with the histologic and phenotypic features of a lymphocytic lymphoma was diagnosed. In view of indolence of lymphoma the patient has not yet started chemotherapy and for the TE, since the number of platelets is not high, she really only antiplatelet drug. In this moment she is only in wacth and wait. *Discussion.* ET is a chronic myeloproliferative disorder characterized by the uncontrolled proliferation of a malignant megakaryocytic with a life expectancy comparable with that of age-matched healthy population. However, the late outcome of ET patients may be negatively influenced by the occurrence of disease transformation into acute leukemia, myelofibrosis with myeloid metaplasia or myelodisplastic syndrome, or by the development of a second solid or hematologic neoplasia. In particular, the occurrence of a lymphoproliferative disorder (LPD) in ET patients has sporadically been reported and widely considered an infrequent event. The concomitant diagnosis of ET and LPD has been documented in three patients while LPD after ET diagnosis has been described in many patients. *Conclusion.* Associations between lymphoproliferative and myeloproliferative diseases are not very frequent.The evolution of such cases is generally characterized bythe suppression of one of the two conditions, and the lymphoproliferative disease generally prevails. The case described is unusual because the two haematological diagnosis of NHL and essential thrombocythemia were carried out at onset and without previous chemotherapy.

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IN CHRONIC MYELOID LEUKEMIA PATIENTS WHO ARE IN MAJOR MOLECULAR RESPONSE AFTER 12 MONTHS OF FIRST-LINE NILOTINIB THERAPY, LOW-LEVEL BCR-ABL KINASE DOMAIN MUTATIONS ARE VERY RARE

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Introduction. Bcr-Abl kinase domain (KD) mutations have been documented in a variable proportion of residual Ph^+ cells in some patients (pts) with stable response to imatinib, although their clinical significance is controversial. Nilotinib is an imatinib derivative more selective and more potent in Bcr-Abl inhibition – with reported major molecular response (MMR) rates of 81-85% at 12 months. We wondered whether low-level Bcr-Abl KD mutations are detectable in pts in MMR on nilotinib. *Methods.* We retrospectively analyzed the samples collected after 12 months of therapy from 8 pts enrolled on the GIMEMA CML working party study of nilotinib 400 mg BID as frontline treatment of CML. These pts had all achieved MMR (>3-log reduction in Bcr-Abl transcript according to the IS) between 3 and 6 months from nilotinib start and had a Bcr-Abl/Abl ratio ranging from 0.009%IS and 0.02%IS at the time of analysis. In all 8 pts, MMR was maintained at last follow up (24 months after nilotinib start), with two pts achieving complete molecular response (CMR; >4.5-log reduction) at 18 months and one at 24 months. Pts were equally distributed across Sokal risk categories (low Sokal risk, n=3; intermediate Sokal risk, n=2; high Sokal risk, n=3). Screening for low level mutations was performed by cloning the Bcr-Abl KD (a.a.240-502) in a bacterial vector and sequencing 100 independent clones for each patient. To rule out false positive results, a mutation was considered to be present in a sample if it was detected on both strands of two or more independent clones. The KD of Abl in 3 healthy individuals was analyzed in parallel. *Results.* Our cloning and sequencing approach showed evidence of Bcr-Abl KD mutations in only 1 out of 8 pts analyzed. In this high Sokal risk patient, a Q346L mutation was detected in 3/100 independent clones, and an additional T315I mutation was present in 2 out of these 3 clones. The Q346L has never been reported in imatinib-resistant pts, neither is it among the mutants emerged in the *in vitro* random mutagenesis screenings for nilotinib-resistant mutations – hence it should be devoid of any clinical relevance. The T315I, in contrast, is known to be highly insensitive to nilotinib both *in vitro* and *in vivo*. Nevertheless, Bcr-Abl transcript level continued to decline in this patient down to CMR (24 months from nilotinib start). The remaining 7 pts scored negative for mutations – showing only evidence of some single, mutated clones as also the three healthy individuals did. *Conclusions.* Our results suggest that a) low level Bcr-Abl KD mutations seem to be very rare in pts in MMR after 12 months of nilotinib therapy – a milestone achieved by the vast majority of pts; b) as hypothesized by some authors, tyrosine kinase inhibitor-resistant mutations at low levels do not always predict for subsequent relapse and should not trigger changes in therapy. Supported by European LeukemiaNet, AIL, AIRC, PRIN, Fondazione del Monte di Bologna e Ravenna.

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IMPACT OF BCR-ABL MUTATIONS ON RESPONSE TO DASATINIB IN ELDERLY PATIENTS WITH IMATINIB-RESISTANT CHRONIC-PHASE CHRONIC MYELOID LEUKEMIA

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Introduction. Mutations of the BCR-ABL kinase domain represent a frequent cause of Imatinib (IM) resistance in chronic myeloid leukemia (CML) patients (pts). Dasatinib (DS) is highly effective against the majority of BCR-ABL mutants detected in pts failing IM. However, little is known about the incidence of mutations in elderly CML pts resistant to IM and on their impact on response to DS in this setting. *Methods.* To evaluate the role of pre-DS BCR-ABL mutations, we analysed 81 pts aged >60 years who received DS for chronic phase (CP) CML resistant to IM. Forty-five pts (55.5%) did not display any BCR-ABL mutation, while 36 (44.5%) had one or more mutations. Twenty-four different mutations were detected in the 36 mutated pts, with 4 cases displaying multiple mutated clones. Most frequent mutations were: M244V (n=7), Y253H (n=4), F317L (n=3), F359V (n=5), M531I (n=3) and H396R (n=3). Overall, these 6 mutations represented 69% of all cases. V299L and T315I mutations insensitive to DS were detected in one case each. *Results.* The mutated and non-mutated groups were comparable for sex distribution, age at DS start, concomitant diseases, medications and cause of IM resistance. DS daily starting dose was higher in mutated pts (140 mg in 24 pts, 100 mg in 11 pts, <100 mg in 1 pt) than in non-mutated ones (140 mg in 13 pts, 100 mg in 27 pts, <100 mg in 5 pts). Consequently, the dose reduction rate, owing to toxicity, was higher in the mutated pts (26/36, 72% *vs.* 19/45, 42%; P=0.01). Final DS dose was almost identical in the two groups (mean dose: 90 mg in mutated and 100 mg in non-mutated), as was the percentage of pts that suspended therapy and the rate of adverse events (including pleural effusion). When considering best response to DS therapy, non-mutated patients did significantly better than mutated ones. Complete cytogenetic responses (CCyR) and/or major/complete molecular responses (MMolR and CMolR) were obtained in 24 of the 39 evaluable non-mutated patients (61.5%) and in 11 of the 35 evaluable mutated patients (31.5%) (P=0.02). The rate of primary resistance was higher in the mutated group (16/36 *vs.* 7/45, P=0.009), and even excluding the 5 pts with mutations unresponsive to DS (i.e. 3 with F317V, 1 with V299L and 1 with T315I), mutated pts had a lower CCyR/MolR rate (11/31 *vs.* 24/39, P=0.05) and more frequently displayed primary resistance (13/31 *vs.* 7/45, P=0.02). On the contrary, secondary resistance (4/36 *vs.* 0/45), severe toxicities (4/36 *vs.* 6/45) and suspensions due to other causes (2/36 *vs.* 5/45) did not significantly differ in the two cohorts. *Conclusions.* Our data suggest that BCR-ABL mutations account for roughly half of IM resistance in elderly CP-CML pts, a proportion similar to that found in the general population. In the elderly setting, however, presence of pre-DS BCR-ABL mutations seems to reduce optimal response to therapy, in terms of lower CCyR and MolR rates.

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PLITIDEPSIN INHIBITS THE GROWTH OF CELLS HARBORING JAK2V617F MUTATION

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Introduction. Plitidepsin (Aplidin®) is a novel cyclic depsipeptide derived from the marine tunicate Aplidium albicans, currently obtained by chemical synthesis, that is under Phase II clinical development. Plitidepsin is effective against a large panel of tumor cells and improves thrombocytopenia of myelofibrotic mice, reduces the frequency of megakaryocytes (Mk), normalizes angiogenesis in the bone marrow, and prevents extramedullary hematopoiesis. *Methods.* Plitidepsin (Aplidin®) was kindly provided by PharmaMar (Madrid, Spain). Quantification of apoptotic cells was accomplished by Annexin-V-FLUOS Staining kit. Cell cycle distribution was analyzed by flow cytometry. The effects of Plitidepsin on cell lines were evaluated in liquid culture and in agar clonogenic assay. MNCs were plated in methylcellulose with cytokines. For the growth of CFU-Mk, $CD34^+$ cells were plated in Megacult collagen medium with lipids. P27 mRNA level was evaluated by RT-QPCR. Single colony genotyping was performed by JAK2 (ASO)-PCR. Protein targets were analysed by Western Blotting method. *Results.* We assessed the effects of Plitidepsin on cell lines harboring homozygous (HEL and UKE-1) or heterozygous (SET2) JAK2V617F mutation and on cells from patients (pts) with myeloproliferative neoplasms (MPN). In 6 days clonogenic assay culture, we found that Plitidepsin prevented cell growth with IC50 values of 1.5±0.05 nM for HEL, 0.5±0.03 nM for UKE-1, and 0.8±0.02 nM for SET2, that were all lower than 2.7±0.3 nM for the BCR/ABL mutated K562 cell line (P<.001 in case of UKE-1 and SET2 cells). Similar results were obtained using a WST1 cell proliferation assay. We observed that 5 and 10 nM Plitidepsin increase significantly apoptosis and cell cycle G0/G1 phase with a correspondent decrease of S phase in SET2 cells. We then evaluated the effects of Plitidepsin on the growth of BFU-E, CFU-GM and CFU-Mk from MPN pts; PMF pts presented significantly lower IC_{50} value than controls (P<.002) and PV (P<.02). We then performed single colony genotyping in presence of 1 nM Plitidepsin. In 4/5 pts we observed a reduction of JAK2-mutated BFU-E and CFU-GM colonies, especially in one we found a decrease from 27% to 5%. Finally, since a correlation between levels of p27(Kip1) and the response of tumor cells to Plitidepsin has been described, we measured p27 levels in different cell lines after exposure to Plitidepsin. We observed that p27 mRNA levels increased 15-fold and 30-fold in UKE1 and HEL cells, respectively, compared to K562 cells after 24 hr with 10 nM Plitidepsin; such an increase was mirrored by a protein content 1.2- to 2-fold greater than baseline in UKE-1 cells at 1.0 and 10 nM Plitidepsin, suggesting that JAK2V617F mutated cells responded to the drug by modulating their p27 levels. *Conclusions.* We provided evidence that Plitidepsin has *in vitro* activity against MPN cells, particularly from PMF pts.

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SINGLE COLONY GENOTYPING FOR JAK2V617F MUTATION ALLOWS BETTER DISCRIMINATION OF PTS WITH THROMBOSIS COMPARED TO ALLELIC BURDEN IN PB GRANULOCYTES

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Introduction. Presence of JAK2V617F mutation and its allelic burden in pts with essential thrombocythemia (ET), or the highest V617F burden in polycythemia vera (PV), have been associated with an increased risk of thrombosis. However, these variables do not allow to correctly categorize all of the pts, as inferred by variability in published series. One possibility is that measuring V617F burden in granulocytes (GN) does not correctly reflect extent of clonal involvement of hematopoietic progenitors (HPs). *Methods.* With the aim to evaluate whether determination of genotype distribution at the level of single HPs could allow better discrimination of ET or PV pts in regards to thrombosis, we studied 26 pts with ET and 14 with PV, all JAK2V617F mutated, half of which had history of thrombosis. *Results.* The mean V617F burden in ET pts with (w/) thrombosis was 31.4% (5-64%) and 31.4% (1%-68%) in those without (w/o); corresponding figures in PV were 71.2% (25-100%) and 60.8% (7-100%). Pts w/o thrombosis in both ET and PV were younger, but the difference

did not reach the significance level; we found no difference in leukocyte or platelet count, hemoglobin value, or gender between pts w/ or w/o thrombosis. Thus, the two populations of pts w/ and w/o thrombosis were comparable for clinical and hematological parameters, including V617F burden. We performed genotyping of single colonies (BFU-E or CFU-GM) obtained from peripheral blood mononuclear cells. A total of 775 and 664 colonies were analyzed in ET pts w/ or w/o thrombosis, respectively, and 508 and 248 in PV pts. ET pts w/ thrombosis had significantly more JAK2 mutated colonies (42%) compared to those w/o thrombosis (19%; P=0.0007), and a greater proportion of the colonies were homozygous (22% *vs.* 6%; P=0.002). These differences were maintained also considering separately BFU-E and CFU-GM; however, the proportion of JAK2V617F mutated and of homozygous colonies was greater among BFU-E than CFU-GM (56% and 38%, and 38% and 11%, respectively, for pts w/ thrombosis and 33% *vs.* 16% and 16% *vs.* 4% for pts w/o thrombosis; all P<0.01). Among PV pts, those w/ thrombosis had significantly more JAK2 mutated colonies (72%) compared to those w/o thrombosis (48%; P=0.0008), and a greater proportion of the colonies were homozygous (61% *vs.* 31%; P=0.0001). As for ET pts, the proportion of JAK2V617F mutated and of homozygous colonies was greater among BFU-E than CFU-GM (72% and 40%, and 65% and 21%, respectively, for pts w/ thrombosis and 79% *vs.* 58% and 71% *vs.* 47%, for pts w/o thrombosis; all P<0.01). *Conclusions.* These data indicate that determination of JAK2V617F allele burden in granulocytes does not appropriately mirror the extent of clonal involvement at the level of HPs, and may explain some unpredictability in the association with thrombotic risk. We also conclude that JAK2V617F mutation favors committent and/or expansion of HPs towards erythroid lineage in both ET and PV.

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INVOLVEMENT OF HEME OXYGENASE 1 IN CML CELLS RESISTANT TO IMATINIB MESYLATE

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Background. The advent of imatinib, a specific inhibitor of BCR-ABL, represents a significant medical advance in CML therapy. However, some patients become resistant or intolerant to imatinib therapy. HO is a rate-limiting enzyme in heme degradation (from hemoproteins and hemoglobin), leading to the generation of free iron, biliverdin and carbon monoxide. In the recent years, HO-1 expression has been reported as an important protective endogenous mechanism against physical, chemical and biological stress. The cytoprotective role of HO-1 has already been demonstrated for several solid tumors and acute leukemias. In addition, HO-1 is considered to play an important role as a survival molecule in CML cells, and an overexpression of HO-1 was found to inhibit apoptosis induced by imatinib. *Methods.* K562 cells were incubated for 24 hrs with imatinib 1 uM alone, or with an inductor of HO-1 (Hemin 50 uM, HE, or Cobalt protoporphyrin, CoPP, 10 uM), or with inhibitor of HO-1 activity (Tin- mesoporphyrin, TIN) or the combination of both. After drug treatment, the viability of cells was evaluated by the ATP-lite1step assay (PerkinElmer). We analyzed several kinase pathways by Taqman low density array, (Applied Biosystems, 7900 Fast Real Time PCR); in addition, we evaluated stress oxidative production by measuring the ROS formation. The results are expressed as mean ± S.E.M. and the statistical analysis was performed using student's t test. A value of P<0.05 was considered as significant. *Results.* The addition of HE or CoPP was able to overcome the inhibitory effect of IM (1 uM) on K562 cells (P<0.002) while the cytotoxic effect of IM was restored by adding an inhibitor of HO-1 (Tin- mesoporphyrin, SnMP, 10 uM) to the combination (P<0.002). The analysis of kinase pathways showed that several kinases were increased when in IM condition was added CoPP while were decreased in IM and IM+TIN treatments. The kinase genes that showed an alteration were Ephrin receptor EphA3 (EPHA3), ephrin receptor EphA5 (EPHA5), insulin receptor (INSR),VEGFR2 (KDR), leukocyte receptor tyrosine kinase (LTK), neurotrophic tyrosine kinase, receptor, type 1 (NTRK1), and SLIT and NTRK-like family, member 6 (SLITRK6), all kinase genes activating mitogenic signals (Table 1). Moreover, IM 1uM was able to increase ROS formation, and this effect was inhibit by HE or CoPP and restored in presence of TIN. *Conclusions.* In conclusion, we observed that induction of HO-1 may activate several mitogenic signals and this activity may represent a mechanism of resistance to IM.

Table 1. Relative qualification of kinase genes expression.

	EPHA3 (fold)	EPHA5 (fold)	INSR (fold)	KDR (fold)	LTK (fold)	NTRK1 (fold)	SLITRK6 (fold)
CTRL	1	1	1	1	1	1	1
Imatinib 1uM	0.03	0	0.13	0	0	0.002	0.05
Imatinib+CoPP	1.89	21	8.42	21.9	92.8	2.89	2.076
Imatinib+Tin	0.04	0.00001	0.2	0.0003	0.00004	0.6	0.01

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ROLE OF SECOND GENERATION TYROSINE KINASE INHIBITORS IN OSTEOBLASTOGENESIS

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Introduction. The BCR-ABL inhibitor imatinib is standard first-line therapy for patients with chronic myeloid leukemia (CML) and has revolutionized treatment of the disease. However, it has been demonstrated that long term treatment of CML patients with IM is associated with altered bone and mineral metabolism with reduction of bone remodeling and increase of bone mineral density. In this perspective, we and others have demonstrated that *in vitro* IM treatment can increase the capacity of hBM-MSCs to differentiate into osteogenic lineage and favour osteoblastogenesis. The mechanisms that are responsible for this effect are not fully understood but an inhibition of PDGF-R (Platelet Derived Growth Factor- Receptor beta) axis has been suspected on the basis of some *in vitro* evidences. However, it is not clear whether the effect of IM on osteoblastogenesis may be entirely due to inhibition of PDGF-R signaling. In order to evaluate if stimulation of osteoblastogenesis is a common feature of other tyrosine kinase inhibitors (TKI) approved for the treatment of patients with CML, we evaluate the osteoblatic differentiation of Mesenchymal Stem Cells derived from bone marrow (BM-MSCs) after *in vitro* treatment with Dasatinib (DA) or Nilotinib (NI) or Bosutinib (BO). *Methods.* Mesenchymal stem cells (hBM-MSCs) were obtained from bone marrow samples of normal healthy adults after informed consent, isolated by density gradient (mononuclear fraction) and cultured in standard medium (SM). hBM-MSCs was induced to differentiate in osteoblastic cells by treatment with osteogenic medium (0.2 mM ascorbic acid, 0.1 μm dexamethasone and 10 mM -glycerophosphate) (OM) with or without DA 2 nM or NI 100 nM or BO 5nM. Expression of osteoblast-associated genes such as osteocalcin (OCN), RUNX2 and Bone morphogenetic protein (BMP-2) were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) at 21 days of culture. *Results.* We found that the addition of DA and to a greater extend NI induced significant (P<0.0007) expression of osteogenic markers mRNA as compared to cultures with SM or OM only. However, treatment with BO did not induce increase of osteogenic markers as compared to controls. *Conclusion.* In conclusion, we showed that besides IM, other TKI such as DA and NI, but not BO, increase osteogenic markers (BMP-2, Runx2 and OCN) mRNA expression in BM-MSCs. Since BO differ from the other TKI because of its low affinity to PDGF-R, these experiments indicate that inhibition of PDGF-R is a major pathway in the induction of osteoblastogenesis by TKI. In this perspective, long term therapy with Bosutinib should not induce perturbation of bone and mineral metabolism and it could represent the treatment of choice for children affected by CML.

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

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Introduction. A crucial role of segmental duplications (SDs) of the human genome has been demonstrated in chromosomal rearrangements associated with several genomic disorders. Limited knowledge is yet available on the molecular processes resulting in chromosomal rearrangements in tumors. The t(9;22)(q34;q11) causing the 5'BCR/3'ABL gene formation has been detected in more than 90% of chronic myeloid leukemia (CML) cases. In 10-18% of CML patients genomic deletions were detected on der(9) chromosome next to translocation breakpoints. The molecular mechanism triggering the t(9;22) and deletions on der(9) is still unknown. Our study presents an experimental evidence of the involvement of SDs in the genesis of the t(9;22) translocation in CML and in the occurrence. *Methods.* We studied 71 cases with der(9) deletions by FISH with appropriate bacterial artificial chromosome clones. The mapping of all breakpoints revealed an evident breakpoints clustering, on both chromosomes 9 and 22, in two regions of about 2 Mb in size. Indeed, these regions contained the breakpoints detected in 90% and in 88% of patients bearing chromosome 9 and 22 deletions, respectively. Bioinformatic analysis of chromosome 9 and chromosome 22 genomic regions involved in the deletions was performed to search for features that could correlate with the breakpoints clustering. *Results.* The most striking result was the fact that both clusters contain a previously described 76-kb duplicon, shared by chromosome 9 and 22 (SD_9/22). The SD_9/22 is the only duplication located inside the breakpoints clustering region on chromosome 9, whereas the chromosome 22 clustering region harbors several duplications. A remarkable feature of the chromosome 9 clustering region was the high frequency of Alu repeats. Accordingly, as expected, the content in LINE sequences of the region was relatively low. Gene distribution analysis of chromosome 9 and 22 showed that both SD_9/22 map inside gene-poor regions. A statistically significant negative association was observed between the number of breaks and the distance from SD_9/22, on both chromosomes 9 (P=0.01) and 22 (P=0.006), respectively. The relationship between the breaks and the interspersed repeats revealed, on chromosome 9, a positive linear regression with Alu repeats (P=0.04), and a negative one with LINEs (P=0.04). Very similar conclusions were obtained by comparing the distance from the SD_9 and the Alu (P=0.03, positive) and LINE distribution (P=0.02, negative). *Conclusions.* In our study the involvement of SDs was proposed to explain the recurrent t(9;22) translocation in CML and the genomic deletions that could accompany the rearrangement. At the light of these findings, the analysis of secondary non-recurrent events could represent a new methodological approachable to identify architectural elements involved in the occurrence of recurrent primary rearrangements in human neoplasia.

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THE ROLE OF SPARC IN CHRONIC MYELOID LEUKEMIA

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Secreted protein acid and rich in cysteine (SPARC) is a multifunctional matricellular glycoprotein, also known as osteonectin and BM-40. This protein has counter-adesive properties, it has effects on cell shape, immune surveillance, and angiogenesis. SPARC is considered a tumor suppressor protein because it inhibits cell proliferation by affecting the PI3K/AKT signaling and by interfering with PDGF-B binding to its receptor. We evaluated SPARC expression (mRNA and protein) in peripheral blood of 32 CML patients at diagnosis and of 10 of them during treatment with Imatinib (IM). We observed a downregulation of SPARC at diagnosis (8 fold as mRNA and 80±2.4% as protein) in respect to healthy controls (P<0.0001). SPARC mRNA increased during IM treatment, especially in patients who reached a molecular remission (MR) (Figure 1.a). For determination of the amount of secreted protein, we analyzed serum of 11 CML patients at diagnosis and 6 patients at 6, 12, 18, 24, and 32 months of IM therapy by ELISA. Secreted SPARC was decreased at diagnosis of 7 fold compared to healthy donors (P<0.05) and increased of 10 fold at 18 months in respect to diagnosis (P<0.0001) (Figure 1.b). We also studied *in vitro* the effect of addition of human recombinant SPARC to IM in K562 leukemic cell line. Cells were exposed to 15 μg/mL of exogenous SPARC for 48 h followed by 24 h of IM 1 μM. Vitality was assessed at day 3 by ATP-lite. IM alone and SPARC induced a mortality of 25±3% and 37±2,3% respectively while their combination resulted additive (mortality of 50±3,3%). We conclude that in CML the tumor suppressor activity of SPARC is downregulated at diagnosis and it has a strong increase during treatment, especially in patients who obtain a molecular remission. In addition, SPARC may sensitize CML cells to IM treatment.

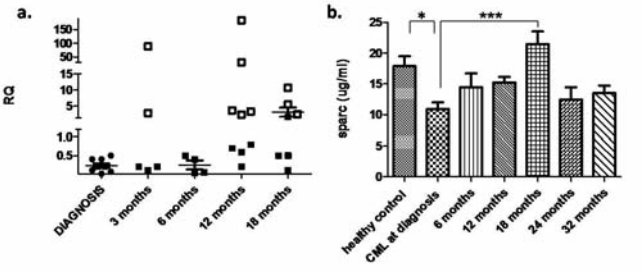

Figure 1. a. SPARC mRNA expression in CML patients at diagnosis and during IM treatment. Empty squares represents patients who reached molecular remission within 18 months of IM treatment. b. Secreted SPARC evaluated at diagnosis and during IM therapy by ELISA.

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CHRONIC EOSINOPHILIC LEUKEMIA WITH ETV6-NTRK3 FUSION TRANSCRIPT IN AN ELDERLY PATIENT AFFECTED WITH PANCREATIC CARCINOMA

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Introduction. ETV6-NTRK3 fusion oncoprotein, also known as TEL-TRKC, which functions as a constitutively active protein tyrosine-kinase, is frequently detected in non-hematologic malignancies, like congenital fibrosarcoma, congenital mesoblastic nephroma and secretory breast carcinoma. A fusion transcript variant including exons 1-4 of the ETV6 gene had so far been reported only in a single case of AML M2. The AML-associated ETV6-NTRK3 variant has been reported to confer IL-3 independent growth to the murine hematopoietic cell line Ba/F3 and to cause a rapidly fatal myeloproliferative disease in a murine bone marrow transplantation model. *Methods.* We describe the case of a 82-year old woman affected with locally advanced and metastatic pancreatic adenocarcinoma, who presented with low-grade fever, bone pain and marked fatigue. The laboratory investigations revealed leukocytosis with marked eosinophilia and thrombocytopenia. Even if an eosinophilic leukemoid reaction secondary to the metastatic pancreatic cancer was initially suspected after having excluded alternative causes of reactive eosinophilia, extensive morphological, cytogenetic and molecular examinations were performed on both peripheral blood (PB) and bone marrow (BM) samples. *Results.* The morphological examination of PB smear revealed marked eosinophilia accompanied with circulating myeloid immature precursors and blasts (3%), together with BM trephine biopsy documenting remarkable proliferation of leukemic eosinophil granulocytopoiesis. Extensive FISH and molecular studies failed to detect PDGFRA, PDGFRB and FGFR1 rearrangements, but the clonality of the myeloid population was confirmed by further cytogenetic and FISH studies. In particular, conventional G-banding showed 46,XX,-7,+8 kary-

otype, while whole chromosome painting and metaphase FISH examinations performed with specific probes showed that ETV6 gene was cryptically rearranged with NTRK3 gene. These latter results were consistent with a submicroscopic translocation t(12;15)(p13;q25). Moreover, the solid cancer-associated ETV6-NTRK3 fusion transcript variant was documented by reverse transcriptase-PCR performed on RNA isolated from the BM sample. Chronic eosinophilic leukemia not otherwise specified according to the revised WHO classification was thus diagnosed. *Conclusions.* ETV6-NTRK3 is the first fusion oncoprotein which has been identified to be expressed in multiple tumor types including those derived from mesenchymal, hematopoietic and epithelial cells. It has been proposed that ETV6-NTRK3 oncogenic activity probably relies on transforming mechanisms which are independent of lineage constraints and which do not directly affect differentiation programs. Our case description widens the spectrum of myeloid malignancies in which this rare and possibly underestimated molecular abnormality may be documented.

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PRELIMINARY DATA ON SERUM THROMBOXANE B2 (TXB2) IN ASA-RESISTANT PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS (MPN)

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Introduction. Thrombosis is the most common cause of morbidity and mortality in Essential Thrombocythemia (ET) and Polycythemia Vera (PV). ASA is efficient in reducing these complications in these patients as well as in general population. However, in general population some patients, both under primary and secondary prevention treatment, develop thrombotic complication: the Aspirin-non-responders are about 30% of treated patients. Laboratory aspirin-resistance has been found evaluating the ability of aspirin to block platelet aggregation *in vitro* and the platelet production of TxB2. At present, few is known about aspirin-resistance in patients with MPN and we found interesting to evaluate aggregation under Arachidonic Acid (AA) and serum TxB2 in MPN patients treated with ASA. *Material and methods.* We studied 64 MPN patients (mean age 60±14 y, 26 PV, 38 ET, 31M/33F, mean platelet 614±323×10^9/L) treated with Aspirin (100 mg/die)(MPN-ASA), 10 untreated MPN patients (mean age 58 ± 15 y, 3 PV, 7 ET, 6 M/4 F, mean platelet 585±120×10^9/L)(MPN-basal) and 27 healthy subjects sex and age matched, platelets 256±120×10^9/L, were used as controls (normal). In 10 of these controls the study was performed also after 7 days treatment with ASA 100 mg/day (normal ASA). We performed aggregation under AA (1mM) with Born method. TxB2 was evaluated using an ELISA kit (Cayman Chemical, USA). TxB2 values were expressed both as total serum TxB2 content (pg/mL) and as ratio between serum TxB2 and platelet number in whole blood (pg/10-8plts). Statistical analysis was performed using SPSS 16.0 software. We used the student T test to compare the mean values expressed as mean ± standard error. *Results.* Platelet counts of MPN patients were statistically higher (P=0.01) than those of the controls. 18 (29%) MPN patients were ASA-resistant on the basis of aggregation with AA, while all the controls had a normal aggregation pattern. Serum and platelet TxB2 in normal were respectively 22054 ± 4578 and 8751±1847, in MPN-basal 44033±18906 and 6156±2585, in normal-ASA 1144±162 and 421±60, in MPN-ASA 11059±1936 and 2349±649. No difference was observed comparing serum and platelets TxB2 in controls and MPN patients without ASA while, in treated cases, serum TxB2 was statistically higher (P=0.04) in MPN than in normal and the statistical significance disappeared considering platelet TxB2. *Conclusions.* Aspirin resistance in MPN patients is as frequent as in normal subjects on the basis of the data of the literature. The small number of our controls does not permit to drive definitive conclusions. However, we surmise that the higher TxB2 found in MPN patients serum is related to the high platelet number as suggested by the platelet TxB2 levels. Our data seem to confirm previous results in urine-TxB2.

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JAK2V617F MUTATION AND ALLELE BURDEN: CORRELATION WITH MORPHOLOGIC AND CLINICAL FEATURES AND RESPONSE TO THERAPY IN PATIENTS WITH ESSENTIAL THROMBOCYTEMIA

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Introduction. The activating Janus kinase 2 mutation (JAK2V617F) has been detected in most patients with Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). Notably, JAK2V617F mutation has been observed in about 50-60% of patients with ET, suggesting a biological heterogeneity of the disease. Evidence suggests that the JAK2V617F mutation is associated with higher erythropoiesis, increased risk of thrombosis and bone marrow fibrosis. *Methods and Results.* Histopathological classification of myeloproliferative neoplasms (MPN) cases was correlated with the allelic burden of JAK2V617F using direct sequencing and/or semiquantitative real time PCR allelic discrimination assay. In particular, we assessed the frequency of JAK2V617F mutation in 261 ET patients and the mutation was detected in 162 cases (62%). Clinical and laboratory findings and histomorphological features of ET patients were compared to mutational status. Patients with JAK2V617F mutation presented with significantly higher haemoglobin level and lower platelet count than wild-type patients. Where available, bone marrow (BM) biopsy from ET patients was analysed. The biopsy of cases with JAK2V617F mutation showed a higher marrow cellularity with marked erithroid hyperplasia and a smaller number of total and large megakaryocytes than wild-type cases. Interestingly, the JAK2V617F mutated cases showed a higher number of dysplastic megakaryocytes and a lower number of stag-horn and clustered megakaryocytes than wild-type cases. No difference in marrow fibrosis was seen. Finally, a correlation with thrombotic events will be studied during the follow-up of about 40 patients. *Conclusions.* Our results confirm that ET patients with JAK2V617F mutation have a PV-like phenotype (high haemoglobin level and hypercellular marrow). In addition, they show that the JAK2V617F status is strongly associated with a number of changes of the megakaryocyte lineage (hyperplasia, dysplasia, size, nuclear morphology and clustering of megakaryocytes), as detected by the BM biopsy. Therefore, both JAK2V617F mutational status and BM histology could characterize subsets of ET patients with different phenotype, although the significance of the quantification of JAK2V617F allele burden during follow-up still remains to be determined.

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IMATINIB/MELPHALAN COMBINATION INCREASES KILLING OF CHRONIC MYELOID LEUKEMIA CELLS BY DOWNREGULATION OF ANTIAPOPTOTIC GENES

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Introduction. Imatinib (IM) has become the drug of choice for first line therapy in the treatment of chronic myeloid leukemia (CML). However, a relatively small percentage of CML patients develop resistance to IM and there is a need for alternative strategies. We have already demonstrated that a sequential exposure of K562 cells to IM followed by Melphalan (MEL), induces a greater cell death: inhibition of BCR/ABL activity by the TKI increased cytotoxicity of MEL by increasing the DNA damage induced by this drug and by decreasing the time for DNA repair at the G2/M checkpoint. This synergistic activity was also confirmed in primary cells from bone marrow of 6 CML patients at diagnosis. The IM/MEL combination was the most effective treatment in reducing the total number of CFU-GM and BFU-E colonies (P<0.05 *vs.* IM alone) and especially the number of BCR/ABL copies (P<0.01 *vs.* IM). (Giallongo C. *et al.*, submitted). *Methods.* We analyzed several apoptotic pathways on K562 cells by TaqMan Low Density Arrays to evaluate the possible mechanism of action of IM/MEL combination. *Results.* Treatment with MEL alone induced expression of genes that activate caspase-8 (FASL,

FAS and FADD of 1000, 400 and 40 fold respectively) and caspase-9 (APAF and PYCARD 20 and 75 fold respectively). It also induced genes involved in activation of mitochondrial apoptotic pathway (BAD, BAK1 and BAX 7×10^{6}, 40 and 40 fold respectively) but it was counterbalanced by activation of an anti-apoptotic pathway (BCL2, BCL-xL and BIRC8 80, 12 and 50 fold respectively) and activation of genes involved in NFkB activity such as NFKB1, NFKB2, IKBKE and RELB (7, 50, 150 and 800 fold). In the TNF superfamily system MEL both up-regulated an apoptotic pathway, TRAIL (1600 fold) and its receptors DR4 and DR5 (30 and 25 fold) with consequent activation of caspase-8, and an anti-apoptotic one, TNF gene (800 fold) and its receptor TNFR2 (20 fold), leading to activation of NFKB. In addition, MEL activated the anti-apoptotic genes BIRC1 (1500 fold), BIRC3 (3000 fold) and, especially, BIRC7 (>109 fold). IM alone is able to down-regulate all above mentioned anti-apoptotic genes. Unexpectedly, IM/MEL combination did not dramatically increase expression of apoptotic genes, rather, the association silenced the anti-apoptotic signaling induced by MEL alone. *Conclusions.* The greater cytotoxic effect observed with IM/MEL combination is probably due to a shift towards the pro-apoptotic gene expression profile. In fact, MEL alone induces both pro- and anti-apoptotic genes while IM alone is able to down-regulate anti-apoptotic pathways.

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IMPACT OF LEUKOCYTOSIS ON THROMBOTIC RISK AND SURVIVAL IN 532 PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA: A RETROSPECTIVE STUDY

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Introduction. Established risk factors for thrombosis in Essential Thrombocythemia (ET) include age (≥60 years) and previous vascular events, while trombocytosis seems to play a minor role. Recently, also leukocytosis has been considered in risk stratification in ET patients (pts). *Methods.* We report a long-term retrospective study on 532 ET pts (205 males and 327 females) followed for a median of 7.6 yrs (range: 0.5-32.5). Overall, 64 pts (12%) developed 95 thrombotic events during follow-up. *Results.* The median age at diagnosis of 64 yrs (range:16-95). In all, 86% had received cytoreductive drugs, mainly hydroxyurea, and 92% antiplatelet drug (aspirin) alone (11%) or associated with cytoreductive treatment. The JAK2V617F mutation assay was carried out for 324 pts and 202 (62%) were positive. Multivariable analysis of risk factors for thrombosis confirmed the value of age and previous events but not of JAK2V617F mutation and of platelets, leukocytes, haemoglobin and hematocrite values at baseline. However, using the Cox proportional hazards model, also a WBC value of 11×10^{9}/L, corresponding to the fourth percentile value, significantly correlated with a higher thrombotic risk, together with the high risk condition (P=0.047 and P=0.002, respectively). The cumulative probability of thrombosis was significantly higher in high-risk pts with leukocyte number over the fourth percentile value (11×10^{9}/L), while low-risk pts with leukocytosis and high-risk with lower leukocyte count had the same thrombotic risk. Overall, 123 (23%) pts died at a median age of 81 yrs (range 51-100), for a median survival of 24 yrs. Three independent parameters were noted as prognostic factors for survival in multivariate analysis: age>60 yr, leukocytosis>11×10^{9}/L and haemoglobin level below normal values. Taking into account these parameters, a model for survival was constructed in order to define three groups of risk: high (3 risk factors), medium (1 or 2 risk factors) and low (no risk factors). Median survivals were 9, 16 yrs and yet undefined in the three groups, respectively (P<0.001). *Conclusions.* The inclusion of leukocytosis (>11×10^{9}/L) in risk stratification identified a cohort of low-risk pts with leukocytosis with a vascular risk comparable to that of high-risk pts with lower leukocytes. Leukocytosis also selected a subgroup of high-risk pts with a thrombotic risk significantly higher than pts classified at high risk according to standard criteria. Moreover, leukocytosis negatively affected survival. Leukocytosis seems to play a role in determining thrombosis and long-term outcome.

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MYELOPROLIFERATIVE NEOPLASMS PHILADELPHIA NEGATIVE DIAGNOSTIC WORK UP

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Introduction. The aim of this study was to create a diagnostic itinerary for Myeloproliferative neoplasms Philadelphia negative (MPN) in the Haematology Unit of Florence. *Methods.* From April 2008 to April 2010 we collected a total of 216 cases.126 cases resulted MPN. 75 male (60%), 41 female (40%). (Range 25-82 years, median age 50 years). The diagnostic itinerary included the following tests: physical examination, several blood tests, (including serum tryptase in suspect of mastocytosis), X-ray chest scan, abdominal ecography scan, bone marrow biopsy, karyotype, evaluation of BCR/abl by FISH analysis, flow cytometry (for mastocytosis), molecular biology (JAK2 mutation, MPL, cKit, FIP1L/PDGFRa). In all the bone marrow specimens we performed both a morphological study (GIEMSA, H&E), istochemestry (PERLS and Gomori silver stain) and immunohistochemestry analysis (GPA, CD34, CD61, MPO, tryptase for mastocytosis). Expression rate, growth and features of dysplasia were evaluated for all haematopoietic series (granulocytic, erythroid, megakaryocytic). For megakaryocytic lineage, megakaryocytes size, istotopography and the presence of clusters were analized. Marrow microenvironment, was characterized performing analysis of fibrosis grading, presence of sinusoids, lymphoid aggregates and fat. *Results.* On the basis of bone marrow evaluation we found the following diagnosis: 31 Primary Myelofibrosis (PM) (25%), 33 Polycytemia Vera (PV) (26%), 45 Essential Trombocitosis (ET) (36%), 17 Systemic Mastocytosis (SM) (13%). The median expression of $CD34^{+}$ cells was: 2.9% in PM, 2,6% in PV, 2,2 % in ET, 2.6% in SM. All Primary myelofibrosis showed a different grade of fibrosis, while only in two cases of PV, three cases of ET and ten cases of Mastocytosis some degree of fibrosis was detected. Bone marrow vascularisation was present in 21/31 PM cases, 5/33 PV, 3/45 TE, 3/17 mastocytosis. We analized the correlation between bone marrow results and other tests, finding a discordance with the final diagnosis in 13 cases on 116, (7 PV and 6 ET), corresponding to 11% of cases. In that discordants cases, the bone marrow biopsies resulted only in a moderate lineage hyperplasia. However, all the seven biopsies with erythropoietic hyperplasia were $JAK2^{+}$, while 1/6 ET was $JAK2^{+}$, and 6/6 MPL negative. In the five JAK2 negative ET, the final diagnosis was based on minor criteria. All mastocytosis showed a multifocal, dense infiltration of mast cells (>15%) strongly tryptase positive, and 14/17 presented cKit D816V mutation (83%). *Conclusions.* Our results showed the benefit of a diagnostic work up in terms both of diagnostic accuracy and patient satisfaction.

Chronic Myeloid Leukemia II

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STUDY OF MEGAKARYOPOIESIS AND PROPLATELET FORMATION IN MYELOPROLIFERATIVE DISORDERS

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Introduction. Myeloproliferative neoplsms (MPNs) are clonal disorders that include primary myelofibrosis (PMF), polycythemia vera (PV) and essential thrombocytemia (ET). 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 MPNs have been described, their pathogenesis is not thoroughly elucidated. The general objective of this research is to study the alterations of megakaryopoiesis in MPNs 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 25 patients diagnosed with PMF and 15 patients diagnosed with ET/PV. 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 alpha-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.* PMF Mks presented smaller size and increased nuclear/cytoplasm ratio with respect to the other MPNs and controls. Moreover, only PMF presented decreased numbers of polyploid Mks (>8N) with respect to controls. Interestingly, analysis of Mk output revealed that the percentage of $CD41^+$ cells with Mk morphology was significantly increased in pre-fibrotic PMF and ET/PV with respect to PMF that were comparable to controls. Further PPF analysis showed an important functional defect of PMF derived Mks in extending proplatelets, while ET/PV derived Mks presented increased numbers of PPF with respect to both PMF and controls. Moreover morphological analysis revealed profound differences in the architecture of PPF in between MPNs and with respect to controls. In PMF derived PPF the most evident alteration was a significantly reduced proplatelet branching. On the opposite, ET/PV derived PPF presented an enormous increase of bifurcations with respect to other MPNs and controls. Interestingly, PPF numbers and features positively correlated with platelet numbers observed in patients. *Summary and conclusions.* Mks from patients affected by MPNs presented both quantitative and qualitative abnormalities of *in vitro* differentiation and maturation. All together, these results suggested that platelet production in MPNs may not only depend from increased number of Mks, but from intrinsic differences in Mk biology and PPF.

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CHRONIC MYELOID LEUKEMIA WITH THE RARE E6A2 BCR-ABL: AGGRESSIVE DISEASE PROGRESSION AND PECULIAR GENE EXPRESSION PROFILE

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Introduction. More than 90% of patients with CML show BCR-ABL transcripts with a β3/α2 or β2/α2 fusion, translated into the p210 BCR-ABL. CML with p190 BCR-ABL or p230 BCR-ABL are less frequent. Atypical BCR breakpoints outside these cluster regions have also been described. A very rare fusion transcript joining the exons 6 of BCR to exon 2 of ABL (e6a2) has been reported in 10 cases of CML, most with an aggressive clinical course and Imatinib resistance, in single cases of CMML,T-ALL, $AMLPh^+$ and acute basophilic leukemia. We describe a case of CML with the rare BCR-ABL transcript e6a2, resistant to Imatinib treatment showing an aggressive clinical course. *Case report and methods.* A 63 -year-old man patient was referred to our hospital in July 2009 with a clinical picture consistent with CML. Cytogenetic and FISH analysis documented t(9:22); no additional chromosomal abnormalities was found. Real-time quantitative PCR (Taqman, Applied Biosystem) performed on cDNA obtained from bone marrow at diagnosis (according to EAC Protocols) did not detect MBCR-ABL. Neither m-BCR-ABL nor micro-BCR-ABL transcript were found. Because of the discrepancy of cytogenetic findings and molecular results, RT-PCR was performed using several forward primers located in various upstream exons in BCR gene (exon 2,4,6,8,9,10) and a reverse primer in ABL exon 3. PCR products obtained were directly sequenced (Big Dye chemistry, Applied Biosystems) identifying the rare e6a2 transcript variant. Imatinib was given to patient at a dosage of 400 mg/day, for about two months but it was ineffective and hydroxyurea had to be added in order to control hyperleucocytosis. We optimized a quantitative RT-PCR for detection of e6a2 transcript and molecular monitoring of the MRD: a very high level of transcript was found at diagnosis and at BCR/ABL monitoring. Mutational analysis sequencing the ABL region was performed: no mutations or polymorphisms in the ABL kinase domain were found. In September 2009 the patient was admitted to hospital with a WBC count of 220×10^9/L and five days later he died for a massive hemorrhagic stroke. cDNA from diagnosis was used for a gene expression analysis (Low-Density Arrays,TaqMan MicroFluidic Card) *Results and comments.* We identified a rare e6a2 CML associated with aggressive clinical features and Imatinib treatment resistance. In a precedent study (Cammarata G *et al.*, 2007), we investigated the molecular events involved in innate resistance to Imatinib in CML comparing the expression profile of 380 genes on "responder"(R) and "non responder" (NR) patients identifying a set of 26 genes differentially expressed. In order to understand the mechanism involved in e6a2 CML resistance to Imatinib we compared e6a2 sample to the two category (R and NR) using the subset of 26 genes. The e6a2 patient was co-clustered with R patients. When we compare the expression of all the 380 genes profiles we found a peculiar gene profile. The mechanism involved in e6a2 resistance to imatinib appears different from innately resistance observed in CML patients. Expression of e2a6 CML is quite different from all the others CML, in agree with the aggressive clinical phenotype.

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DELETION OF L3MBTL1 IN 20Q- MYELOID DISORDERS: A FUNCTIONAL ABNORMALITY

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L3MBTL1 represents the human homologue of the drosophila L(3)MBT Polycomb Group tumor suppressor gene, which is located on 20q12, within a region commonly deleted in patients with PV, MDS and AML. We demonstrated that L3MBTL1 encodes a chromatin binding protein that recognizes mono- and di-methylated lysine residues in histone H4 (K20) and histone H1 (K26) (Trojer 2007 and Kalakonda 2008). After crystallizing the structure of the MBT domains (Wang Structure 2003), we identified the ability of L3MBTL1 to compact chromatin (Trojer *et al.* Cell 2007) and to function as transcriptional repressor (Boccuni JBC 2003). We also demonstrated the expression of L3MBTL1 within the human hematopoietic $CD34^+$ cell compartment (MacGrogan 2004), suggesting that L3MBTL1 may represent a candidate target gene in 20q- disorders. To define its role in hematopoiesis we knocked down L3MBTL1 by lentiviral expression of shRNAs in primary hematopoietic stem/progenitor (i.e. $CD34^+$) cells isolated from human cord blood and also overexpressed full length L3MBTL1-HA c-DNA in three 20q- hematopoietic cell lines using retroviral infection. We observed that knock-down of L3MBTL1 promotes the premature differentiation of erythroid-committed cells and recruits more progenitor cells to differentiate toward the erythroid lineage. Consistent with this, L3MBTL1 levels decrease during hemin- or erythropoietin -induced erythroid differ-

entiation, suggesting a specific role for the downregulation of L3MBTL1 in enforcing the cell fate decision towards the erythroid lineage. L3MBTL1 knock-down enhances the sensitivity of hematopoietic cells to erythropoietin (EPO), with increased Epo-induced phosphorylation of STAT5, AKT, and MAPK as well as detectable phosphorylation of these substrates in the absence of Epo. In contrast,overexpressing L3MBTL1 in 20q- cells results in a block of erythroid differentiation and downregulation of p16INK4a, a CDKi that is normally upregulated in PV patient samples. Our data suggest that haploinsufficiency of L3MBTL1 contributes to the development of 20q- disorders, by promoting activation of pro-growth signaling pathways and by regulating the erythroid commitment of hematopoietic stem cells. Ongoing studies are aiming to investigate how L3MBTL1 functions to regulate "stemness" and the lineage differentiation of HSPCs, and how its expression cooperates with the kinases that are found to be active in patients with myeloproliferative diseases.

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PROTEOMIC MODIFICATIONS IN CHRONIC MYELOID LEUKAEMIA CELLS TREATED WITH IMATINIB AND VALPROIC ACID

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Chronic myelogenous leukaemia (CML) is characterized by the oncogenic fusion protein BCR/ABL, a constitutive active tyrosine kinase initiating several signal transduction pathways, with consequent increase of proliferation and block of apoptosis. Imatinib is an effective therapy for chronic phase CML, but patients may become irresponsive because of development of resistance caused most frequently by bcr/abl point mutations, but also by mechanisms independent from bcr/abl. Treatment of CML cells with histone deacetylase inhibitors (HDACi) induced proteasomal degradation of BCR/ABL, resulting in a synergistic effect with imatinib. It is becoming evident that deacetylases are active not only in chromatin remodelling but in a wider range of cellular activities. We demonstrated that CML human cell line LAMA Resistant to imatinib expressed a different protein phenotype respect to LAMA Sensitive cells. Treatment with imatinib plus HDACi valproic acid (VPA), induced a significant modification in the acetylome and phosphoproteome and we identified, by mass spectrometry analysis, as targets of post-translational modifications, proteins like HSP90 and hnRNPs. We confirmed here the modification of these proteins in CML human cell-line with different mechanisms of resistance to imatinib (KBM5 and KCL22) and in primary cells of 10 patients with CML during clinical treatment, at different phases of the disease. Total protein extracts were analysed by western blot with specific antibodies (anti-HSP90, HSP70, hnRNP A2/B1, hnRNP K) and, contemporary, by Two-Dimensional Electrophoresis and Mass Spectrometry analysis (MALDI-TOF). Signal transduction pathways were studied by flow-cytometry analysis: cells were fixed, permeabilized, incubated with coniugated to Alexa-Fluor488 anti phospho-ERK1/2 or anti phospho-STAT-5 antibodies and analysed by cytometer. Our preliminary results showed that in KBM5 Resistant cells, expression of HSPs and hnRNPs was not modified by treatments. After *in vitro* exposure of CML primary cells to imatinib, the expression of these proteins was extremely variable, depending on the phase of the disease and the clinical resistance to imatinib. In a case of primary resistant cells, we observed an high level of the HSP90 expression and *in vitro* treatment with imatinib and imatinib plus VPA inhibited this protein. These cells did not express hnRNP K and hnRNP A2/B1, but VPA *in vitro* exposure lead to theirs re-expression. In a case of sensitive cells, we observed, by western blot, that hnRNP K was expressed only after treatment *in vitro* with imatinib plus VPA. Moreover, the analysis by Two-Dimensional Electrophoresis showed a global shifting of proteins to lower pH after *in vitro* treatments, probably because of post-translational modifications (PTMs). Our preliminary observations indicate that the study of proteome by mass spectrometry and confirmation with western blot is important to characterize markers correlated with resistance to imatinib.

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HIGH JAK-1 MRNA LEVELS MAY BE PROGNOSTIC OF POOR RESPONSE TO INTERFERON-ALFA TREATMENT IN PATIENT WITH ESSENTIAL TROMBOCYTHEMIA

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Introduction. Interferon-alfa (IFN-α) is used in a wide range of clinical conditions such as chronic viral hepatitis, renal cell carcinoma, melanoma, lymphoproliferative and myeloproliferative disease, id est Polycythemia Vera, Essential Trombocythemia (ET) and Idiopathic Myelofibrosis. In Ph-negative chronic myeloproliferative neoplasia (MPN Ph^-), it seems the only treatment able to induce complete hematological remission and keep patients off therapy. Yet IFN-α induces different clinical responses in MPN Ph-, as happens in chronic hepatitis C. In order to predict IFN treatment responsiveness, we evaluated the expression of specific genes involved in the IFN-α Receptor pathway, which signal cross-talks with Jak-STAT pathway under erythropoietin and thrombopoietin receptors. In particular, IFN receptor mediates its signaling through the activation of two kinases (Jak-1 and Tyk-2), leading in turn to the phosphorylation of STAT-1 and STAT-3, which work as transcriptional factors for SOCS proteins. IFN-α inhibits erythropoiesis and megakaryocytopoiesis by the induction of SOCS1 and 3, which bind and directly inhibit Jak-2 and STAT-5, favoring their degradation in the proteasome. *Methods.* Jak-1, Tyk-2, STAT-1, STAT-3, SOCS1 and SOCS3 mRNA expression was explored by RT-quantitative real-time PCR using SYBR® Green detection. Data were normalized as follows: [mRNA normalized copy number (NCN)=mRNA IFN-α related gene/mRNA control gene*104]. The molecular analysis was performed on 21 bone marrow samples of patients with ET at diagnosis (according to WHO criteria) who had been classified for IFN treatment response by their levels of platelet count after six months' treatment with IFN-α at a dose of 3MU for three times a week. In particular, Rapid Responder patients (RR) were defined by a platelet count less than 400×10^9/L, unlike Slow/No Responders (SNR). *Results.* We observed a statistically significant higher level of Jak-1 expression in the 9 SNR (171113±115402 NCN) compared to the levels observed in the 12 analyzed RR (56700±15605 NCN; P=0.0008). SNR (49260±27499 NCN) had also higher level of STAT-3 expression than RR (35165±13056 NCN; P=0.01). Age, sex, spleen volume, presence of Jak-2 V617F mutation were not different between the two patient groups. Only hemoglobin levels were slightly higher in RR (P=0.05). To further evaluate our findings, we analyzed the expression levels of the above-cited genes on the patients' samples taken at follow up. After IFN- treatment SOCS1 and SOCS3 expression increased with IFN induction of STAT-1 and 3, only in RR, allowing the inhibition of megakaryocytopoiesis, independently of the mutational status of Jak-2. Instead, in SNR, SOCS1 and 3 levels decreased concurrently to Jak-1. *Conclusions.* Although these data need to be confirmed in a larger cohort of patients, we suggest that the high expression of Jak-1 plays a role in the altered pathway of SNR patients. Furthermore Jak-1 levels could represent a prediction marker of this behavior.

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DISABLED GENE IS INVOLVED IN CML PROGRESSION AND ITS EXPRESSION LEVEL AT DIAGNOSIS CAN PREDICT MAJOR MOLECULAR RESPONSE (MMR) TO IMATINIB THERAPY

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Introduction. The role of Bcr-Abl in the pathogenesis of Chronic Myeloid Leukemia (CML) is well established, however, the mechanisms involved in CML progression remain poorly understood. By making use of our model of Drosophila Melanogaster (Dm) transgenic for human Bcr-Abl we have identified Dab1 and Dab2 as genes involved in CML progression. Dab codes for a non receptor tyrosine kinase (TK) acting downstream of many RTK. Dab1 is a large common fragile site gene,

involved in neural migration, Dab2 codes for an adaptor protein implicated in RTK signalling, endocytosis, cell adhesion and, importantly in the regulation of Wnt signalling. The aim of the study was to investigate the role of Dab1/2 in CML progression. *Methods.* Dab1/2 expression was analyzed by RQ-PCR in 94 samples from 82 CML patients (34 PB and 60 BM). Among those patients 55 were collected at diagnosis (19 enrolled in TOPS study), 9 patients in CP collected at the time of TKI resistance, 7 in AP and 11 in BC, In addition 21 healthy donors (10 PB and 11 BM) were included. In 18 patients genes expression was analyzed during remission as well. Protein level was investigated by WB and Immunofluorescence. In addition, HEK293T cells were transfected with Dab to gain insight in the effects of Dab1/2 on cell proliferation. *Results.* We found that in CML patients Dab1/2 expression levels were significantly decreased in either BM or PB (P<0.002 and P<0.0004) as compared to healthy subjects. In blast crisis Dab1/2 transcript levels are increased compared to CP (P<0.001). In addition Dab1/2 expression is further increased in patients resistant to TKI therapy. In TKI sensitive patients achieving remission the transcript levels returned comparable to normal controls. Data analysis of patients included in TOPS studies shows that Dab1 values at diagnosis are lower among those achieving MMR by 12 months compared to those without MMR although this difference does not appear significant (P=0.15). By analyzing all together the CP patients enrolled in different protocols based on imatinib as front line therapy, this difference became significant (P=0.01). The protein levels, analyzed by Western Blot and immunofluorescence, demonstrated a concordance with the transcript levels. *Conclusions.* Although preliminary, our results show a significant decrease of Dab1/2 expression in CP CML thus suggesting their role in the pathogenesis mainly through the reduction of cell adhesion and by favouring proliferation. Surprisingly, patients resistant to TKI therapy or in AP and BC express higher Dab1/2 levels. We are conducting further studies to better explain these data. Since it has been demonstrated that Wnt signalling pathway is active during BC and may contribute to resistance through the expansion of the leukemic stem cell pool we can form the hypothesis that Dab1/2 are implicated in CML progression and resistance mainly for their role in activating Wnt signalling. This hypothesis will be better investigated.

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IDENTIFICATION OF RAB5 AS A GENE INVOLVED IN CHRONIC MYELOID LEUKEMIA (CML) PROGRESSION AND TKI RESISTANCE

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Background. The role of Bcr-Abl in the pathogenesis of CML is well established, however, the mechanisms involved in progression remain poorly understood. By making use of our model of Drosophila Melanogaster (Dm) transgenic for human Bcr-Abl we have identified Rab5 as a gene involved in CML progression. Rab5 gene is involved in lysosomal mediated protein recycle and degradation through the regulation of clatrin coated vesicles. Many tyrosine kinase receptors are switched off through this lysosomal mediated mechanism. In addition Rab5 mediated endosomal transport is required for Wnt signal thus suggesting a possible role in the leukemic stem cell maintenance. *Methods.* Rab5 gene expression was measured by RQ-PCR in 90 samples from 80 CML patients (32 PB and 58 BM). Among those, 53 are collected at diagnosis (19 of 53 patients have been enrolled in TOPS study). In addition, 9 samples from in CP patients have been collected at the time of imatinib (IM) resistance, 7 in AP and 11 in BC. In 14 patients, genes expression was analyzed during remission and 28 healthy donors (7 PB and 21 BM) were evaluated as control. Rab5 protein expression was investigated by WB and Immunofluorescence. HEK293T cells were transfected with Rab5 plasmid and proliferation was assessed. *Results.* We found that in CML patients Rab5 expression was significantly decreased in either BM or PB (P<0.001 and P<0.0001) compared to healthy subjects. Interestingly, Rab5 is significantly upregulated during progression of the disease (P<0.0001 and P<0.0001) and further increased in patients IM resistant (P<0.0001 and P<0.0001). At the time of remission, the transcript levels returned to normal values. The analysis of samples from TOPS trial have shown a trend that Rab5 levels are lower at diagnosis among those patients achieving MMR by 12 months, when compared to those non achieving MMR on 400 mg, but that difference was not statistically significant (P=0.2). Among those randomized to receive IM 800 mg the difference at diagnosis was statistically significant (P=0.04). Patients analyzed at the time of resistance presented significantly increased expression (P<0.0001). The protein levels, analyzed by Western Blot and immunofluorescence demonstrated a concordance with the mRNA transcript levels. *Conclusions.* Although preliminary, our results show a significant decrease of Rab5 expression in CP CML thus suggesting a role Rab5 in the pathogenesis of the disease mainly through its activity of TK recycling, including BCR-ABL. Surprisingly, patients resistant to TKI therapy or in AP and BC express higher Rab5 levels. Since it has been demonstrated that Wnt signalling pathway is active during BC and may contribute to resistance through the expansion of the leukemic stem cell pool we can form the hypothesis that Rab5 mainly regulates CML progression and resistance through its contribution in Wnt signalling activation. We are conducting further studies to better explain these data.

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

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

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BORTEZOMIB SPECIFICALLY TARGETS A SUBSET OF HYPOXIA-SELECTED CML STEM CELLS

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Introduction. We previously demonstrated that severe hypoxia selects LSC from CML or other leukaemias and that hypoxia-adaptation is a common feature of HSC and LSC. We also found that BCR/Abl protein is suppressed (although mRNA is not) in hypoxia-selected CML cells, so that these cells are independent of BCR/Abl signalling for their maintenance. Hypoxia-resistant LSC of CML thereby resulted refractory to Imatinib-mesylate (IM) due to the lack of its molecular target. This study was targeted to determine the effects of the proteasome inhibitor Bortezomib (BZ) on hypoxia-selected, IM-refractory LSC of CML. *Methods.* Cells of the stabilized CML line K562 were used. BCR/Abl protein expression was determined by SDS-PAGE/western blotting. The maintenance of stem cell potential in hypoxic (0.3% O2) primary cultures (LC1) was assessed by the Culture-Repopulating Ability (CRA) assay on the basis of the capacity of LC1 cells to repopulate secondary liquid cultures (LC2) incubated in normoxia. *Results.* The addition of BZ at time-zero (t0) of incubation in hypoxia (as well as in normoxia) markedly reduced the number of viable cells, whereas that at day 1 of hypoxia was completely ineffective. In hypoxic LC1 treated with BZ from t0, BCR/Abl suppression was faster than in untreated cultures, where BCR/Abl was still well expressed at day 2 of incubation. In cells treated with BZ at day 1, BCR/Abl suppression was delayed to day 3, indicating that it was driven by the proteasome, and also that BZ was devoid of aspecific toxicity. In hypoxia, before its suppression, BCR/Abl was cleared from cytosol to accumulate into the nucleus in its enzymatically-inactive form. Treatment with BZ at day 1 did not interfere with nuclear translocation of BCR/Abl, extending its survival therein. The CRA assay was carried out with cells recovered from hypoxic LC1 at days 2, 3 or 7. Cells recovered at day 2 started to repopulate LC2 immediately, due to the maintenance of BCR/Abl, to peak at day 10. LC2 repopulation by cells from day-3 LC1 was delayed, starting from day 7 and peaking at day 21. BZ did not alter either of these kinetics. The kinetics of LC2 repopulation by cells from day-7 hypoxic LC1 was identical to that of day-3 LC1 cells, but in this case BZ (added at either t0 or day 1) significantly reduced LC2 repopulation with respect to untreated controls. *Conclusions.* Incubation of K562 cells in hypoxia for 1 day protects completely cells bulk from the toxic effects of BZ. In hypoxia, BCR/Abl translocates into the nucleus and is suppressed, at least in part, via proteasome. BZ delays, but not prevents, disappearance of BCR/Abl from nucleus. The CRA assay defined 3 progenitor subsets: A, progenitors selected in hypoxic LC1 at day 2, BCR/Abl+ and insensitive to BZ; B, LSC selected at day 3, BCR/Abl- and insensitive to BZ; C, LSC selected at day 7, BCR/Abl- and insensitive to BZ. Thus, BZ seems selectively active on the CML progenitor subset which is typically refractory to IM.

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A NEW STATISTICAL METHOD TO STUDY STANDARD DOSE, HIGH DOSE, LOW DOSE, AND DISCONTINUATION OF IMATINIB IN PATIENTS AFFECTED BY PH' POSITIVE CHRONIC MYELOID LEUKEMIA

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Introduction. Imatinib mesylate (IM) therapy is effective in patients with chronic myeloid leukemia (CML). However, the opportunity of its discontinuation or dose variation in patients who experience sustained molecular response is debated. We describe our single institution experience in patients with undetectable or major molecular response levels of BCR-ABL transcript who reduced or discontinued IM therapy. We performed a new statistical model to study the BCR/ABL variation according to IM doses. *Methods.* Between 2000 and 2009, we have treated 89 CML patients in chronic phase. Each patient's treatment history was subdivided into time periods at constant dosage. Fifty-six patients were followed-up, for a total of 257 periods at constant dosage. At the end of each period, cytogenetic and/or molecular response were evaluated. Twenty-one progressions were recorded: 11 molecular, 5 cytogenetic and 5 of both types. The association between progression (molecular, cytogenetic or either) and treatment dose was assessed with the aid of generalized estimating equations (GEE) models, i.e. regression models designed to account for correlation due to repeated measurements over time on the same subject. *Results.* We found no association between dose and progression, not even after accounting for period length. Treatment interruption was not associated to an increased risk of progression. No association with a higher risk of progression was found for periods at reduced dosage (<400 mg/daily). 10 patients discontinued IM:4 are still in complete molecular response and out of treatment; 6 patients reassumed IM due to molecular progression but promptly reachieved molecular complete response. *Conclusions.* It is unclear whether IM can "cure" chronic myeloid leukaemia and whether this therapy can be safely stopped or if the dose can be adjusted in patients with complete cytogenetic and major molecular response. Our experience suggests that withdrawal or dose reduction of IM therapy in CP CML patients after achievement of a complete or major molecular response is not associated with a higher risk of progression. The selection of resistant clones after IM exposure and the emergence of Ph'-negative clones with secondary cytogenetic abnormalities are matter of concern, particularly in patients receiving IM for long time. The improved quality of life while off of therapy and the prompt response to resumed IM therapy suggest that the subset of patients who have sustained complete or major molecular response may be candidates for intermittent therapy. This statistical model can be applied to the therapy with second generation TK-inhibitors that are on studying for CML.

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GLUCOSE AVAILABILITY IN HYPOXIA REGULATES THE SELECTION OF CML PROGENITOR SUBSETS WITH DIFFERENT RESISTANCE TO IMATINIB-MESYLATE

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Introduction. We previously showed that clonal Chronic Myeloid Leukaemia (CML) cell lines are highly heterogeneous populations, where incubation under severe hypoxia (0.3% O2) selects hypoxia-resistant BCR/Abl-independent Leukaemia Stem Cells (LSC) and suppresses clonogenic progenitors (CFC). Accordingly, hypoxia-selected LSC exhibited complete "primary" resistance to imatinib-mesylate (IM), a feature highly relevant to the estabilishment of Minimal Residual Disease. The refractoriness of CML stem/progenitor cells (LSC/LPC) to IM was explored in relation to their resistance to hypoxia or its combination with glucose shortage, as a basis to design therapeutical approaches targeted to the long-term eradication of leukaemia. *Methods.* Hypoxic (0.3% O2) CML cultures were established with different cell densities and glucose concentrations and treated or not with IM. BCR/Abl expression was assessed by western blotting. BCR/abl transcription was determined by RT-PCR. The maintenance of stem cell potential in hypoxic primary cultures (LC1) was assessed by the Culture-Repopulating Ability (CRA) assay on the basis of the capacity of LC1 cells to repopulate secondary liquid cultures (LC2) incubated in normoxia. *Results.* By varying cell density and glucose concentration in culture, it was possible to identify hypoxia-resistant LSC/LPC subsets endowed with different kinetic properties and IM-resistance. Glucose availability in hypoxia appeared to regulate the balance between the LSC and LPC phenotypes. BCR/Abl-protein was suppressed in hypoxia following a kinetic paralleling that of glucose concentration decrease in culture medium, so that hypoxia-resistant cells maintained BCR/Abl-protein expression until glucose was available. LPC surviving merely hypoxic conditions were immediately recruitable to clonal expansion upon transfer to growth-permissive secondary cultures in normoxia. Such a property, due to the prompt rescue of BCR/Abl signaling, as confirmed using an inducible BCR/Abl expression system, was paralleled by LPC sensitivity to IM. LSC selected under hypoxia/ischemia were instead capable of delayed clonal expansion only and refractory to IM. IM-resistant LSC were also selected in hypoxia/ischemia from primary BCR/Abl-positive leukemia cells. These LSC are suitable to home *in vivo* within hypoxic stem cell niches and to represent the CML cell subset responsible for MRD and relapses of disease. *Conclusions.* We demonstrated that different combinations of hypoxia and glucose shortage differently modulate BCR/Abl expression

and result in the selection of of hypoxia-resistant, BCR/Abl-protein–positive LPC sensitive to IM, as well as of ischemia-selected, BCR/Abl-protein–negative LSC refractory to IM.

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NILOTINIB 400 MG BID IN EARLY CHRONIC PHASE PH+ CHRONIC MYELOID LEUKEMIA: RESULTS AT 2 YEARS OF A PHASE II TRIAL OF THE GIMEMA CML WORKING PARTY

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Background. Imatinib (IM) 400 mg daily is the standard treatment for Ph+ chronic myeloid leukemia (CML) in early chronic phase (ECP). According to the IRIS trial, the rate of progression to advanced phase and the rate of events are higher during the first period of treatment (during the first 2 years: 10.8% and 4.3%, respectively). Moreover, only 3% of patients who achieved a complete cytogenetic response (CCgR) progressed to advanced phase. In a phase III trial (ENESTnd, ASH Meeting 2009), nilotinib at both 300 mg and 400 mg bid induced significantly higher and faster rates of major molecular response (MMR) and CCgR compared with imatinib 400 mg daily, with a favourable tolerability profile. *Aims.* To investigate the dynamics and deepness of molecular response, the stability of responses, the outcome and the tolerability, in ECP Ph+ CML patients treated with nilotinib 400 mg BID, with a minimum follow-up of 24 months (results at 12 months have been previously published, Rosti *et al.*, Blood 2009). *Methods.* A multicentric phase II trial was conducted by the GIMEMA CML Working Party (ClinicalTrials.gov. NCT00481052). Molecular response was evaluated by RT-Q-PCR (PB), according to the international scale. Complete 24 months data will be presented on site. *Results.* 73 patients have been enrolled; median age 51 years (range 18-83), 45% low, 41% intermediate and 14% high Sokal risk. The cumulative CCgR rate within 12 months was 100%. The CCgR rate was 78% at 3 months and 96% at 6,12 and 18 months. The median duration of CCgR was 18 months. The rates of MMR at 1, 2, 3, 6, 12, 15, 18 and 21 months were 3%, 21%, 52%, 66%, 85%, 87%, 87% and 87%, respectively. At 12 and 18 months, 7% and 16% of patients tested negative with nested PCR, respectively. One patient only progressed to advanced phase (ABP) at 6 months, with T315I mutation. Adverse events (AEs) were mostly grade 1 and 2 and manageable with appropriate dose adaptations. The proportion of patients with interruptions decreased during the study: 45%, 22% and 7% during the periods 0-6 months, 7-12 months and 12-18 months, respectively. At 18 months, the last daily dose was 800 mg, 400 mg and 200 mg in 68%, 25% and 7% of evaluable patients, respectively. Four adverse events accounted for the great majority of dose interruptions: bilirubin increase, skin rash and/or pruritus, amylase and/or lipase increase, transaminases increase. The hematopoietic toxicity (grade 3-4) was negligible. Two patients went off treatment after 9 and 15 months due to recurrent episodes of amylase and/or lipase increase (no pancreatitis). *Conclusions.* At 2 years, an increasing number of patients achieved transcript-undetectable status; responses to nilotinib, either CCgR and MMR, remain stable. No progressions to advanced phase have been observed during the second year. *Acknowledgements.* European LeukemiaNet, COFIN, Bologna University, BolognAIL.

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LA MUTAZIONE A1059E IN JAK2 NEI PAZIENTI CON ERITROCITOSI IDIOPATICA SECONDARIA ALLA LEUCEMIA LINFOBLASTICA ACUTA

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Acute lymphoid leukemia (ALL) comprise a group of neoplastic diseases that arise from multiple genetic alterations. Therefore, immunophenotypic cytogenetic and molecular technique is able to identify distinct subgroups of patients often endowing specific prognostic features. In this study, we evaluated 6 adult patients (mean age 28±16.4 years) affected by pre-B ALL who, after achievement of continuous complete remission on antracyclin-containing aggressive polychemotherapy regimen, developed a significant erythrocytosis (pre-ALL-E). This patients showed sustained hematocrit values >50% often managed with phlebotomy. Eleven healthy subjects, 24 myeloproliferative syndromes, 2 CML and 3 B-ALL were also included in this study as negative control. All the known causes of secondary erythrocytosis were excluded in the 6 pre-ALL-E-patients. Indeed, despite the use of very sensitive methods such as the Surveyor DHPLC Analysis (Wave System 4500 HT-HS, Transgenomic), we did not detect the canonical V617F mutation of Jak2 gene usually found in more than 95% of patients with Polycitemia Vera (PV). Therefore, we decide to screen for mutations all genomic sequence of JAK mRNA (ex1-25) and the DNA region spanning over exons 12 to 14, where the majority of already described mutations lie. We also searched for the presence of alternative spliced Ikaros and AID isoforms in samples taken from the patients at the time of acute leukemia and subsequently during the erythrocytosis phases. Interestingly, our results showed that the five out six pre-ALL-E-patients consistently have a single novel mutation of the Jak2 gene, the A1059E, that is localized at the exon 23. Noteworthy, the protein regions encoded by exon 23 modulate Jak2 activation status and this mutation may be important to maintain JAK2 constitutively activated, thus interfering with the JAK/STAT pathway. Furthermore, four out of six patients expressed the Ik4a isoform, already known to be associated with an intrinsic genomic instability, and five out of six patients expressed a spliced isoforms of AID which also correlated with genetic instability. *Conclusions.* In this study we described a novel mutation in tyrosine kinase domain of JAK2 which seems to correlate with the erythrocytosic phenotype in patients with a previous ALL. This mutation could have occurred in the contest of an intrinsic genomic instability due to the presence of Ik4a isoform and spliced AID. The question of whether the chemotherapy could have selected a pre-existing mutated clone or the cytotoxic agents have produced the mutation directly has to be still answered.

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IMATINIB 400 MG VS 800 MG DAILY AS A FRONT-LINE TREATMENT OF SOKAL HIGH-RISK PH+ CHRONIC MYELOID LEUKEMIA: 4-YEAR RESULTS OF A PROSPECTIVE RANDOMIZED STUDY OF THE GIMEMA CML WORKING PARTY

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Background. The Sokal risk formulation, elaborated 25 years ago and based on patients treated with conventional chemotherapy, still plays a major role in chronic myeloid leukemia (CML) prognostication, even in

the imatinib (IM) era: Sokal high-risk patients need further therapeutic strategies. IM 400 mg daily is the standard treatment of early chronic phase (ECP) CML. Several observations suggested that high-dose IM may be more effective than standard doses. The European LeukemiaNet (ELN) published in 2009 the results at 1 year of a randomized study comparing 400 mg *vs.* 800 mg IM daily in ECP, untreated, Sokal high-risk patients (216 pts - Italy, Nordic Group, Turkey and Israel provided patients): briefly, patients treated with high-dose IM showed the same response rates, either cytogenetic and molecular, at each milestone. *Aims.* to describe the italian patients enrolled in the ELN study and to evaluate if patients treated with high-dose IM, notwithstanding the same level of responses in the brief term, have or not different long-term outcome. *Methods.* a subanalysis of the italian patients, enrolled within the multicentric nationwide phase 3 trial CML/022 conducted by the GIMEMA CML Working Party (ClinicalTrials.Gov NCT00514488), was performed. Cytogenetic response was evaluated by chromosome banding analysis (CBA) of marrow metaphases, and by FISH analysis of marrow cells in case of insufficient metaphase number. Molecular response was evaluated by RT-Q-PCR (PB), according to the international scale. Definitions: failures: according to European LeukemiaNet criteria; events: failure or treatment discontinuation for any reason. All the calculations have been made according to the intention-to-treat principle. Overall survival (OS), progression-free survival (PFS), failure-free survival (FFS), and event-free survival (EFS) were calculated by the Kaplan-Meier method. *Results.* Between January 2004 and April 2007, 41 Italian Hematologic Centers enrolled 112 patients: 58 patients treated with 400 mg and 54 patients treated with 800 mg IM daily. Baseline characteristics of the 2 groups were comparable. The median follow-up is currently 48 months (range 2-72 months). Either responses and outcome are detailed in table 1: no response and outcome differences between the two arms were significant at any time point. Overall, 40% of patients discontinued IM in the standard-dose arm, compared with 50% of patients in the high-dose arm. *Conclusions.* Based on an intention-to-treat analysis, this subanalysis does not suggest a significant benefit of 800 mg over 400 mg in terms of outcome and does not support the extensive use of high-dose IM (800 mg daily) in front-line therapy of high-risk ECP CML patients.

Table 1. Response and outcome.

	400 mg ARM (N=58)	*800 mg ARM (N=54)*	*P*
CCgR at 6M	66%	52%	NS
MMR at 6M	38%	30%	NS
CCgR at 12M	66%	63%	NS
MMR at 12M	43%	43%	NS
CCgR at 18M	71%	59%	NS
MMR at 18M	48%	48%	NS
4-year EFS	58%	52%	NS
4-year FFS	69%	68%	NS
4-year PFS	89%	82%	NS
4-year OS	88%	88%	NS

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INCIDENCE OF SECOND MALIGNANCIES IN 559 PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB FRONTLINE: DATA FROM THE GIMEMA CML WORKING PARTY

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Background. Imatinib, a TKI inhibitor that revolutionized CML therapy, is now approaching ten years since its commercialization. Until now, even in complete durable molecular response, discontinuation of imatinib is not recommended, and imatinib remains a life-saving drug to be taken chronically. Thus, long-term side effects represent an important issue. Roy *et al.* (Leukemia 2005) reported an unexpected incidence of second malignancies in patients treated with imatinib after interferon (6/189 patients, 3.2%; urinary tract cancer: 4/6); in contrast, an analysis performed by Novartis in 9518 patients treated with imatinib all over the world did not provided evidence for an increased overall incidence of second malignancies (Leukemia 2006). However few data are available from independent studies, and particularly in imatinib-only treated patients. On that basis we evaluated the incidence of second malignancies in patients treated with imatinib frontline, enrolled in 3 multi-centric independent trials in Italy. *Methods.* Overall, 559 patients have been enrolled between May 2003 and April 2007 in 3 concurrent clinical studies of the GIMEMA CML Working-Party: CML/021, Imatinib 800 mg in intermediate Sokal risk patients (Clin Trials Gov. NCT00514488); CML/022, Imatinib 400 mg *vs.* 800 mg in high Sokal risk patients (Clin Trials Gov. NCT00510926); CML/023, observational, Imatinib 400 mg. The median age was 52 (extr.18-84) years; 308 patients (55%) were ≥ 50 years; 66 patients were ≥70 years (12%).

Table.

Patients	*Diagnosis of CML*	*Age*	*Second Neoplasm (2nd Neop.)*	*Diagnosis of 2nd Neop. (months)*	*Distance CML-2nd. Neop*	*Status of CML*	*Alive*
1	10/2004	69	Biliar duct	11/2007	37	CCyR/MMR	No
2	10/2004	50	Breast	02/2009	52	CCyR/MMR	Yes
3	10/2005	76	Breast	05/2007	18	PCyR	No
4	09/2004	61	CNS	06/2007	33	CCyR/MMR	No
5	10/2005	60	CNS	12/2007	26	CCyR/MMR	No
6	05/2004	53	Colon	07/2004	2	n.a.	No
7	05/2005	63	Colon	03/2007	22	CCyR/MMR	No
8	03/2006	60	Colon	05/2006	2	n.a.	No
9	11/2004	56	Oesophagus	10/2006	23	CCyR/MMR	No
10	10/2005	74	Kidney	01/2007	15	CCyR/MMR	Yes
11	03/2005	64	NHL	09/2005	6	CCyR/MMR	No
12	04/2005	77	NHL	04/2006	12	CCyR/MMR	Yes
13	03/2005	64	NHL	07/2005	4	CCyR/MMR	No
14	06/2005	70	Pancreas	05/2006	11	CCyR/MMR	No

Results. With a median follow-up of 42 months (extr. 1-64 months), 14 patients (2.5%) developed a second malignancy: 3 colon cancer, 3 high grade non-Hodgkin lymphomas, 2 breast cancer, 2 brain cancer, 1 esophagous cancer, 1 biliary duct cancer, 1 pancreatic cancer and 1 bladder cancer. All patients were older than 50 years (median 63.5, extr. 50-77 years); the median follow-up of these patients is 30 months (extr. 4-53 months); four of these neoplasia (2 colon cancer and 2 NHL) were diagnosed within 6 months of the beginning of imatinib therapy; 11/14 and 10/14 patients had achieved a complete cytogenetic response and

major molecular response, respectively, at the time of the second malignancy diagnosis. 11/559 (2%) patients died for the second neoplasm progression. According to epidemiologic data (Registro Tumori) in Italy, incidence of neoplasm varies from 1% in the range of age of 50-69 years and 3% for patients over 70 years. *Conclusion.* In this population of CML patients imatinib treated, it seem that the incidence of second neoplasm do not differ from the expected incidence of neoplasm in the national population. In particular, in contrast to what previously reported, no augmented incidence of urinary tract cancers was observed.

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THROMBOTIC RISK OF JAK2V617F MUTATIONAL STATUS IN ESSENTIAL THROMBOCYTEMIA COMPARED WITH CONVENTIONAL RISK FACTORS: AN ANALYSIS OF 218 PATIENTS

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Introduction. Thromboembolic disease (TE) is a major cause of morbidity and mortality in Essential Thrombocytemia (ET). Recently JAK2V617F mutational status and allele burden seem to add prognostic significance to standard risk factors; furthermore it was reported an interesting association between baseline leukocytosis and thrombosis. The aim of the study was to evaluate such factors in a series of patients with ET. *Methods.* Patients were recruited from the Ancona Hematology Clinic database for ET; clinical and laboratory data were collected at time of diagnosis and correlated with the occurrence of TE. In addition a subset of the study patient underwent qualitative and quantitative PCR for JAK2V617F. Standard statistical methods were applied to test significance of associations between thrombosis and other variables. *Results.* 218 patients with ET were studied (median age 59, range 18-90; 71 males and 147 females). At diagnosis the median of platelets, hemoglobin and leukocytes were 776×10^9/L, 13.9g/dL and 8.4 ×10^9/L respectively. At diagnosis a history of remote thrombosis was recorded in 27 patients (12,4%). 110 patients were typed for the presence of JAK2V617F: 61(55.5%) were JAK2 wild-type and 49 (44.5%) were JAK2V617F positive. Quantitative analysis was performed in 42 cases: 6 (14.3%) were homozygotic and 36 (85.7%) heterozygotic. At diagnosis, a total of 17 patients (7.8%) had TE: 8 (3.7%) arterial thrombosis (AT) and 9 (4.1%) venous thrombosis (VT). During follow-up 25 patients experienced TE: 13 VT (6%) and 12 AT (5.5%). Gender, age, hemoglobin, platelets, leucocyte count, splenomegaly, increased cellularity of bone marrow, bone marrow fibrosis and common vascular risk factors did not affect the probability of thrombotic events at diagnosis and during follow-up. On univariate analysis a significant increase of thrombosis as presenting manifestation was registered in patients with a history of remote thrombosis (OR=10.0, 95% CI 4.1-24.5; P<0.001) and in those with JAK2V617F mutation (OR=3.73, 95% CI 1.06-13.05; P=0.032). Considering JAK2 wild-type ET as a reference group, the risk of thrombosis was 1.14 (95% CI 0.97-1.33; P=0.07) and 1.42 (95% CI 0.80-2.51; P=0.06) for JAK2V617F heterozygous and homozygous patients respectively. The patients with both JAK2 mutation and a history of remote thrombosis have a OR of 24.44 (95% CI 3.07-194.66; P=0.01) in comparison with wild-type patients without a history of thrombosis. During follow-up, vascular events were predicted again by a history of remote thrombosis (OR=2.3, 95% CI 1.01-5.35; P=0.05) but not by JAK2V617F mutational status (OR=1.24, 95% CI 0.43-3.62; P=0.76). *Conclusions.* The present analysis suggests that the thrombotic risk is higher in the JAK2V617F positive patients and is further increased by a past history of thrombosis. The opportunity of combine such criteria must be defined in prospective trials.

Myelodysplastic Syndromes

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EFFECT OF ERYTHROPOIETIN TREATMENT ON PHOSPHOLIPASE C BETA1 SIGNALLING ON LOW-RISK MDS PATIENTS

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Introduction. Phosphoinositide-phospholipase (PI-PLC) C β1, PI-PLCgamma1 and Akt are key enzymes in nuclear inositide pathways, implicated in cell cycle and differentiation, and have been demonstrated to be involved in the MDS progression to AML. Erythropoietin (EPO) is an effective treatment of anemia in 40-60% of low-risk MDS, often inducing a prolonged response. Since the activation of the EPO receptor has been correlated to the PI3K/Akt axis, and consequently to either PI-PLCbeta1 or PI-PLCgamma1 signalling, we aimed to investigate the role of lipid signalling pathways during EPO therapy. In fact, in patients who are refractory or lose response to EPO there could be a specific activation or inhibition of pathways involved in both cell cycle and differentiation. *Methods.* In this study we examined the effect of EPO treatment (40.000-80.000 U s.c., once a week) on lipid signal transduction pathways in MDS patients and cell lines. The study included 16 patients (IPSS risk: low or intermediate-1), with a favourable response to EPO in 8/16 (50%) of the cases. We firstly assessed the PI-PLCbeta1 allelic gene status, then quantified PI-PLCbeta1 mRNA and protein expression by Real-Time PCR, immunocytochemistry and flow cytometry. The effect of EPO was also evaluated in terms of β-Globin and Glycophorin A expression. *Results.* 5/16 (31%) low-risk MDS patients showed the PI-PLCbeta1 mono-allelic deletion: 2 of them had a rapid evolution into AML, whilst the remaining 3 cases were refractory to EPO treatment. Responder patients showed an increase in β-Globin and Glycophorin A expression, as well as Akt/PI-PLCgamma1 pathway, whereas most of the patients refactory to EPO displayed a slight decrease in p-Akt levels and an activation of PI-PLCβ1 signalling, so that these patients seem to counteract the lack of one PI-PLCbeta1 allele by increasing PI-PLCbeta1 expression. *Conclusions.* Our results, although obtained in a small number of cases, confirm the possible role of PI-PLCβ1 in the EPO signalling, mainly affecting cell differentiation. Moreover, our data suggest that the PI-PLCβ1 mono-allelic deletion is associated with a worse clinical outcome and/or a lack of response to EPO treatment, even in low-risk MDS patients who apparently have a good response profile for EPO (recent diagnosis, absence of long-term transfusion dependence, low or intermediate-1 IPSS risk, serum EPO levels<500 U/L). In fact, in our series, patients with the PI-PLCbeta1 mono-allelic deletion had an unfavourable outcome (either a rapid evolution into AML or refractoriness to EPO treatment). Moreover, our findings indicate that also Akt/PI-PLCgamma1 pathways are critical for cell survival and differentiation of MDS cells treated with EPO. Therefore, these inositide signal transduction pathways could be targeted for the development of innovative therapeutic approaches for MDS patients.

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TELOMERE SHORTENING IN LOW-RISK MYELODYSPLASTIC SYNDROME

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Introduction. Telomeres are regions of highly repetitive GC-rich DNA sequence at the end of eukaryotic chromosomes that act to protect loss of genetic information due to incomplete DNA replication at the ends of chromosomes in late S phase. Telomeres are progressively shortened with cell divisions and trigger cellular senescence in somatic cells. Most stem cells and cancer cells are able to proliferate indefinitely, because of a telomere maintenance mechanism as activation of telomerase, a ribonucleoprotein that synthesizes telomeric DNA sequences and almost universally provides the molecular basis for unlimited proliferative potential. Loss of telomerase function produces short telomeres,

potentially resulting in chromosome recombination, end-to-end fusion and recognition as damaged DNA. Regulation of hTERT expression depends on different transcription factors that bind hTERT promoter and positively or negatively control hTERT expression. In particular the c-Myc and Mad1 network can regulate hTERT expression through the binding to E-box binding sites. In this study we evaluated telomerase enzymatic activity (AT), telomeres length and hTERT expression, in mononuclear cells (MNC) from bone marrow at diagnosis of 45 patients with Low Risk (IPSS) Myelodysplastic Syndrome. We also evaluate the expression of c-myc, mad-1 and p53 transcription factors acting over hTERT promoter. These results are compared with the control (normal bone marrow, n=10) to increase our knowledge upon telomere maintenance mechanism and correlation to the clinical features in MDS patients. *Methods.* The telomerase activity (AT) was quantified using a real-time PCR-based telomeric repeats amplification protocol. All gene expressions were determined by real-time PCR. Telomere length was analyzed by Telomere Restriction Fragments (TRFs) length method. *Results.* Compared with the controls, in 18 patients (40%) AT was significantly increased and hTERT was significantly over-expressed. mad1 expression was significantly higher in all MDS samples than in controls, no significantly differences were found for c-myc and p53 expression. TRFs length was significantly lower than in controls. *Conclusions.* Our data indicates a dual behaviour in Low Risk MDS patients regarding AT and hTERT expression, even TRFs length was homogeneously low. We are evaluating a possible correlation of AT and hTERT expression with other prognostic factors than IPSS, disease-free survival and over-all survival.

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THE VASCULAR ENDOTHELIAL GROWTH FACTOR INDEX (VEGF-I) HAS A PROGNOSTIC IMPACT IN MYELODYSPLASTIC SYNDROMES

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Introduction. Angiogenesis, a multiphasic process resulting in growth of new capillaries from preexisting vessels, depends on the balance between pro- and anti-angiogenetic factors. The main pro-angiogenetic factor, Vascular Endothelial Growth Factor (VEGF), realizes its action through the interaction with specific tyrosine-kinase receptors. We evaluated microvessel density (MVD), a surrogate marker of angiogenesis, and the immunohistochemical expression of VEGF in bone marrow biopsies (BMBs) of MDS patients, and their correlation on survival. *Methods.* 68 patients affected by MDS (9 RA, 4 RARS, 16 RCMD, 6 5q- syndrome, 23 RAEB-1, 10 RAEB-2 according to WHO 2008 classification; IPSS: 28 low, 20 int-1, 15 int-2 and 5 high; WPSS: 14 very low, 14 low, 12 intermediate, 18 high and 10 very high) and 25 healthy controls were studied. MVD was evaluated through CD34 immuno-histochemical reactivity, calculating the average number of vessels in 5 HPF microscopic fields in the "hot spots" areas. The expression of VEGF was calculated as the percentage of positive cells on the total of nucleate marrow cells. To avoid bias related to variations in the cellularity of BMBs, we estimated the VEGF index (VEGFi), defined as the cellularity of the BMB specimen multiplied by the fraction of $VEGF^+$ cells [(% of BM Cellularity % of VEGF+ Cells)/10.000]. Taking into account a cut off corresponding to the mean -/+ 2 SD of VEGFi in MDS patients, a high (H-VEGFi) and a low (L-VEGFi) VEGFi class were determined. *Results.* The MVD in MDS patients was higher than in the healthy controls (P=.015). VEGFi levels of normal controls were significantly lower than those of H-VEGFi MDS patients (P<.0001). The distribution of the VEGFi levels was not different among IPSS classes, while higher VEGFi were observed in patients with higher WPSS score (P=.03). VEGFi correlated with transfusional need in the first year from the diagnosis (P=.04). L-VEGFi MDS patients had a significant better survival compared to high class patients (P=.0024). At multivariate analysis, VEGFi predicted survival independently from IPSS, but not from WPSS (P=.007). *Conclusions.* We showed that markers of neo-angiogenesis, namely MVD and VEGFi, are overexpressed in the BMBs of MDS patients. In addition VEGFi is a prognostic factor independent from IPSS, whereas it correlates with transfusional need in the first year from MDS diagnosis and with WPSS. This finding gives additional support to the importance of transfusional need in defining the prognosis of MDS patients. VEGFi can be incorporated into the diagnostic work-up and determinated therefore at diagnosis, besides providing an accurate prediction of survival in MDS patients.

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NPM1 GENE ABNORMALITIES IN MYELODYSPLASTIC SYNDROMES WITH LONG ARM CHROMOSOME 5 DELETION

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Introduction. The deletion of 5q (5q-) is a frequent clonal chromosomal abnormality in patients with MDS. MDS patients with 5q- as a sole chromosome alteration have a good prognosis. By contrast, when detected in the context of a complex karyotype, the 5q- aberration is associated with adverse prognostic outcome in both MDS and AML. The NPM1 gene is located in chromosome 5q35 and is disrupted in a number of translocations observed in haematological malignancies. In mouse model NPM1 is haploinsufficient for regulating centrosome duplication as NPM1 heterozygous (NPM +/-) cells show aberrant centrosome numbers, genomic instability and aneuploidy. We analyzed NPM1 status in terms of deletion, methylation and mutations in 53 patients with MDS or MDS-sAML carrying the 5q- abnormality as a sole chromosomal alteration or with other chromosome defects. *Methods.* Cytogenetic methanol/acetic acid fixed pellets and DNA samples were available from 53 unselected patients with a diagnosis of MDS (48 cases) or MDS-sAML (5 cases) with 5q abnormalities. Cytogenetic and FISH studies for the 5q- abnormality were performed according to the standard methods used in our laboratories. NPM1 FISH analysis was carried out with bacterial artificial chromosome (BAC) (RP11-117L6 5q35.1 chr5:170,746,724-170,770,492). Detection of NPM1 mutations was carried out by RT-PCR with fluorescente primers followed by capillary gel electrophoresis. The methylation status of the promoter of NPM1 gene was determined by methylation-specific PCR. Bone marrow DNA from healthy donors was used as a negative control and human male genomic DNA universally methylated for all genes was used as a positive control for methylation-specific assays. *Results.* Methylation analysis of the NPM1 gene promoter disclosed unmethylated CpG islands in all the 53 samples analyzed including patients with isolated 5q- and with complex karyotype. The mutational studies of NPM1 exon 12 showed wild type NPM1 in all patients. FISH analysis of the NPM1 locus revealed deletion of one copy of the gene in 7 out of 30 cases with complex karyotype and in 0 out of 23 of those with isolated 5q- (P=0.0148). Six out of the 7 cases with deleted NPM1, 5q- and complex karyotype showed >10% blasts at presentation. *Conclusions.* Although not contained within any common deleted region on 5q, NPM1 can be deleted in 5q- cases with large chromosomal deletions. In agreement with previously reported findings, neither NPM1 hypermethylation, nor mutations were found in patients with 5q- MDS. Our findings suggest that NPM1 haploinsufficiency may have a role in myeloid malignancies associated with large 5q- deletions contributing to the phenotype of MDS, most likely inducing genetic instability. Since an increasing number of genes on 5q seem to contribute to the MDS development, it will be interesting to explore how haploinsufficiency for multiple genes may cooperate to the development of genomic instability and MDS pathogenesis.

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IRON CHELATION THERAPY WITH DEFERASIROX IN TRANSFUSION DEPENDENT MYELODYSPLASTIC SYNDROME PATIENTS. FIRST REPORT FROM THE PROSPECTIVE MDS0306 GIMEMA TRIAL. CLINICALTRIAL.GOV IDENTIFIER NCT00469560

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Introduction. The recent development of a safe and efficient once daily oral iron chelator (Deferasirox, Exjade®) made possible regular chelation therapy in transfusion dependent MDS patients. However in this category of patients the reported clinical experience is limited to the selected population enrolled in official pre-registration clinical trial. For this reason the GIMEMA group developed a phase IIIb prospective trial to test safety and efficacy of Deferasirox in a large population of MDS patients comparable to general population. *Methods.* One hundred and fifty-nine transfusion dependent IPSS low-intermediate1 risk MDS patients were enrolled. Analysis was performed on 113 patients: ongoing patients were excluded. Baseline characteristics were the following (data are expressed as median with upper and lower quartile unless specifically indicated): median age was 72 years (range 24-87); 44 were IPSS low risk and 69 Intermediate1; duration of transfusion dependency before treatment was 20 months (11-36) corresponding to 38 (22-71) packed red blood cells transfusions received. Serum ferritin was 2000 ng/mL (1471-3000). Charlson and CIRS comorbity scores were 1 (0-1) and 0.2 (0.1-0.5), respectively. Patients started treatment with the standard 20 mg/kg Deferasirox dose but dose adjustments on clinical indications were allowed. *Results.* 60 patients (53%) prematurely interrupted the study (drop out), 53 patients completed the planned year of treatment and remaining patients are ongoing. In logistic model for drop out rate high scores of comorbidity (CIRS) were resulted as significant risk factors (P=0.0165). Drops out were mainly related to disease progression (16%) unrelated causes mainly aging related (20%) and drug related toxicity (11% of the entire population). Main causes of toxicity related drop out were increase of creatinine and gastro-intestinal disturbance. Out of 113 patients analyzed for adverse events 4 (3%) presented grade >2 drug related adverse events. The most frequently reported SAEs were pneumonia, diarrhea, increase of transaminases, cerebral haemorrhage. SAEs with suspected relationship with study drug were diarrhea and increase of transaminases. Figure 1 reports serum Ferritin evolution in the 53 patients who completed the protocol with a statistically significant decrement during the 12 months follow up (P<0.001). Analysis of quality of life is ongoing. *Discussion.* Preliminary results from the GIMEMA MDS0306 study confirmed feasibility of Deferasirox therapy in transfusion dependent MDS patients. Drop out rate, toxicity related drop out and severe side effects were similar to those reported in other trials even if the present population presented clinical characteristics of more advanced disease and age. The rate of progression is coherent with prolonged disease story. Serum ferritin behavior confirms Deferasirox efficacy. The serum ferritin reduction was more evident in the more heavily overloaded population indicating successful iron depletion in this group of patients as clinically requested.

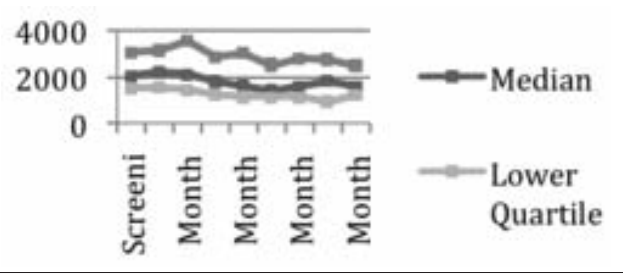

Figure 1. Ferritin (NG/ML).

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INFLUENCE OF BONE MARROW FIBROSIS ON RESPONSE OF INT-2/HIGH RISK MDS PATIENTS TO 5-AZACITIDINE

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Introduction. Azacitidine (AZA) induces responses and prolongs overall survival compared with conventional care regimens in patients with high-risk myelodysplastic syndromes (MDS). Risk factor that may influence the response to AZA are still largely unknow. Bone marrow fibrosis is a known negative prognostic index and could alter the efficacy of 5-azacitidine therapy. *Methods.* We analyzed 88 MDS patients (72% of them was high risk MDS) treated with subcutaneous 5-Azacitidine 75 mg/kg/day for 7 days every 28. Mean number of cycles was 7.4 (range: 1-54). Mean age was 70 yrs (26-85), 63% were male. Response to azacitidine treatment was evaluated according to IWG 2006 criteria as CR, PR, HI, SD, DP. Overall response rate according IWG criteria 2006 was 45.5%, stable disease was obtained in 25.3% of MDS patients. In 16 patients response was not evaluable at the time of analysis because they received less than 4 azacitidine cycles. The grade of fibrosis and its extent were evaluated semiquantitatively in archival slides stained by Gomori silver impregnation according to European consesus on gradingof bone marrow fibrosis and assessment of cellularity criteria (58,2% of patients was grade 0; 32,9% grade 1 and 7,6 grade 2). *Results.* Overall response of patients with grade 0 fibrosis was 60%, and for patients with grade 1 and 2 was 72% (P>0.05). All the patients with grade 2 fibrosis reached HI except one, that reached SD. In details: response of patients with grade 0 was: CR 5%, PR 5%, HI 27%, SD 23% and DP 40%. Response of patients with grade 1 and 2 was: CR 4%, PR 8%, HI 28%, SD 32% and DP 28%. Response of patients with grade 1 only was: CR 5%, PR 10%, HI 19%, SD 33% and DP 33%, Differences between groups are not statistically significant. *Conclusions.* Bone marrow fibrosis does not seem to influence the response of MDS patients to 5-azacitidine therapy.

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A STUDY OF KIR GENOTYPE OF ITALIAN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA PATIENTS

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Paroxysmal Nocturnal Haemoglobinuria (PNH) is a very rare haematopoieis disorder characterised by the expansion of a stem cell bearing a somatic mutation in the phosphatidyl-inositol glycan-A (PIG-A) gene, which is involved in the biosynthesis of the glycosyl-phosphatidyl-inositol (GPI) anchor. Haemolytic anaemia, thrombophilia and cytopenia characterise this disease. A number of data suggest the inability of PIG-A mutation to account alone for the clonal dominance of the GPI-defective clone and for the development of PNH. In this context, the occurrence of immune-mediated mechanisms have been hypothesized. Our previous data (Hum Immunol 2008, 69:202-6) revealed the association of PNH with the HLA class I haplotype B*1402, Cw*0802 as well as with the extended Mediterranean haplotype A*33, B*1402, Cw*0802, DRB1*0102. NK cells are critical components of the innate immune response. They may also drive, shape and regulate the activity of the adaptive immune compartment. Killer-cell Immunoglobulin like Receptors (KIR) represent, with their known HLA ligands, a key component in the regulation of NK response. To analyse the biological mechanisms underlying PNH pathogenesis we are addressing the analysis of KIR genotype, whose role in the regulation of immune response and self-tolerance has been established. KIR distribution in 53 patients affected by PNH (estimated to represent almost the half of all Italian PNH patients) and in 64 controls of the same ethnical origin was analysed by PCR-SSP typing. In order to increase the number of controls bearing the HLA class

I haplotype (B*1402,Cw*0802), 14 normal donors bearing such HLA haplotype have been selected and additionally enrolled in the study. No significant associations with the KIR haplotype A or B as well as with specific KIR alleles has been observed. Independent segregation of HLA (6p21) and KIR (19q13.4) genes raises the possibility that any given individual may express KIR molecules from which no ligand is present or vice versa. The analysis of KIR-HLA matching in PNH patients, as compared with controls, showed significant differences only in the 3DL1/Bw4 matching. Indeed, a significant decrease in 3DL1/Bw4 mis-matching has been appreciated in the B*1402,Cw0802 subgroup (3DL1 without Bw4 was observed in 50% of controls as compared with 8.33% of PNH patients; P<0.05). When considering the occurrence of weak interactions, as represented by the presence of T80 Bw4 alleles, such observation become more consistent (72.22% in controls versus 19.66% in PNH patients; P<0.01). In addition, a significant decrease in genotypes showing 3-4 activating genes (46.67% versus 21.95%; P<0.01) accompanied by an increase in the frequency of genotypes showing 1-2 activating genes (35% *vs.* 56.09%) has been observed in B*1402,Cw0802- PNH patients, as compared with controls.

Acute Leukemias I

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EFFICACY, FEASIBILITY AND SAFETY OF NELARABINE SAVAGE THERAPY IN ADULT RELAPSED OR REFRACTORY T CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) OR LYMPHOBLASTIC LYMPHOMA (T-LBL): THE BOLOGNA EXPERIENCE

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Introduction. T-ALL represents 15% of childhood and 25% of adult ALL. Despite a cure rate of almost 80% in children and adolescents, adult ALL remains a difficult disease to cure, due to a high risk of relapse. Effective treatment of relapsed acute T-ALL is limited with a low CR rate, a high treatment-related mortality, and a very low prolonged disease-free survival. Nelarabine is a pro-drug of ara-G, approved by the FDA for the treatment of T-ALL and T-LBL that have not responded to or has relapsed after treatment with at least 2 chemotherapy regimens. Similar to other nucleoside analogues, Nelarabine acts by inhibiting DNA synthesis and inducing apoptosis in malignant cells. Our aim was to evaluate safety profile and efficacy of Nelarabine treatment as savage therapy in 9 adult relapsed or refractory T-ALL or T-LBL. *Methods.* After obtaining an informed consent, ten patients (median age 31 years, range 19-37, M/F= 9/0) affected by T-ALL (N=6) and T-LBL (N=4) received a savage therapy with Nelarabine (median cycle=1, range 1-3). Nelarabine was administered at standard adult dosage (1500 mg^2 on days 1, 3 and 5, every 21). Eight patients were relapsed after two previous chemtotherapy regimens, including allogeneic bone marrow transplantation; the remaining two patients were primary resistant to standard induction treatment. *Results.* Five out of ten patients obtained a complete morphological remission (4 T-ALL patients and 1 T-LBL patient), whereas a partial remission was documented in three cases, with an overall response rate of 80%. Median duration of complete response was 6 weeks (range 3-6 weeks). Nelarabine was well tolerated, and no significant adverse events were registered. Extra- hematological neurological toxicity, not clearly related to the drug, occurred in two cases, determining, in one patient a complete and irreversible paraplegia, and in the second one a condition of mental confusion (grade III), which resolved after few days. *Conclusions.* In our experience Nelarabine was successfully administered in such a high risk patients population. The drug showed a relevant efficacy and a good safety profile. *Acknowledgments.* This work was supported by European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, PRIN 2008, Ateneo RFO grants, Project of integrated program (PIO), Programma di Ricerca Regione - Università 2007-2009.

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AMP-DEPENDENT KINASE SIGNALING IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA: THERAPEUTICAL IMPLICATIONS

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Introduction. Over the past 20 years, survival rates of T-ALL patients have improved, mainly because of advances in chemotherapy protocols. Despite these improvements, we still need novel and less toxic treatment strategies targeting aberrantly activated signaling intermediates which increase proliferation, survival, and drug-resistance of T-ALL cells. One such intermediate is represented by the mammalian target of rapamycin (mTOR). mTOR exists as two complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Allosteric mTOR inhibitors (rapamycin and its analogues) mainly target mTORC1 and have displayed some promising effects in preclinical models of T-ALL. mTORC1 activity can be inhibited by activating AMP-dependent kinase (AMPK). AMPK activators include AICAR and the antidiabetic drug, metformin. It is now emerging that metformin displays antineoplastic activity in preclinical settings of solid tumors and its efficacy is being currently evaluated in patients. Here, we have analyzed the therapeutic potential of AMPK activators in T-ALL cell lines and pediatric patient

lymphoblasts. *Methods.* We have used a panel of T-ALL cell lines including CEM-R cells [which overexpress high levels of the membrane transporter, 170-kDa P-glycoprotein], MOLT-4, Jurkat, RPMI-8402, and BE-13 cells. Cell lines and patients samples displayed mTORC1 activation as documented by the levels of p-4E-BP1 and p-S6 ribosomal protein. *Results.* MTT assays indicated an IC_{50} for metformin at 48 h ranging from 1.6 to 5.6 mM in cell lines. We also investigated AICAR, whose IC_{50} ranged from 0.7 to 2.6 mM at 24 h. In patient samples metformin IC_{50} was 0.6-0.9 mM at 96 h, whereas AICAR IC50 was 0.7-1.0 mM at 96 h. Consistently with mTORC1 inhibition, metformin caused autophagy, as demonstrated by transmission electron microscopy and western blot analysis with antibodies to beclin 1 and LC3B.Western blotting documented increased levels of Thr 172 p-AMPKalpha in CEM-R, BE13, and RPMI8402 cells treated with metformin, indicating AMPKalpha activation. In contrast, no AMPKα activation was seen in Jurkat cells. As a further proof of AMPKalpha activation, Ser 792 Raptor phosphorylation was seen in CEM-R and RPMI8402 cells. However, we detected decreased phosphorylation levels of 4E-BP1, S6 ribosomal protein, and p70S6K in all the cell lines treated with metformin, indicating mTORC1 signaling inhibition. Interestingly, Ser 473 p-Akt levels decreased in Jurkat cells treated with metformin, suggesting that mTORC1 inhibition could be due to downregulation of IRS1/PI3K/Akt signaling, as recently demonstrated in breast cancer cell lines. Remarkably, metformin targeted the side population (identified by Hoechst 33342 staining and ABCG2 expression) of T-ALL cell lines, which might correspond to leukemia initiating cells *Conclusions.* Our results suggest that mTORC1 inhibition by means of AMPK activators could be a promising option for the treatment of T-ALL.

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COMPLEX CHROMOSOMAL ANOMALIES AND CXCR4 EXPRESSION IN *DE NOVO* ADULT ACUTE MYELOID LEUKEMIAS (AML)

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Introduction. In adult AML the complex karyotypic alterations at the diagnosis characterize a sub-group of patients with worse prognosis. It is also known that the complete immunophenotypic characterization contributes to define the prognosis of *de novo* adult AML. Stromal cell-derived factor-1 (SDF-1) is a homeostatic chemokine that is constitutively secreted by marrow stromal cells. SDF-1 signals through CXCR4, which plays an important role in hematopoiesis, development and organization of the immune system. Prognostic impact of CXCR4 expression levels on the neoplastic cells has been demonstrated in breast cancer, renal cell cancer and AML. *Methods.* We investigated the expression of the chemokine receptor CXCR4 on bone marrow blast cells in a group of adult de novo AML with complex chromosomal anomalies. We have observed 32 young adult *de novo* AML patients (median age: 44 years, r: 25-60) in the last 4 years, who presented complex chromosomal anomalies in bone marrow blood. On the basis of FAB classification the patients were considered LMA-M5 (10 pts.), LMA-M2 (9 pts.), LMA-M4 (6 pts.), LMA-M1 (4 pts.) and LMA-M0 (3 pt.). Six patients showed internal tandem duplications of FLT3; 15 patients presented hyperleukocytosis (WBC> 40×10^9/L). The clinical outcome was that one of a "high risk" AML; at the present only two patients are still alive in CR (+30 months and +36 months). *Results.* We found at the diagnosis, in all cases, an high CXCR4 expression on leukemic blasts, as defined by CXCR4 mean fluorescence intensity ratio thresholds of more than 5.*Conclusions.* Several studies have shown the prognostic significance of the expression of differentiation myeloid markers at the diagnosis of adult de novo AML. However, specific immunophenotype expression patterns associated with complex chromosomal anomalies are still unknown. Further studies are warranted to confirm the correlation between complex chromosomal anomalies and "CXCR4 over-expression" immunophenotypic pattern.

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A SIMPLE CLINICAL PROGNOSTIC SCORING SYSTEM FOR NEWLY DIAGNOSED ACUTE MYELOID LEUKAEMIA PATIENTS WITH NORMAL KARYOTYPE: A RETROSPECTIVE ANALYSIS ON 530 CASES

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Objectives. the prognosis of patients with normal karyotpye acute myeloid leukaemia (NK-AML) is highly variable and can be influenced by several clinical and biological variables. Nevertheless, some biological data may be conflicting and difficult to combine with the clinical ones. *Methods.* in order to propose a simple scoring system, we retrospectively analysed the clinical data of 337 patients newly diagnosed with NK-AMLs, aged 65 years, consecutively treated in eleven haematological Italian Centres from 1990 to 2005. Univariate and multivariate analysis on event free survival and overall survival (EFS and OS) were performed. Factors found to be significant in univariate analysis were tested in multivariate analysis. A numerical score was derived from the regression coefficients of each independent prognostic variable. The Prognostic Index Score (PIS) for each patient was then calculated by totalling up the score of each independent variable. Patients could thus be stratified into low-risk (score=0-1), intermediate-risk (score=2) and high-risk group (score ≥3). The score obtained in this group of patients (training set) was then tested on 193 patients with newly diagnosed with NK-AMLs, aged ≤65 years, enrolled in the GIMEMA LAM99p clinical trial (validation set). *Results.* the clinical variables that were independent prognostic factors on EFS in the training set of patients were: age >50 yrs (regression coefficient: 0.39, HR 1.5, score=1), secondary AML (regression coefficient: 0.90, HR 2.5, score=2) and WBC $>20\times10^9$/L (regression coefficient: 0.83, HR 2.3, score=2). For what concerns the OS, the same variables showed the followings statistical data: age > 50 yrs (regression coefficient: 0.48, HR 1.6, score = 1), secondary AML (regression coefficient: 0.99, HR 2.7, score=2) and WBC $>20\times10^9$/L (regression coefficient: 0.87, HR 2.4, score=2). In the training set of patients, the median EFS was 22, 12 and 8 months in the low, intermediate and high-risk group (P<0.0001). The median OS was not reached in the low-risk group and was 20 and 10 months in the intermediate and high-risk group (P<0.0001). In the validation set of patients, the median EFS was 66, 16 and 3 months in the low, intermediate and high-risk group (P<0.0001). The median OS 66, 16 and 4 months in the low, intermediate and high-risk group (P<0.0001). *Conclusions.* this simple and reproducible prognostic score may be useful for clinical-decision making in newly diagnosed patients with NK-AML, aged less than 65 yrs.

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QUANTITATIVE ANALYSIS OF MENINGIOMA-1 (MN1) GENE EXPRESSION FOR EVALUATION OF MINIMAL RESIDUAL DISEASE IN PATIENT WITH ACUTE MYELOID LEUKEMIA WITH NORMAL KARYOTYPE

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Introduction. Molecular characterization of acute myeloid leukemia (AML) is essential for management of therapy. Molecular markers are necessary for prognostic stratification and monitoring of minimal residual disease (MRD), including early detection of relapse. Unfortunately,

only 30% of AML patients (pts) presents a disease-specific molecular signature. So, in the last years, efforts have been made to detect a "universal" marker for AML. WT1 gene is over-expressed in about 80% of all AML cases, and has been successfully used for MRD monitoring. More recently, the meningioma 1 (MN1) gene has been found to be over-expressed in AML with inv(16), and high MN1 levels seems to have prognostic impact in cytogenetically normal (CN) AML pts. To test the possible role of MN1 in AML, we first studied MN1 expression in a group of CN AML pts, evaluating the role of MN1 as marker of MDR in cases over-expressing WT1, by comparing the levels of these two genes at various stage of disease. We then analysed MN1 expression in a group of WT1-negative AML pts, to determine if MN1 could be used for MDR assessment in pts lacking specific molecular markers. *Methods.* To assess MN1 expression, we designed a quantitative PCR assay with TaqMan chemistry, using ABL as housekeeping gene. The assay has been localized on the junction between two exons of MN1. The standard curve was prepared with 5 serial dilutions of RNA from a patient with inv(16) AML; the assay efficiency resulted 0.98. To determine the relative quantification of MN1 expression, we used the 2- Ct method, based on a calibrator obtained on a control group of 12 healthy donors. Then we analysed the expression of MN1 in 23 pts with CN AML over-expressing WT1, comparing the 2 markers at different time points (diagnosis, after chemotherapy, before and after transplantation). Finally, we tested MN1 expression in 11 cases of CN AML with normal WT1 levels. *Results.* Median expression level (2- Ct) of MN1 in healthy controls was 0.37 (range: 0.15-3.44). Among the 23 pts with CN AML and high WT1 expression, the proportion of cases with MN1 level >3.44 was 39% (9 pts), with a median value of 24.72 (range: 3.48-64.74). After chemotherapy, 8/9 pts responded and presented MN1 and WT1 levels within the normal values, while one resistant patient over-expressed both MN1 (17.85) and WT1. One of the eight responding pts relapsed after transplant; both MN1 and WT1 raised, but the former increased to pathological values earlier. Among the 11 AML pts with low WT1 at diagnosis, only one case (9%) over-expressed MN1 (6.76); this patient died during induction therapy. *Conclusions.* MN1 was over-expressed in about 30% of CN AML pts, and its levels correlated well with clinical course and other molecular marker (where available), thus representing a potential candidate gene for MRD assessment. In our small cohort of AML without WT1 over-expression the proportion of cases positive for MN1 was unexpectedly low (only 9%), therefore MN1 could not be used to monitor MRD in pts without other molecular markers.

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CXCR4 EXPRESSION ON LEUKEMIC CELLS IN AML CORRELATES WITH LEUKOCYTOSIS, EXTRA-HEMATOLOGIC DISEASE AND NPM1 MUTATIONS BUT NOT WITH PROGNOSIS

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Introduction. CXC chemokine receptor 4 (CXCR4) is the receptor for stromal-derived factor 1 (SDF1α); the SDF1/CXCR4 axis is involved in the trafficking of leukemic cells and its over-expression has been associated with hyperleukocytosis, FLT3-ITD and poor prognosis in AML. *Methods.* We have evaluated CXCR4 expression by flow cytometry on bone marrow leukemic cells in non-M3 AML. CXCR4 expression was assessed by the following staining: CD34 FITC/CXCR4 (CD184) PE/CD45 PerCP/CD33 or CD117 APC. CD45 PerCP was exploited to allow gating of cell populations. MoAb CXCR4 PE was purchased from Becton-Dickinson. 50,000 events per tube were acquired through FACSCalibur flow cytometer and analyzed through Infinicyt software. CXCR4 was explored on: i) whole leukemic population, CD45/side scatter-gated; ii) $CD34^+$ cells, in AML cases in whom $CD34^+$ cells represented at least 1% on entire leukemic population. CXCR4 expression was determined as mean fluorescence intensity corrected for background fluorescence (MFI) determined by a control. Cytogenetics was assessed by conventional karyotyping and FISH for inv(16), t(8;21) and MLL; mutations of NPM1 were revealed by immunohistochemistry on trephine biopsy and/or by PCR; FLT3-ITD was evaluated by PCR. *Results.* Since 2005 to 2010, 93 consecutive non-M3 AML patients (pts) were studied. Median age was 52 (16-70). Cytogenetics was available for 88 pts. According to genotype, they were subdivided as follows: a) t(8;21) # 5; c) inv16 # 8; d) normal karyotype # 44, of whom 20 NPM1-wt and 24 NPM1-mutated; e) MLL rearrangement # 1. According to SWOG, 13 and 6 patients had high- and intermediate-risk (other than normal) karyotype respectively. Conventional karyotyping resulted lack of growth in 11 cases. Median CXCR4 MFI on whole blasts was 12.37 (1.04-141.05). We classified patients in 2 groups according to CXCR4 MFI lower (1) or higher (2) than the median. Group 2 showed significantly higher WBC (45520/μL *vs.* 8650/μL; P<0.001), absolute PB blast count (30135/μL *vs.* 2904/μL; P<0.001), LDH (1.95 *vs.* 1.4 normalized for ULN; P=0.001), incidence of hepatosplenomegaly and/or extra-hematologic disease (43% *vs.* 19%; P=0.011). Furthermore, group 2 displayed an higher frequency of NPM1 mutations (46.6% *vs.* 21.3%; P=0.01), while no differences were seen regarding FLT3-ITD. The expression of CXCR4 did not show any correlation with DFS and OS. Focusing the analysis on $CD34^+$ AML (74 of 93 patients showed at least 1% $CD34^+$ on whole leukemic population), median CXCR4 MFI on $CD34^+$ was 8.73 (0.79-125.09); no differences emerged with respect neither to any of the previous clinic-biological features nor to prognosis. *Conclusions.* In this homogenously treated cohort of AML patients we describe the correlation of CXCR4 expression with WBC and extra-BM localization, thus confirming its role in leukemic cells trafficking, and with NPM1 mutations. It is worth of note that the expression on whole blasts, rather than on merely $CD34^+$ cells, correlated with such features. Unlike previously reported, an independent prognostic value of CXCR4 did not emerge from our analysis.

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MIR-155 UP-REGULATION IN FLT3 POSITIVE AML INVERSELY CORRELATES WITH EXPRESSION OF MYELOID-SPECIFIC TRANSCRIPTION FACTORS

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Acute myeloid leukaemia (AML) arises from myeloid progenitor cells that are arrested at early stages of differentiation. AML is a cytogenetically heterogeneous disorder with acquired recurrent chromosomal alterations such as translocations, inversions, deletions, trisomies, and monosomies detected in about 55% of adult patients. In the remaining 45% of cases of normal karyotype AML, a number of novel molecular abnormalities, such as the internal tandem duplication (ITD) or mutation (D835) of FLT3 gene, mutations of NPM1 gene, mutations of CEBPA gene and partial tandem duplication of the MLL gene have been described. Several studies have shown that genome-wide gene expression profiling can clearly distinguish the major cytogenetic groups, so providing a better understanding of the underlying disease biology. Despite this progress, studies focusing on known genes will likely not suffice to uncover the molecular puzzle of AML. The integration of a whole genome approach including non-coding RNAs analysis may lead to an improved understanding of AML biology. MicroRNAs (miRNAs) are a class of small noncoding RNAs that negatively regulate protein expression of specific mRNA by either translational inhibition or mRNAs degradation. There are several indications that miRNAs might be a new class of regulators involved in human cancer and has been observed that distinct patterns of miRNA expression reflect different developmental lineage and different genetic categories of AML. I a previous work we performed quantitative real-time RT-PCR to study the expression of 365 known human miRNA in a cohort of 29 primary selected AML characterized by common cytogenetic and molecular alterations and we identified distinctive miRNA expression patterns in some genetic groups. According to the recent literature we found a strong up-regulation of mir-155 in normal karyotype AML carrying FLT3 mutations (Cammarata G.*et al.*, Am J Haematol 2010); so we decide to investigate expression levels of this miRNA in a larger cohort of 31 FLT3 AML patients (27 ITD and 4 D835) and 33 AML of other genetic categories. We found a 37,259 fold up-regulation of mir-155 in the first group compared to the second (P=0.0001). Several studies demonstrated that miR-155 directly repressed a broad range of target mRNAs implicated in myeloid hyperplasia and/or hematopoiesis. Using available prediction target algorithms we selected hypothetical mir-155 regulated genes such as PU.1 and CEBPbeta, both genes codify for lineage specific transcription factors, indispensable for normal myeloid development. In the same cohort of 64 patients we

found an inverse correlation between mir-155 expression levels and its predicted targets: PU.1 (Fold=0,471; P=0,005), CEBPbeta (Fold=0,414; P=0,008). Based upon our current study, miR-155 appears to play a role in malignant haematopoiesis targeting the expression of central myeloid specific transcription factors that may contribute to block differentiation. Future functional analysis will better clarify the role of mir-155 in AML pathogenesis and its molecular mechanism in the inhibition of myeloid differentiation.

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14Q32/MIRNA CLUSTERS LOSS OF HETEROZYGOSITY IN ACUTE LYMPHOBLASTIC LEUKEMIA IS ASSOCIATED WITH DEREGULATION OF BCL11A

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Genome-wide analyses of DNA copy number abnormalities and loss of heterozygosity (LOH) have provided important insights into the pathogenesis of newly diagnosed acute ALL. If one assume that LOH may impact on the mechanisms of leukemogenesis, definition of its real role is to be considered of interest: one intriguing model may be that cryptic deletions may influence gene expression pattern by loss of microRNA or as a consequence of gene dosage. The present study evaluated the loss and expression level of miRNAs 14q32 clusters in ALL patients with cryptic deletions at 14q32 chromosomal band to investigate their involvement in this disease. We performed LOH analysis of 4 microsatellite markers located on chromosome 14q32: D14S65 (at 97,6 kb), D14S1426 (at 100,6 kb), D14S985 (at 101,2 kb) and D14S292 (at 104,5 kb). Near this region maps Ig heavy chain (IGH) locus involved in recombination mechanism during B cell differentiation. Errors in this mechanism could lead to loss of small DNA fragments detectable by LOH analysis. Fifty patients were evaluated for LOH analysis. balanced 14q32 allelotypes were found in 39 cases, 14q32 imbalance was detected in 11 case. Patients showing imbalance at 14q32 included 3 cases with hyperdiploidy karyotype and microsatellite amplification and 8 cases showing LOH. In 14q32 lie large clusters of MicroRNAs (miRNA), spanning from 101.3 to 101.5 kb. MiRNA are small noncoding RNAs recently found involved in haematological malignancies that have been shown to regulate the expression of other genes in a variety of eukaryotic systems and have been identified as a novel mechanism in gene regulation by base pairing with their target mRNAs. Using a quantitative real-time PCR assay specific to the mature miRNA we studied the expression levels of 18 miRNAs located in these 14q32 clusters. We performed a comparative analysis of the two population LOH-ve and LOH+ve and we observed that all miRNA evaluated, except miR-410, showed a lower level of expression in the LOH+ve group (fold change ranging from 0.001 to 0.24). Moreover 4 miRNAs, miR-382 (decreased fold 0.001 P<0.01) , miR-412 (fold 0.03 P=0.04), miR-433 (fold 0.012 P<0.01) and miR-127 (fold 0.03 P=0.01) resulted down-regulated at statistical REST analysis. In an effort to evaluate the effects of miRNAs down-regulation we identified a common potential mRNA target of studied miRNAs. Using available prediction programs such as TargetScans, Miranda, PicTar and Diana-lab we selected BCL11A gene as target of miR-544, miR-376a, miR-494, miR-432 and miR-410. BCL11A is a Kruppel-like transcription factor that have been shown to be required for B cell development and its de-regulated expression is associated with B cell malignancies. We found that BCL11 showed a significant inverse correlation with miRNA expression resulting up-regulated in patients LOH+ve (9.7 fold, P=0.013 at REST analysis) suggesting that miRNAs de-regulation may have a potential role in the genesis and evolution of lymphoblastic leukemia. These results suggest that 14q32/miRNA clusters LOH may be another mechanism involved in lymphoid B cell transformation and differentiation and therefore, could be used as a diagnostic marker and therapeutic target in subsets of ALL.

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GENETIC POLYMORPHISMS IN INNATE AND ADAPTIVE IMMUNITY-RELATED GENES FOR DEFINING THE RISK OF INVASIVE FUNGAL INFECTION IN ITALIAN ONCO-HEMATOLOGIC PATIENTS DURING STANDARD CHEMOTHERAPY REGIMENS

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Introduction. Invasive Fungal Infection (IFI) is a main cause of morbidity and mortality in hematologic patients following either standard chemotherapy or allogeneic stem cell transplantation (alloSCT). Growing evidences have so far highlighted a role for polymorphisms in genes involved in the innate and adaptive immune responses, in defining the risk for the development of IFI, mainly invasive Aspergillosis (IA) and in alloSCT patients. Thus, we studied whether selected polymorphisms in genes related to the immune responses against fungi (i.e. IFNγ, IL10, CD14, TLR2, TLR9, MD-2, IL6) may influence the risk for hyalohyphomycosis in an Italian cohort of hematologic patients undergoing standard chemotherapy. *Methods.* SNP genotyping was performed by means of RFLP, Allele-Specific PCR and direct sequencing. Our study comprised a cohort of 112 hematologic patients including 18 cases of proven hyalohyphomycosis, namely 11 IA, 3 fusariosis, 4 zygomicosis, and 22 cases of probable IA defined according to the EORTC/MSG criteria. The control group consisted of 72 patients not developing hyalohyphomycosis in a 4-years follow-up after the diagnosis of the hematologic malignancy. A number between 35 and 42 cases with at least one matched control was required for odd ratios of 2.5, with 80% power and 5% type I error rate, for a minimum allelic frequency of 10% (Quanto software, NEJM 2008). *Results.* All the SNPs analyzed showed no statistically difference in genotypic distribution between cases and controls. Three out of the 4 IL10 SNPs analyzed (A-1082G, C-819T and A-592C in the promoter region), showed high linkage disequilibrium (Haploview software, Bioinformatics 2005), allowing haplotype analysis in our cohort. Haplotype ATC was significantly over represented among cases with respect to the controls (P=0.031). Haplotype GCC, by contrast, was over represented among controls (P=0.07). According to logistic regression analysis, the former haplotype could represent a covariate influencing IFI risk (OR=2.75; 95%CI 1.03-7.38). *Conclusions.* It has been reported that genetic predisposition results in higher risk for IA in alloSCT setting. In particular, recent studies have reported that either a TLR4 haplotype of the donor or a SNP in IL10 gene promoter (G-containig genotypes -1082) seems to be a predisposing factor for IA. Our study seems to suggest that the IL10 ATC haplotype is associated with the development of IFI. Thus a genetic predisposition may be a risk factor not only for IA, but also for the other invasive hyalohyphomycosis, and not only for patients undergoing alloSCT, but also for subjects undergoing standard chemotherapy. Validation of these findings in a wider cohort would improve patient risk stratification, identifying those patients deserving antifungal prophylaxis since the induction phase of treatment.

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LOSS OF THE TUMOR SUPPRESSOR GENE CDKN2A/ARF IS FREQUENTLY DELETED BUT NOT MUTATED IN BCR-ABL1-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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Background. This locus 9p21, containing the p16/CDKN2A (cyclin-dependent kinase inhibitor 2a) tumor suppressor gene and two other related genes, p14/ARF and p15/CDKN2B, is a major target in the pathogenesis of a number of human tumors. *Patients and methods.* In order to assess whether and how it is inactivated in adult BCR-ABL1-positive ALL, we studied 112 adult patients: 78 (70%) were de novo ALL, 15 (13%) were unpaired relapsed cases and 19 (17%) were paired relapsed cases. Their median age was 53 years (range: 18-76) and their median

blast percentage was 90% (range, 18-99). Affymetrix single nucleotide polymorphism (SNP) arrays were used to identify at a high resolution copy number changes on 9p21. Mutation screening of all exons by cloning and subsequent sequencing were also performed. *Results.* SNP array analysis revealed CDKN2A/ARF and CDKN2B genomic alterations in 33% and 24% of diagnosed patients, respectively. In 70% of cases, deletions were limited to CDKN2A/CDKN2B genes, whereas in 30% they also affected neighbour genes and/or the entire chromosome 9. In order to assess whether CDKN2A loss is responsible for progression, 34 patients were analyzed at the time of relapse and a significant increase in the detection rate of CDKN2A/ARF loss (53%) compared to diagnosis (P=0.04) was found. In contrast, CDKN2B deletions were found to be not significantly different between diagnosis and relapse (41% *vs.* 24%, P=0.07). The mutation screening of all exons showed that the 9p21 locus is rarely affected by point mutations, since we only identified the D146N and the R128 in the exon 2 of CDKN2A/ARF and the P83 silent mutation in the exon 2 of CDKN2B gene. These mutations were mutually exclusive and were found in only single cases. *Conclusions.* Loss of the tumor suppressor gene CDKN2A/ARF by genomic deletions is a frequent event in adult Ph^+ ALL and it is involved in disease progression.

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BAX/BCL-2 RATIO IS A SURROGATE OF MULTIPLE BIOLOGIC PROGNOSTIC PARAMETERS IN ACUTE MYELOID LEUKEMIA

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Introduction. The unbalance between anti-apoptotic (bcl-2) and pro-apoptotic (bax) proteins has been already recognized as a key prognostic tool explaining the high rate of resistance and treatment failure in acute myeloid leukemia (AML) (Del Poeta, Blood, 2003; Curr Cancer Drug Targets, 2008; Br J Haematol, 2010). The current availability of effective pro-apoptotic compounds such as bortezomib and arsenic trioxide moved us to re-analyze bcl-2 and bax expressions making correlations with other crucial biologic prognostic factors. For this purpose, we tested a large series of 420 non M3 AML patients (pts), median age 63 years, treated with intensive chemotherapy regimens. The aims of our research were: 1) to correlate bax/bcl-2 ratio, as a measure of spontaneous apoptosis, with the differentiation and maturation antigens (CD34, CD15, CD14), with cytogenetics, with NPM1, with FLT3-ITD and with the multidrug related protein (MRP); 2) to evaluate whether bax/bcl-2 ratio was able to dissect normal karyotype with regard to prognosis, and finally 3) to confirm bax/bcl-2 ratio as an independent prognostic factor. *Methods.* Bcl-2 and bax were assessed by multicolor flow cytometry and bax/bcl-2 ratio was obtained by dividing mean fluorescence intensity (MFI) of bax/MFI bcl-2. The threshold of positivity was set at the median value >0.35. *Results.* Fifty-nine percent of pts were bax/bcl-2 positive. There was a strict correlation between higher bax/bcl-2 and FAB M2-M4-M5 subsets (P<0.0001). Higher bax/bcl-2 ratio and CD34 negativity (P<0.0001) or CD15 (P=0.0003) or CD14 positivity (P=0.0005) were closely associated, indicating that an elevated apoptosis is frequently found within a more mature and differentiated leukemic phenotype, also along the monocytic lineage. Moreover, a significant correlation was found between higher bax/bcl-2 and normal karyotype (P<0.0001) or, on the contrary, between lower bax/bcl-2 and MRP positivity (P=0.00007). Noteworthy, a significant relationship was demonstrated between higher bax/bcl-2 and NPM1 positivity/FLT3-ITD negativity (P=0.0002). A higher complete remission (CR) rate was found in pts with higher bax/bcl-2 (68% *vs.* 32%, P<0.0001). Overall survival (OS) and disease-free survival were longer in pts with higher bax/bcl-2 (18% *vs.* 0% at 3.5 years, P<0.0001 and 19 *vs.* 0% at 2.7 years, P=0.00007). Furthermore, within the normal karyotype subgroup (243 pts), higher bax/bcl-2 was associated with higher CR rate (76% *vs.* 24%, P<0.0001) and longer OS (16% *vs.* 0% at 3.5 years, P=0.00001). The independent prognostic value of bax/bcl-2 was confirmed in multivariate analysis with regard to CR (P=0.00007) and OS (P=0.0009). *Conclusions.* Bax/bcl-2 ratio may be used as a surrogate of important biologic prognostic factors, thus recognizing the central role of the apoptosis on AML prognosis and consequently focusing the future therapeutic strategies on small pro-apoptotic molecules, also in combination with conventional treatments.

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SERUM FERRITIN AS A PROGNOSTIC MARKER IN YOUNG ADULT ACUTE MYELOID LEUKEMIA

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Background. Different studies have demonstrated an iron overload contribution to post-transplantation liver toxicity, infectious events and poor survival in patients undergoing hematopoietic stem cell transplantation for haematological malignancies. *Aims*. So far, in the clinical setting of adult acute myeloid leukaemia (AML) there is no evidence of the possible role of iron in response and survival rates. We studied the role as a prognostic factor of pre-treatment serum ferritin in young adult AML. *Methods.* The study sample included 43 consecutive adult de novo AML patients (17 males and 26 females, median age 46 years, range 16 to 60 yrs). The serum ferritin level was determined at onset of the disease in each case. According to the FAB criteria the subtypes were: 3 M0, 29 M2, 6 M4, 4 M5, 1 M6. M3 subtypes were excluded from the analysis. NPM, FLT3 and cytogenetic evaluation was performed for all cases. The NPM mutation was present in 16 patients (37%) and 16 (37%) harboured the FLT3 alteration (ITD: 9 (21%); D835: 7 (16%)). Sixteen (37%) patients were in the unfavourable cytogenetic group, 8 (19%) in the favourable group and 19 (44%) presented a normal karyotype (NK). The patients were subdivided into two groups according to serum ferritin values (<800 *vs.* >800 ng/mL). Student's t-test or the Mann-Whitney test was performed for comparisons of means. Two-tailed Fisher's exact test was used to compare categories. Overall survival (OS) was measured from the time of diagnosis to death or last follow-up visit and was calculated using the Kaplan-Meier method; the log-rank test was used to compare survival curves. Logistic regression was performed for multivariate analysis. Only p values <0.05 were considered statistically significant. Results. Seventeen (40%) patients showed a ferritin serum value >800 ng/mL. Compared with the <800 ng/mL group, patients with serum ferritin >800 ng/mL were more frequently non responders to chemotherapy (35 *vs.* 73%, P=0.003) and they had a shorter OS (235 *vs.* 657 days, P=0.006). Moreover, patients with serum ferritin >800 ng/mL showed a higher frequency of documented infections during induction treatment (35% *vs.* 4%, P=0.001). At multivariate analysis, FLT3, NPM, Cytogenetic and Ferritin value (< or >800 ng/mL) all showed a statistical correlation with the response rate (P=0.02; P=0.05; P=0.03; P=0.03, respectively). Conclusions. The results of our study suggest a link between serum ferritin and AML prognosis. Further studies are needed to confirm the utility of serum ferritin as a prognostic marker in the adult acute myeloid leukaemia setting.

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PEDIATRIC-LIKE INTENSIFIED THERAPY IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: A SINGLE CENTRE EXPERIENCE

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Introduction. Acute lymphoblastic leukemia (ALL) shows different outcome in children and adults, with event-free-survival (EFS) rates of 70-80% and 30-40% at 5 years, respectively. Results apparently improved in young adults/adolescents aged 15-21 years, with de novo ALL, when treated with pediatric intensive regimens rather than with typical adult regimens and clinical studies are ongoing in older patients, toxicity related-therapy seeming the limiting issue. We report a single centre experience on adult ALL treated with an intensive pediatric-inspired schedule, aiming to assess its tolerability and efficacy. *Methods.* From 11/07 to 06/09, 11 ALL patients were treated at our Center according to modified AIEOP-LAL2000 regimen. Treatment consisted of 7 days steroid pretreatment, and 4 drugs 78-days induction (phase IA and phase IB) after which high risk patients were treated with 3 polychemotherapy blocks, while intermediate and standard risk went on 8-weeks consolidation and subsequent delayed intensification. Allo-SCT was planned for all

patients with HLA-matched donor; 2-years maintenance therapy was given to the others. Median age was 31 years (17-47). *Results.* 9/11 patients completed the phase IA, 2 being out for toxicity (grade IV infection and intestinal occlusion). 7(64%) obtained a complete remission (CR); 2 were refractory. However, 1 of them subsequently achieved CR after blocks, for an overall response rate of 73% (8/11). 7 patients then completed the 28-days phase IB. Median induction duration was 92 days (82-136). Delays were mostly due to extra-hematological toxicity, the commonest being gastrointestinal (n=12), infective (n=7) and thrombotic (n=3). Delays were accumulated in both induction phases without significant difference between IA (median 18.5 days, 4-37) and IB (median 17 days, 9-66), despite an absolute number of AE superior in IA versus IB (12 *vs.* 5). 3/8 patients received consolidation therapy; 2/3 then received allo-SCT. The median duration of consolidation was 51 days (22-94). Conversely, 5/8 received blocks: 1/5 dropped out after 1 block due to grade III renal failure. All the other 4 received allo-SCT. The median CR duration was 17 months (7+-35+); 2 patients relapsed, both after allo-SCT. With a median follow-up of 23 months (8-36) 8/11 (73%) are alive, 7 in CR (4 undergone allo-SCT). 3 patients dead, 1 in CR for infection after allo-SCT, 2 for relapsed/refractory disease. *Conclusions.* Though in a small series, pediatric-like intensive chemotherapy seemed to be feasible in adult ALL. Extra-hematological toxicity, however, caused significant treatment delays during induction. Finally, the overall outcome appeared promising, though longer follow-up and larger populations are needed to draw definitive conclusions. *Acknowledgements.* BolognAIL, EuropeanLeukemiaNet, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB2006, PRIN2008, Ateneo RFO,Project of Integrated Program(PIO),Programma di Ricerca Regione, Università 07-09.

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THE INCIDENCE AND CLINICAL VALUE OF QUANTITATIVE MONITORING OF PREFERENTIALLY EXPRESSED ANTIGEN OF MELANOMA (PRAME) AS A MARKER OF DISEASE ACTIVITY IN ACUTE MYELOID LEUKEMIA (AML)

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Introduction. AML is a clinically and molecularly heterogeneous disease. Cytogenetic and/or molecular studies cannot be applied to 60%-70% of AML patients with normal karyotype (NK-AML). It's recently have been found exon-12 nucleophosmin (NPM) gene mutations in 60% of NK-AML (about one third of all adult AML) that are characterized by cytoplasmic localization of NPM protein (NPMc). The remaining 40% of patients with NK-AML still need to be molecularly characterized. Several molecular markers have been recently proposed, which could improve patient risk-stratification. In particular, PRAME has been previously associated with a shorter relapse-free survival and overall survival in AML. *Methods.* We analyze PRAME expression by RT-QPCR by using a specific TaqMan Assay, in 22 patients affected by de novo NK-AML, with 8 out of 22 patients characterized by positivity for NPMc. Data were normalized as follows: [PRAME mRNA normalized copy number (NCN)=mRNA PRAME/mRNA GUSb*104]. PRAME levels were either extremely low or undetectable in control samples (PBMC from 20 healthy donors), with a median of 0,04±1,7 mRNA NCN. Samples were considered positive for PRAME expression if their expression level was one log higher than the mean level of expression reported for the control group (more than 0,4 PRAME mRNA NCN). *Results.* Overexpression of PRAME was found in all 22 analyzed patients, with a mean relative expression of 27.6±445.6 PRAME mRNA NCN in whole NK-AML group of patients. Specifically, 6 out of 8 patients with NPMc AML (75%) expressed more than 2 log of PRAME mRNA copies compared to the control group, with a median of PRAME expression of 25.6±74.3 PRAME mRNA NCN at diagnosis. The same patients were then molecularly monitored for PRAME expression during the follow-up period of 3-9 months, after starting conventional chemotherapy, and the derived data were compared with the NPM fluctuation expression level. In particular, the MMR assessed by monitoring PRAME mRNA shown to be in agreement with data obtained currying-out a RT-QPCR for cNPM. Importantly, we demonstrated that PRAME mRNA overexpression could be observed in all 14 patients with wt NPM (35±338 median PRAME mRNA NCN). In particular, the high SD observed in this group of patients was related to the fact that 6 out of 14 studied patients expressed more than 3 log of PRAME mRNA copies compared to the control group (790±587 PRAME mRNA NCN). *Conclusions.* Therefore, RT-QPCR of the PRAME gene may be useful for detecting MMR in AML patients, especially in NK patients. Thus, a greater molecular study need to be realized in randomized clinical trial to assess the statistical power of PRAME monitoring in either NPM mutated or wt AML patient population, to stratify patients in specific risk category. Moreover, we have previously established that *in vitro* generated PRAME-specific T cells could eliminate leukemic cells, suggesting that this approach could be effective even in AML patients.

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CHARACTERIZATION OF GENETIC TRANSLOCATIONS INVOLVING PAX5 GENE IN PEDIATRIC B-CELL-PRECURSOR-ALL: IDENTIFICATION OF NEW FUSION GENES AND DISCOVERY OF A NOVEL ZINC-FINGER CODING GENE

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Introduction. PAX5 is a transcription factor essential for B-cell development. Recently, it has been found as frequent target of abnormalities in B-cell precursor ALL cases, showing point mutations, deletions or chromosomal translocations. The functional role of these lesions is poorly understood, but their identification is increasing in new cases and will help the comprehension of the leukemogenesis. Among the translocations, numerous fusion genes are described, in which PAX5 is fused to different gene partners, such as kinases, structural proteins and transcription factors. The most frequent lesion is PAX5/TEL gene. The aim of the present study is to identify and characterize PAX5-translocations in Italian cohort of BCP-ALL pediatric patients, to understand the impact of these genetic entities in leukemia. *Methods.* Cytogenetics: karyotyping techniques; FISH using contigs of fosmids and BACs. Molecular Biology: RACE and RT-PCR; Affymetrix whole-genome Cyto 2.7M arrays. *Results.* We screened by karyotyping techniques the Italian cohort of BCP-ALL pediatric patients enrolled in AIEOP protocol, to identify cases with genetic abnormalities in region 9p13, thus potentially involving PAX5 gene. The involvement of PAX5 in 16 selected cases was assessed by FISH analysis, using contigs of fosmids. Twelve cases showed PAX5 deletions while 4/16 cases had a PAX5 translocations. We focused our attention on these 4 cases and, by 3' RACE-PCR on mRNA, we cloned the fusion genes and identified the partner gene by sequencing. In two cases, carrying the translocation t(7;9)(q11;p13), we identified the fusion gene PAX5/AUTS2, characterized by a breakpoint different from the previously reported single case. A third patient carried a dic(9;12)(p13;p13), and RACE-PCR revealed a new genetic entity where PAX5 was fused to SOX5 transcription factor. The fourth patient was carrying a translocation t(9;12)(p13;q34), in which PAX5 was fused to an unknown gene, encoding for a zinc-finger protein, newly discovered in our study. The coding sequence of this new gene was predicted by bioinformatics, and its expression verified by RT-PCR in patients cells, as well as in leukemic cell lines. All the identified fusion genes were in frame. All these four cases were also analyzed by SNPs array analysis, revealing that no significant cooperative genetic lesions are present in addition to the translocation event. *Conclusions.* Genetic abnormalities involving PAX5 gene are identified among the Italian cohort of BCP-ALL pediatric patients. In particular, we identified four new in frame fusion genes; in two cases, the partner gene AUTS2 was already known but the fusion had a different breakpoint. In one case, we identified a new transcription factor partner gene. Interestingly, one case was characterized by PAX5 fused to a previously unidentified gene, encoding for a zinc-finger molecule.

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COMPARATIVE MOLECULAR ANALYSIS OF THERAPY-RELATED AND *DE NOVO* ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction. An increasing number of therapy-related APL (t-APL) cases have been reported in the past few years. These may arise both as a consequence of chemotherapy for a primary tumor (in particular after anthracyclines administered for breast cancer) or, alternatively, following treatment of non-malignant conditions (such as multiple sclerosis, MS) with other topoisomerase II inhibitors like mitoxantrone (MTZ). We have recently characterized at the genomic level, a series of t-APL developed after MS treatment showing distinct distribution of breakpoint clustering in this subset with respect to *de novo* APL cases. Here we extended our molecular characterization by analyzing the mutational status of FLT3 and TET2 genes in t-APL as compared to *de novo* cases. *Methods.* PML/RARa isoforms, TET2 and FLT3 gene mutational status were investigated in 12 patients with t-APL and 8 patients with *de novo* APL. The main clinical and biological features of APL cases are given in Table 1. Specific primers were used to amplify the entire coding region of TET2 by PCR amplification and mutational screening was carried out by direct sequencing. All chromatograms were compared with the TET2 w/t DNA sequence (GenBank NM_001127208) using NCBI/alignment program. *Results.* RT-PCR showed a long PML-RARa isoform type in all 12/12 t-APL and in 3/8 de novo APL cases, respectively (P=0.003). As regarding the analysis of TET2, only one non-synonymous mutation in a de novo APL case was detected after excluding for common SNPs present in public databases. Four synonymous mutation were found in 2 de novo and 1 t-APL. Finally, the TET2 coding sequence was wt in 2 cases (1 de novo and 1 t-APL) as shown in Table 1. Mutations in the FLT3 were found in 3 t-APL and 2 *de novo* cases. *Conclusions.* Compared to de novo cases, APLs arising after chemotherapy show a higher incidence of bcr1 PML/RARa isoform. As to TET2 mutations, our results in a limited series suggest that alterations in this gene may not play an important role in this leukemic subset. Finally, the strong association of bcr1 isoform with t-APL combined with the low frequency of FLT3 mutations, suggests a different pathogenetic pathway in t-APL and *de novo* APL.

Table 1. Molecular features of de novo APL and t-APL patients.

Patient ID	*APL type*	*Primary disease*	*Molecular analysis PML/RARa isoform*	*FLT3-ITD*	*FLT3-D835*	*TET2* Substitution	SNP ID	Type of substitution	Amino acid change
UPN 1	t-APL	Non-Hodgkin Lymphoma	BCR1	w/t	w/t	c.G1064A	rs61744960	non-synonymous	p.G355D
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 2	t-APL	Multiple sclerosis	BCR1	w/t	w/t	w/t			
UPN 3	t-APL	L.S. syndrome*	BCR1	w/t	mut	c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 4	t-APL	Multiple sclerosis	BCR1	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
						c.A5333G	rs62621450	non-synonymous	p.H1778R
UPN 5	t-APL	Multiple sclerosis	BCR1	w/t	w/t	c.C1088T	rs17253672	non-synonymous	p.P363L
						c.T5162G	rs34402524	non-synonymous	p.L1721W
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 6	t-APL	Breast cancer	BCR1	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 7	t-APL	Multiple sclerosis	BCR1	mut	w/t	c.C1088T	rs17253672	non-synonymous	p.P363L
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 8	t-APL	Hodgkin Lymphoma	BCR1	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 9	t-APL	Multiple sclerosis	BCR1	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
						c. C423T	/	synonymous	/
UPN 10	t-APL	Multiple sclerosis	BCR1	w/t	w/t	c.G1064A	rs61744960	non-synonymous	p.G355D
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 11	t-APL	Multiple sclerosis	BCR1	w/t	mut	c.G1064A	rs61744960	non-synonymous	p.G355D
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 12	t-APL	Prostate cancer	BCR1	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 13	de novo APL	/	BCR3	w/t	w/t	c.G1064A	rs61744960	non-synonymous	p.G355D
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 14	de novo APL	/	BCR3	w/t	w/t	c.C2928A	/	synonymous	/
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 15	de novo APL	/	BCR1	mut	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
						c.A48C	/	synonymous	/
						c.A96C	/	synonymous	/
UPN 16	de novo APL	/	BCR3	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
						c.C65T	/	non-synonymous	p.P22L
UPN 17	de novo APL	/	BCR3	w/t	w/t	c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 18	de novo APL	/	BCR1	w/t	w/t	c.T5162G	rs34402524	non-synonymous	p.L1721W
UPN 19	de novo APL	/	BCR3	mut	w/t	c.T5162G	rs34402524	non-synonymous	p.L1721W
						c.A5284G	rs2454206	non-synonymous	p.I1762V
UPN 20	de novo APL	/	BCR1	w/t	w/t	w/t			

** Lewis sumner syndrome*

Acute Leukemias II

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IN VITRO CYTOTOXIC ACTIVITY OF BIOACTIVE FUNGAL METABOLITES ON LEUKEMIC CELLS

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Introduction. Despite improvements occurred in the last decades in the acute leukemia treatment, clinical results are still reporting, in a proportion of patients, disease relapse and refractoriness, prompting development of innovative therapies. Several publications have described the properties of substances derived from mushrooms and over fifty species (such as Lentinula edodes or Trametes versicolor) have yielded compounds that exhibit immunological and anti-tumor activities. The latter has been demonstrated *in vitro* by mushroom polysaccharides, and particularly by beta-glucans via apoptosis induction. However, the mechanisms underlying this activity are still unclear. *Methods.* In the present study, we examined the *in vitro* effects of bioactive compounds extract from T. versicolor on cell cycle changes (Acridine Orange, Propidium Iodide) and apoptosis induction (MTT, annexin V, caspase activation, mitochondrial membrane depolarization) of various leukemic cell lines. Fractions used in these preliminary experiments are represented by a mixture of exopolysaccharides and exoproteins, i.e. polisaccharides and proteins, which are secreted into the culture media during fungus growth. *Results.* Results obtained on the myeloid leukemic cell line OCI-AML3 demonstrated that proteins induced a marked growth inhibition due to a striking apoptosis induction, as demonstrated by an increase of the subG1-pick, after 24 hours of liquid culture, from 4.64±1.1% in the culture control to 45.11±37.6%, 72.08±47.1% and 74.2±35.2% in the presence of 10 mgr, 50 mgr and 100 mgr of proteins, respectively. Higher concentrations or longer times of exposure were required to get the same cytotoxic effects on the human lymphoid resistant cell line CEM-R, with apoptosis levels increasing from 15.9% at 24h to 63% after 48h of culture in the presence of 10 mgr of proteins. When we analyzed the cytotoxic effects of the polisaccharides we observed that this fraction resulted from 2- to 10-times less active than the protein one. In fact, in the OCI-AML3 cell line, after 24h of liquid culture, the percentage of apoptotic cells only moderately increased from 4.02±0.33% (control) to 4.95±0.17%, 8.78±4.0% and 33.82±10% in the presence of 10 mgr, 50 mgr and 100 mgr of polisaccharides, respectively. *Conclusions.* In summary, our preliminary data demonstrate a cytotoxic activity of bioactive fungal compounds on leukemia cells, suggesting that both fractions contain substances involved in these effects, even though significant differences in activity were recorded. Further studies are ongoing to biochemically characterize the bioactive compounds and subsequently to explore the molecular mechanisms underlying their anti-leukemic activity.

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TARGET MOLECOLAR THERAPY IN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS BY COMBINED BCL-2 AND MTOR INHIBITION

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Introduction. Acute lymphoblastic leukemia (ALL) cells are frequently characterized by deregulation of the apoptotic machinery. Other investigators and us have previously observed that ABT-737 (kindly provided by Abbott Laboratories), a Bcl-2/Bcl-xL (BH3 mimetic) inhibitor, exerts potent cell growth inhibition and apoptosis induction in cell lines and primary samples, especially on those lacking Mcl-1 overexpression (resistance factor). Since ALL is also characterized by the aberrant activation of mTOR and related signalling pathways, mTOR combined inhibition by CCI-779 has been further evaluated in the present study. *Methods.* Analysis of the cell-cycle: Acridine Orange technique. Apoptosis levels: Annexin-V positivity. Proliferation assay: MTT test. Protein expression: western blot (WB) analysis. *Results.* In MOLT-4 cells, ABT-737 induced a dose and time-dependent growth inhibition (IC_{50}=198nM), while at higher concentrations (250-500 nM) it induced apoptosis. In contrast, CEM-S, CEM-R, JURKAT, DAUDI and RAJI cell lines (IC_{50} ranging between 5.4 μM to 1.6×10^{20} μM) proved resistant. WB analysis revealed that all of them shared Mcl-1 overexpression. When we explored the effects of CCI-779 on the aforementioned cell lines, only minor cytotoxic effects were found at higher concentrations (IC-50 ranging between 0.5 μM to 28.2 μM), as demonstrated in the MOLT-4 cells which showed a biphasic dose response with a flat curve (35-55% growth inhibition) at concentrations ranging between 1 nM and 5000 nM (IC50=9.87 μM). To investigate the activity of ABT-737 plus CCI-779 on the resistant phenotypes, we exposed JURKAT cells to the above combination (each of them at 1000 nM). A significant (P=0.04) induction of apoptosis was found, as measured by an increase of the sub-G1 peak, to 47.7%±5.9 (CCI-779 + ABT-737) compared to the effects of the single agents (17.4%±1.5 and 4.2% ±1.5 in the presence of ABT-737 and CCI-779, respectively). Similarly, when we exposed the CEM-R cells to the drug combination (5000 nM), a strong apoptosis induction (sub-G1 peak=79.8%) was observed, as compared to the single agents (38.4% and 2.5% with ABT-737 and CCI-779, respectively). WB analysis revealed that CCI-779 exposure, particularly when combined with ABT-737, induces a Mcl-1 decrease in both cell lines. Primary cells, obtained from 9 ALL patient, treated with of ABT-737 (ranging from 50-100 nM) and CCI-779 (ranging from 5000 to 10000 nM) alone or in combination, showed an increase of the sub-G1 peak in 6/9 and in 3/9 samples exposed to ABT-737 and to CCI-779, respectively, while synergistic effects on apoptosis induction were found in 3/9 cases. *Conclusions.* The combined use of both inhibitors exerts synergistic cytotoxic effects in JURKAT and CEM-R cells, mediated by Mcl-1 down-regulation. Further studies are ongoing to define the preferential activity of this combination to overcome resistance on primary refractory ALL cells.

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RASGRP1/APTX RATIO STRONGLY CORRELATES WITH CLINICAL RESPONSE AND SURVIVAL IN AML PATIENTS TREATED WITH TIPIFARNIB-BORTEZOMIB COMBINATION

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Background. We conducted a phase I-II study aiming to assess efficacy and toxicity of tipifarnib-bortezomib treatment in elderly AML patients. RASGRP1/APTX genetic ratio, which is associated with treatment response in patients treated with tipifarnib alone, was tested. *Methods.* Eighty patients were enrolled with secondary-AML: 14 had high risk cytogenetics; 42 were previously untreated. Seventy-five patients were treated. *Results.* Nine patients achieved complete remission (CR), 1 patient obtained a partial response (PR) and in 2 cases an hematological improvement (HI) was documented for an overall response rate (ORR) of 19%. Median time to response was 112 days, corresponding to 4 cycles (range 2-14). 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), peripheral blood (PB) or both. 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 <8, while the response rate was 43% [how many were CR?] in patients with RASGRP1/APTX >8 (P<0.0001). Finally, RASGRP1/APTX levels significantly correlated with OS (P=0.001) with a median OS of 490 days and 162 days in patients with RASGRP1/APTX >8 and <8 respectively. *Conclusion.* We conclude that the clinical efficacy of the combination of tipifarnib and bortezomib was evident. We confirmed that the RASGPR1/APTX BM or PB level is an effective predictor of response and survival and our now studying the response of such patients to tipifarnib, alone.

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BROAD COPY NEUTRAL-LOSS OF HETEROZYGOSITY REGIONS AND RARE RECURRING COPY NUMBER ABNORMALITIES IN KARYOTYPICALLY NORMAL ACUTE MYELOID LEUKEMIA GENOMES

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Background. Approximately 40-50% of AML are karyotypically normal (KN-AML) and prognostically heterogeneous, showing various molecular alterations. Loss of heterozygozity (LOH) is an allelic imbalance in polymorphic loci, resulting from genomic losses or gains leading to unequal ratios of parental alleles. Genetic polymorphisms based on copy number changes (germ-line CNVs) are present in all tissues of the subject and are not different for size and structural features from somatic copy number alterations (CNAs) accumulated in cancer cells, selectively present in the leukemic clones. *Aims.* To test the ability of the last generation of Affymetrix single nucleotide polymorphism (SNP)/CNV platform (SNP Array 6.0) to distinguish somatic tumour-associated CNAs and LOHs from germ-line CNVs and LOHs and to identify possible recurring genomic abnormalities. *Methods.* 19 patients have been studied, 9 females, 10 males (median age 42 years, range 25-70) using bone marrow aspirates collected at diagnosis (Dx) and at the first complete remission (R), defined as karyotypically-normal on the basis of standard metaphase cytogenetics (MC). Thus, we re-analysed them using the SNP Array 6.0 Assay kit (Affymetrix, Santa Clara, CA) and HapMap 270 DNA as reference. Signal intensity was analysed using Genotyping Console Version 3.0.1. We obtained quality control (QC) call rates in excess of 90% in all cases and MAPD <0.4. In 11 cases we obtained a bone marrow sample at the remission phase of comparable high quality, used to obtain a first estimate of tumor-associated CNAs. All Dx segments not overlapping for more that 30% of their size the corresponding R segments were included in a list of somatic CNAs. *Results.* Only 1 case out of 19 showed a high number of submicroscopic CNAs, both gains and losses, whose somatic origin and association to the tumour were confirmed by the comparison to the remission sample. In remaining cases, we observed few recurring CNAs per sample (Table 1). However, a high prevalence of CNAs in the KN-AML population was detected, thus providing new hints towards identification of cooperating mutations. In particular, our results point to the region 3p14.1-p12.3 as a target for the identification of driver mutations in AML. An extensive search of all tumour-associated CN-LOH regions >1 Mb revealed only 3 broad regions (terminal 12Mb of 22q, terminal 27Mb of 1p and the whole chromosome 21) in three patients out of 19 (16%). All CN-LOH segments lower than 10 Mb were not tumour-associated, as shown by their presence in corresponding matched R samples. CN-LOH of the whole chromosome 21 was responsible for homozygosity of a missense mutation (R80C) of RUNX1/AML1. *Conclusion.* Our study confirms that a relative submicroscopic copy number stability, with low recurrence of specific CNAs and broad CN-LOH regions, characterises KN-AML.

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WT1 OVEREXPRESSION AT DIAGNOSIS IS THE BEST INDEPENDENT PREDICTOR OF FAVORABLE OUTCOME IN DE NOVO NON-M3 AML PATIENTS

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Introduction. Besides cytogenetics, FLT3 and NPM mutations and expression of WT1 and BAALC exert a relevant role in predicting complete remission and outcome of AML patients. *Methods.* A cytogenetic and molecular profile was performed in 100 consecutive untreated non M3 denovo AML patients receiving induction chemotherapy (standard induction in 41 patients, fludarabine-ara C-idarubicin and low dose gemtuzumab ozogamicin in 59 patients) followed by standard consolidation therapy, with the aim of predicting CR rate and long term outcome. Expression levels were obtained by Real-Time-PCR upon normalization on Abl expression. *Results.* 55 patients were younger than 60 years (median age 46) and 45 were older (median age 69). Intermediate or favourable karyotype was detected in 81 patients (81%), unfavorable karyotype in 19 patiens. FLT3: 21 patients (21%) had ITD and 7 (7%) had exon 20 mutations. WT1: in 26 patients (26%) expression was >2365 (>75th percentile); in 74 <2365 (74%); NPM (A and B): the genes were mutated in 37 patients (37%) and non mutated in 63 (60%); BAALC: in 49 patients expression was <1000 (49%), in 51 >1000 (51%). Molecular profile was similar in the younger and in the older subgroups of patients (p NS). In the whole cohort of patients CR rate was influenced by age (P .0002), cytogenetics (P .002), WT1 expression (CRs: 21/26 in pts with WT1 >2365; 41/74 in pts with WT1 <2365, respectively, P 0.02) NPM gene mutations (P 0.009), BAALC expression (CRs: 36/49 pts with BAALC <1000; 26/51 in pts with BAALC >1000, respectively, P 0.02) The presence of FLT3 ITD or FLT3 mutations at exon 20 did not affect CR rate. In pts with not unfavourable karyotype (81 pts, 48 younger than 60) both WT1 overexpression and NPM mutations retained a favourable impact on CRrate. In the whole cohort WT1 overexpression, younger age and not unfavourable karyotype had a significant positive impact on EFS and OS both in univariate and multivariate analysis. In the subgroup of younger patients with not unfavourable karyotype WT1 expression retained its predictive value on EFS and OS (see Figure 1A and 1B). In this subset of patients FLT3 ITD too showed a significant impact on EFS (P 0.034) but only in the univariate analysis. In elderly patients only karyotype significantly influenced OS (P 0.001). *Conclusions.* In the whole cohort of denovo AML patients age, karyotype, WT1 and BAALC expression and mutational status of NPM gene predict the probability of achieving CR. In the subgroup of patients with not unfavourable karyotype high WT1 expression and NPM gene mutations were associated with higher CR rate. OS and EFS were affected by age, cytogenetics and WT1 expression at diagnosis. In the subgroup of younger patients with not unfavourable karyotype WT1 overexpression was the best predictor of favourable outcome.

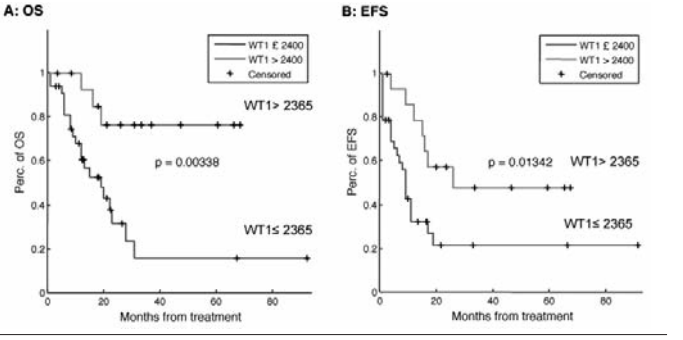

Figure 1. *De novo* AML, age < 60, not unfavorable karyotype.

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ANALYSIS OF FLT3-MUTATIONS IN 186 ADULT NON-M3 AML PATIENTS CONSECUTIVELY TREATED AT THE HEMATOLOGY CENTER, "SAPIENZA" UNIVERSITY OF ROME

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Introduction. Molecular analyses indicate that many types of AML have cytogenetically undetectable mutations with prognostic impact. Somatic mutations of FLT3 involving internal tandem duplications in the juxtamembrane domain (FLT3/ITD) or point mutations affecting the second tyrosine-kinase domain (FLT3/TKD) are among the most frequent mutations detected in AML with normal karyotype. While the adverse impact of FLT3/ITD in non-M3 AML patients is established, the prognostic significance of FLT3/TKD is uncertain. In addition, the best post-remission therapy is still an open question. The aim of our study was to evaluate the prognostic impact of FLT3/ITD and FLT3/TKD in 36 adult AML patients consecutively treated at our Institute with a homogeneous AML protocol. *Patients and methods.* The analysis included 36/186 adult (median age 45 years) non-M3 AML patients with FLT3 alterations referred to our Institute from 1999 to 2007 and treated with a homogeneous and intensive AML protocol. The FLT3 analysis was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) of both exons 14-15

and 20. Digestion with ECO RV enzyme was employed to discover FLT3/TKD affecting codons 835/836. Twenty-eight patients harbored an FLT3/ITD (22 in heterozigosis with the normal allele and 6 with lack of normal allele), while 8 had an FLT3/TKD. *Results.* After induction chemotherapy, 17/28 (61%) FLT3/ITD patients obtained a CR, 6/28 (21%) had a refractory disease and 5 (18%) died during induction; all FLT3/TKD patients obtained a CR. After CR, 11/17 (65%) FLT3/ITD patients were transplanted (10 autografts and 1 allograft), while 6 (35%) did not undergo a transplant procedure due to early relapse (n=2) or ineligibility (n=4). Only 2 patients are alive: 1 in CCR 77 months after an autograft, while the other, who relapsed 7 years after an autograft, is alive in II CR more than 2 years later. Regarding the 8 patients with FLT3/TKD, 5/8 (62%) were transplanted (3 autografts and 2 allografts), 3/8 (37%) did not receive a transplant due to inelegibility. Four patients (50%) are in CCR at a median time of 88 months since diagnosis. The 2-year projected OS of FLT3/ITD patients is significantly shorter compared to FLT3 wild-type (WT) and FLT3/TKD patients (7% *vs.* 44% *vs.* 50%, respectively; P=0.0001); the 2-year projected DFS of the ITD positive patients is also significantly lower (5.8% *vs.* 47% *vs.* 50%, respectively; P=0.0006), and the median duration of remission is 8, 40, 16 months, respectively. No significant differences were observed between FLT3/TKD and WT patients, both in terms of OS and of DFS. *Conclusions.* Our data confirm the negative prognostic impact of FLT3/ITD both in terms of DFS and survival. New treatment protocols including selective FLT3 inhibitors are under investigation to improve the clinical outcome. There is no evidence from our experience that FLT3/TKD influences DFS and survival compared to WT patients.

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THE HISTONE DEACETYLASE INHIBITOR SODIUM BUTYRATE INDUCES APOPTOSIS IN NPM1-MUTATED AML CELLS

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AML carrying NPM1 mutations [Falini B *et al.*, NEJM 2005;352:254-266] accounts for about one-third of adult AML, shows distinctive biological and clinical features [Falini B *et al.*, Blood 2007;109:874-885] and has been included as a provisional entity in the 2008 World Health Organization (WHO) classification of myeloid neoplasms. In spite of the relatively good prognosis of NPM1-mutated AML, there are still cases that show poorer outcome, especially those associated with FLT3-ITD mutation and elderly patient population.

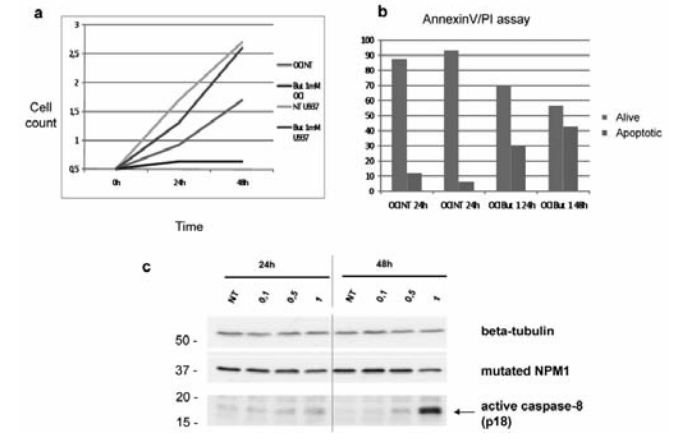

Figure 1.

Therefore new therapeutic strategies need to be explored. Here, we investigated the effect of sodium butyrate, a short-chain fatty acid which has long been known to be a histone deacetylase inhibitor (HDACi) able to induce maturation in normal and tumor cells, in cellular models of NPM1-mutated AML: i) the OCI/AML3 cell line, previously identified as a human AML cell line carrying cytoplasmic mutated NPM1 in the absence of FLT3-ITD; ii) primary AML cells originated from a patient with NPM1-mutated AML bearing FLT3-ITD mutation (MONT1) and propagated as cell line in NOD/SCID mice; and iii) primary AML cells from 4 NPM1-mutated AML patients at diagnosis. In either cell lines or patients' primary AML cells carrying NPM1 mutation, but not in the U937 cell line (not harboring NPM1 gene mutation) used as control, growth arrest and pro-apoptotic effects were evident after 24 hrs and marked after 48 hrs of treatment with doses of drug of 0.5-1 mM (Figure 1a and b). In particular, no signs of differentiation were evident at morphological examination of treated cells. Induction of apoptosis was associated with activation of caspase-8 (Figure 1c), suggesting involvement of the death cell receptors pathway. Levels of either NPM1 mutant or wild-type protein did not appear significantly affected by treatment with sodium butyrate. Further studies are needed to characterise the molecular mechanisms of sodium butyrate-induced apoptosis in NPM1-mutated AML cells.

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THE HSP90 INHIBITOR 17-(ALLYLAMINO)-17-DEMETHOXYGELDANAMYCIN (17-AAG) INDUCES APOPTOSIS IN NPM1-MUTATED AML CELLS

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AML carrying NPM1 mutations [Falini B *et al*, NEJM 2005;352:254-266] accounts for about one-third of adult AML, shows distinctive biological and clinical features [Falini B *et al*, Blood 2007;109:874-885] and has been included as a provisional entity in the 2008 World Health Organization (WHO) classification of myeloid neoplasms. In spite of the relatively good prognosis of NPM1-mutated AML, there are still cases that show poorer outcome, especially those associated with FLT3-ITD mutation and elderly patient population. Therefore new therapeutic strategies need to be explored. Here, we tested whether 17-(Allylamino)-17-demethoxy-geldanamycin (17-AAG), an inhibitor of the molecular chaperone heat shock protein 90 (Hsp90), known to affect proper folding and steady-state level of different proteins and , could have an effect on cell growth and survival of AML cells carrying cytoplasmic NPM1mutated protein. As cellular models of NPM1-mutated AML we used: i) the OCI/AML3 cell line, previously identified as a human AML cell line carrying cytoplasmic mutated NPM1 in the absence of FLT3-ITD; ii) primary AML cells originated from a patient with NPM1-mutated AML bearing FLT3-ITD mutation (MONT1) and propagated as cell line in NOD/SCID mice; and iii) primary AML cells from 4 NPM1-mutated AML patients at diagnosis. In either cell lines or patients' primary AML cells carrying NPM1 mutation, but not in U937 and HL-60 cell lines (not harboring NPM1 gene mutation) used as controls, growth arrest and pro-apoptotic effects were evident after 24 hrs and marked after 72 hrs of treatment with doses of drug starting from 0.5 M (Figure 1a and b).

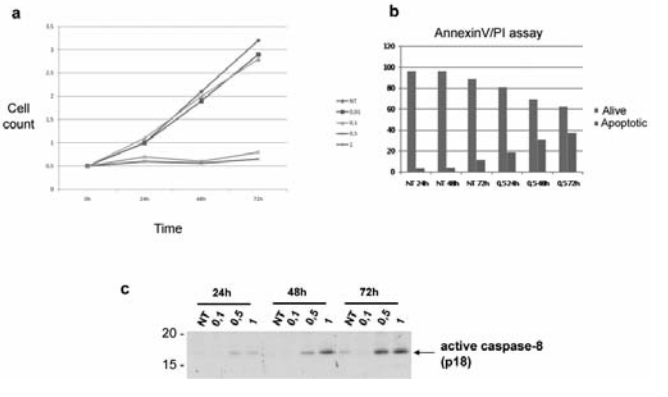

Figure 1.

Morphological analysis showed cytoplasmic vacuolization and apoptotic bodies' formation. As expected, levels of Hsp70 protein in AML cells increased upon 17-AAG treatment, confirming Hsp90 inhibition. Interestingly, activation of caspase-8 (Figure 1c), associated with down-regulation of NPM1 mutant protein in some cases, was documented at Western blot analysis. Although effects on NPM1 mutant and wild-type

protein levels were not evident in all cases, effects on multiprotein complexes established by NPM1 can not be excluded and are under investigation. Our preliminary data suggest Hsp90 inhibitors might represent valuable alternative drugs to study and test in the NPM1-mutated AML setting.

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TRAIL IS INVOLVED IN HYPOXIA-INDUCED APOPTOSIS OF ACUTE MYELOID LEUKEMIA T(8;21)-POSITIVE CELLS

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Introduction. The acute myeloid leukaemia (AML) is a clonal neoplasia of the hematopoietic system characterized by a block of proliferation control and hematopoietic differentiation. 15-20% of AML, defined core-binding factor AML (CBF-AML), is caused by two chromosomic aberration, t(8;21) or inv(16). In particular, t(8;21) AML is defined by the expression of AML1/ETO fusion protein, responsible for HDAC recruitment, determining transcriptional repression of target genes involved in myeloid maturation. We investigated the effects of severe hypoxia on inhibition of proliferation, induction of apoptosis and histone acetylation, and modulation of AML1/ETO expression. Moreover, we deepened the role of TRAIL in the apoptosis induced by hypoxia. *Methods.* We used Kasumi-1 t(8;21)-positive cell line and U937-A/E cell line, characterized by AML1/ETO-inducible expression. Cells were incubated in 0.3% O2 or normoxia for several days, and treated or not with blocking antibody for TRAIL or control antibody of the same isotype. Cells were harvested at different time points and analysed as for their number, proliferation by propidium iodide staining and viability by the annexin-V test, or lysed to undergo western blotting or real-time PCR. *Results.* Hypoxia reduced cell viability of Kasumi-1, that is correlated to a reduction of proliferation, as indicated from the increase of cells percentage in phase G0/G1 and the decrease of that in phases S and G2/M, and an increase of apoptosis. Hypoxia-induced apoptosis is confirmed by caspase-9, -8 and -3 activation. Hypoxia additionally induced AML1/ETO disappearance and acetylation of histone H4. Histone acetylation is induced before the disappearance of fusion protein to indicate that hypoxia function as an epigenetic modulator. When we used U937-A/E cell line, we showed that hypoxia induced a high amount of apoptosis only in AML1/ETO-expressing cells, underlining the relationship between hypoxia, as apoptotic agent, and AML1/ETO as sensitizing factor to a pro-apoptotic condition. Then we showed that the presence of AML1/ETO induced the expression of TRAIL and DR5 mRNA. In AML1/ETO-expressing cells, hypoxia initially determined a further increase of TRAIL expression. Moreover Kasumi-1 cells physiologically expressed TRAIL and DR5 mRNA levels similar to those of U937-A/E AML1/ETO-expressing cells. Although hypoxia maintained stable TRAIL mRNA, TRAIL protein is markedly induced by hypoxia. Finally we pointed out that the blocking antibody for TRAIL significantly reduced hypoxia-induced apoptosis (about 20%) of Kasumi-1 cells and completely impaired that of U937-A/E AML1/ETO-expressing respect to not expressing cells. *Conclusion.* Hypoxia seems to play a relevant anti-leukemic effect, inhibiting cell proliferation, inducing apoptosis and working as an epigenetic modulator. The high sensitivity of AML1/ETO-positive cells to the apoptotic effect of hypoxia involves TRAIL extrinsic pathways of apoptosis.

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MOLECULAR ANATOMY OF IGH REARRANGEMENT AND CDR3 STRUCTURE IN B-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

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Many studies have shown a preferential usage of specific immunoglobulin heavy chain (IgH) genes in B-CLL and the correlations between the molecular characteristics of IgH rearrangement and the outcome of the malignancy are currently in use in the clinical management of CLL. Few studies have reported on the Ig gene usage in B-cell ALL, all indicating an overusage of the VH6-1 gene. The privileged usage of the VH6-1 segment was previously identified as a component of the rather restricted human fetal antibody repertoire (Schroeder *et al.*, 1987). Development stage-specific gene usage and changes in the third high variable complementary-determining region (CDR3) structure have been reported (Berman *et al.*, 1991; Souto-Carneiro *et al.*, 2005). CDR3 is generated through a gene recombination during the early stage of B-cell differentiation (pro-B) and the increase in length of the CDR3 during ontogeny is particularly noteworthy. CDR3 length increase seems to be related to the choice of gene usage, level of TdT activity and nucleotide excision in the coding genes. In ALL, the structural characteristics of the IgH CDR3 remain unknown. In our study, 157 de novo B-lineage ALL enrolled in the GIMEMA ALL protocols between 2005 and 2009 were examined for the immunological cell receptor genes. Among them, 133 complete IgH rearrangements were identified, sequenced and studied for the CDR3 molecular structure. IgH rearrangements were studied for V-D-J gene usage, mutational status, functionality, CDR3 length, V-J distance, N addition and number of trimmed nucleotide at the V, D and J junctions. The CDR3 molecular characteristics were analyzed according to the IMGT criteria. To perform comparative analisys between leukemic and normal IgH rearrangements, we used two control groups (adult peripheral normal lymphocytes and bone marrow pro-B cells). Looking at the homology with the germline, we found, as aspected, 130/133 rearrangemets showing plus than 98% homology; 110/133 were non-productive (86 of them were out of frame (OF)). The percentage of OF rearrangements was 64.7% according to that of] pro-B cells reported by Rao *et al.*, 1999. IgHV usage in ALL patients was compared to our peripheral control group and to previously published data (Brezinschek *et al.*, 1995). In agreement with the literature, we found a VH6-1 gene overuse and a VH3-23 underuse in ALL compared to normal peripheral lymphocyte. This may be due to a different maturative stage; in fact, some differences between immature and mature B-cells may be attributed to the numerous positive and negative selection checkpoints. To avoid the bias introducted by selection mechanisms, we compared unproductive rearrangements (therefore unselected) for gene usage between ALL and the pro-B control group and found no differences in any VH genes. CDR3 featureas of ALL and the control group are summarized in Table 1. Similarly, we found no difference in CDR3 molecular features between ALL and pro-B cells (Table 1); moreover, ALL rearrangements showed the same CDR3- adult type features reported by Souto-Carneiro *et al.*, 2005. Our results suggest that the differential VH6-1 and VH3-23 gene usage in B-lineage ALL may due to B-cell differentiation selection mechanisms. Moreover, the molecular anatomy of IgH rearrangements seems to show no correlation with leukemogenesis; nevertheless, its role in identifying leukemic cells remains a powerfull tool for the evaluation of minimal residual disease.

Table 1. Non productive rearrangements analyzed in this study.

	N°	Mean bp CDR3 Length* (median)	Mean bp VH-JH Distance* (median)	Erosion V3'-D5' D3'-5'J* (bp)	VHD-DJH N addition* (median)	VHD-DJH with no addition* (n)	Missing CDR3 landmarks (n)	IGHD-IGHD Fusion (n)	P-value
B-ALL	110	60.8 (60.0)	34.2 (33)	2.88-5.1 4.8-6.2	8.9-7.9 (8.0-7.0)	4-1	13	5	NS
pro-B cells	50	61.56 (60.5)	36.03 (33.05)	1.8-5.2 4.5-7.0	10.01-8.83 (6.0-8.0)	4-1	4	4	NS

* Rearrangements missing CDR3 landmarks and with identified IgHD-IgHD fusion were not included

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LOW-DOSE GEMTUZUMAB-OZOGAMICIN AS POST-CONSOLIDATION THERAPY (WITH OR WITHOUT ASCT) IS FEASIBLE IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKAEMIA

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Background and aims. Elderly patients with acute myeloid leukemia (AML) are less able to tolerate intensive chemotherapy regimens and are more likely to have poor-risk cytogenetic abnormalities. In these patients initial complete remission (CR) rates achieved with conventional induction chemotherapy are around 45% to 55%; moreover most of these patients eventually relapse: the relapse risk (RR) is indeed around 80% to 85%. Recently the use of new induction regimens (including new drugs) resulted in better CR rates, but we urgently need to reduce the persistently, unacceptably high RR in this frail population. Gemtuzumab ozogamicin (GO) is a humanized anti-CD33 monoclonal antibody, conjugated to calicheamicin, that is rapidly internalized after binding to CD33. Phase II studies in patients with AML in first relapse showed that GO monotherapy, at 9 mg/m^2, was associated with a 26% ORR in patients aged>60 years. Except for two recently published reports, little is known about the usefulness and the safety of administration of multiple courses of GO, at lower doses, as post-consolidation therapy, when the leukemic burden has been consistently reduced. We evaluated the safety and efficacy of low dose GO in a cohort of AML elderly patients, in CR1, fit for aggressive chemotherapy, after consolidation with both. *Design and methods.* From November 2006 to December 2009 55 patients with non-M3 AML, median age 69 years (range 60-80), were prospectively evaluated according to a simplified Multidimensional Geriatric Assessment, to identify fit from unfit or frail patients; 17 fit patients were eligible for aggressive chemotherapy and received a planned induction schedule, including High Dose-ARA-C for 5 days plus Idarubicin. Then the 11 patients in CR received consolidation with intensive chemotherapy and were planned to receive a further consolidation with ASCT, followed by low dose GO or low dose GO after mobilization failure; GO was administered in both cases at 3 mg/m^2 three times monthly on an outpatient basis. *Results.* 11 patients (61%) achieved CR and 2 achieved PR; 1 had refractory disease and 3 died in aplasia; 3 patients successfully mobilized PBSC and underwent ASCT followed by GO. Among the 8 patients that did not mobilize PBSC, 6 received GO while 2 died due to early relapse. Overall 9 patients received post-consolidation GO (3 after ASCT and 6 after chemotherapy). With a median follow up of 17 months (range 6-30), 4 patients are alive and 3 of them are in continuous CR (only one had been previously received ASCT); 6 relapsed after stopping GO (1,1,2,3,3 and 14 months respectively). The median OS in the 17 pts, evaluated according to the intention to treat criteria is 6 months and disease-free survival (DFS) at 30 months, calculated in the 11 patients achieving the CR, is 30%. *Conclusions.* Our data confirm that post-consolidation therapy with low-dose GO seem to be safe and easily manageable while its impact on the outcome of these patients needs to be evaluated in a larger cohort of patients.

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T-LBL AND T-ALL: DIFFERENCES AND SIMILARITIES DETECTED BY GENOMIC AND TRANSCRIPTOMIC ANALYSES

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Introduction. Childhood T-cell malignancies include T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL). T-LBL represents about one third of non-Hodgkin's lymphomas of childhood, while T-ALL accounts about 20% of all paediatric ALL. The two pathologies are often considered to be different manifestations of the same disease, indeed they share common morphologic and immunophenotypic features at diagnosis but show distinct clinical presentations: bone marrow involvement for T-ALL and mediastinal mass for T-LBL. The recent use of T-ALL type therapy in the treatment of T-LBL has improved the outcome for these patients suggesting that a deeper study of the two pathologies could lead to select an appropriate treatment for T-LBL patients. *Methods.* Toward a better understanding of the biological relationship between T-ALL and T-LBL we performed genome-wide gene expression profiling on 20 T-LBL and 10 T-ALL diagnostic specimens. Genome-wide copy number alteration analysis was also performed on a subset of the samples analyzed by gene expression profiling and characterized for NOTCH1 mutational status. *Results.* The results confirmed that T-LBL and T-ALL share a large number of features but also display distinct transcriptional signatures. This gene signature includes genes involved in chemotactic responses and angiogenesis which may play a role in the different tumor cell localization. The genome-wide copy number analysis detected lesions in all T-ALL and T-LBL patients. Although most aberrations are found in both entities some of them are recurrently detected in T-LBL but not in T-ALL or vice versa. Moreover, more than 38% of the tested genes show a positive correlation between the genetic lesion and the gene expression in at least one dataset (T-LBL, T-ALL or combination of the two), suggesting that gene expression data could be informative on the effects of genetic aberrations. As expected, NOTCH1 mutations were identified in both T-ALL and T-LBL, however the mutational status distinguishes two subgroups based on their transcriptional profiles and genetic aberrations. *Conclusions.* Taken together these results suggest that T-LBL and T-ALL share a large fraction of their biological features; nevertheless each malignancy seems to display also a unique pattern of genetic lesions affecting their transcriptome. However, further validation in a large panel is needed in order to completely understand the genetic basis of these two pathologies.

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BIOLOGICAL FEATURES AND PROGNOSTIC IMPACT OF CRLF2 OVEREXPRESSION IN PEDIATRIC B LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Although in AIEOP-ALL 2000 protocol, early MRD assessment allowed to better recognize high risk (HR) cases, still about 25% of children relapse, with more than half of relapses occurring in the largest subgroup of non-HR patients. Therefore, new prognostic markers are necessary to adapt treatment. Recently, we and others identified a novel subgroup of BCP-ALL with high-level expression of the CRLF2 gene, as a consequence of a translocation at the IGH@ locus, and/or an interstitial deletion, juxtaposing CRLF2 with the promoter of the P2RY8 gene. CRLF2 over-expression appeared to be the driver of STAT activation, either alone or in combination with gain-of-function mutations in Janus Kinases (JAKs), mainly JAK2 R683. In order to estimate the incidence of this abnormality, its prognostic value and the association with patients' biological features, we analyzed CRLF2 gene expression in 317 non-Down Syndrome BCP-ALL children (500 total cases planned), treated according to the ALL-AIEOP 2000 protocol. *Methods.* MRD and chromosomal translocation screening was routinely performed. Gene expression profiling by Affymetrix HG U133 Plus 2.0 arrays; RQ-PCR for CRLF2 expression; High Resolution Melting (HRM) and sequencing for JAK2 mutations, gene Copy Number Analysis by Affymetrix Cyto 2.7M array. *Results.* Out of 317 samples, 42 showed a CRLF2 expression 5 times higher than the overall median (13%). The P2RY8-CRLF2 rearrangement was detected in 7/28 CRLF2-high samples (25%) and in 6/8 considering only the CRLF2-highest (>30 times) expression group (75%). JAK2 mutations were observed in 4/67 cases (2/2 with P2RY8-CRLF2). Comparing CRLF2-high and -low, no significant differences were observed for main features and drug response. No cases with recurrent chromosomal translocations were in the CRLF2-high group. CRLF2-high patients had a lower WBC count at diagnosis (>20,000/ l: 28% *vs.* 37%) and a higher age at diagnosis. Patients with CRLF2-high had a worse 5-year EFS compared to CRLF2-low patients (74±7% *vs.* 79±3%), mainly due to a high-

er cumulative relapse incidence (CRI) (26±7% *vs.* 19±2%). CRLF2-high patients were mainly in the MRD non-HR group. BCP-ALL, non-HR patients with CRLF2-high had an EFS probability of 78±7% compared to 84±3%, with major differences in CRI (23±7% *vs.* 14±2%). Remarkably, 7/7 P2RY8-CRLF2-positive were non HR by MRD; only 6/42 high CRLF2 patients were stratified as HR. *Conclusions.* High level CRLF2 expression was associated with a poor EFS in childhood BCP-ALL treated according to the AIEOP-ALL 2000 protocol, although patients seem to be sensitive to the early *in vivo* response. This effect was mainly related to a higher CRI in non-HR patients with presence of the P2RY8-CRLF2 rearrangement. Once prospectively confirmed in large series, the assessment of CRLF2 status may serve as a new stratification marker.

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SEQUENTIAL ACQUISITION OF BCR-ABL1 FUSION AND IKAROS DELETION IN PHILADELPHIA CHROMOSOME POSITIVE ALL

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Introduction. The pathogenesis of childhood Acute Lymphoblastic Leukaemia (ALL) is presumed to follow a 2-hit model. The transcription factor IKAROS (IKZF1) is deleted in the majority of BCR-ABL1 ALL. This suggests that loss of IKAROS function is an important event in the development of BCR-ABL1 ALL. The sequence of cooperating oncogenic events in BCR-ABL1 ALL is currently unknown. *Methods.* We performed extensive genomic analysis of two pairs of monozygotic, monochorionic twins, one pair concordant the other discordant for Ph + ALL. *Results.* The concordant twins received identical treatment (AIEOP-BFM ALL 2000) but followed a different clinical course. Twin 1 was a good prednisone responder and survived after BMT, whereas twin 2 had a poor prednisolone response, demonstrated persistent high MRD and died after BMT. Whole-genome-wide high density 500K SNP array analysis identified high hyperdiploidy in twin 1 and IKAROS deletion in twin 2. The BCR-ABL1 fusion breakpoint sequence was shared between the concordant twins. Of discordant twin set 1, the patient was treated on the EsPhALL protocol, inclusive of imatinib mesylate. She was a Slow Early Responder (SER) and had high MRD at the end of induction. She received an allogeneic stem cell transplant, but unfortunately relapsed 6 months later and died of progressive disease. SNP array analysis demonstrated deletion of IKAROS, CDKN2A, and PAX5. The healthy co-twin had, in blood, a BCR-ABL1 positive clone (at ~ 10-4) lacking IKAROS deletion. Blood spots of the two twins shared the same BCR-ABL1 genomic sequence. *Conclusions.* This data indicates prenatal origin of BCR-ABL1 fusion and subsequent - presumed post-natal - loss of IKAROS and is compatible with the notion that the malignancy and poor outcome of Ph^+ p190 BCR-ABL1 ALL is driven in some measure by IKAROS deletion.

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TEL-AML1 INCREASES THE SPONTANEOUS MOTILITY BUT IMPAIRS THE CHEMOTAXIS TO CXCL12 IN AN *IN VITRO* MODEL OF PRE-LEUKEMIA

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Introduction. The t(12;21) chromosome translocation generating TEL-AML1 chimeric fusion gene is a frequent initiating event in childhood leukaemia. Its impact is to generate a clone of covert, clinically silent pre-leukemic B cell progenitors. The leukemia arises only following second, post-natal hit/genetic events occurring years later. Moreover, relapse of leukemia is frequently arising from the pre-leukemic clone. Aim of our study is to investigate how TEL-AML1 expression can sustain this covert condition for many years and protect the pre-leukemic clone to effective eradication during the conventional therapies anti-leukemia. In a recent paper we described that the fusion gene rendered the B precursors resistant to the inhibitory activity of TGFβ. Here we want to inquire into other factors that can explain the positive selection of the pre-leukemic clones over the normal counterpart. In particular, given the importance of the interaction with the microenvironment for survival signals for normal and leukemic stem cells, we question if the fusion gene causes changes in cellular adhesive and migratory properties. *Methods.* We used a TEL-AML1 inducible expression system generated by stable transduction of the murine pro-B Ba/F3 cell line and murine PreBI primary cells. *Results.* The expression of TEL-AML1 in Ba/F3 cell line causes alteration in morphology and phenotype: the cells acquire long extensions and several molecules involved in cell adhesion and migration are disregulated. Moreover, we find that TEL-AML1 significantly impairs the chemotactic response to CXCL12 in transwell migration assays *in vitro*, although the expression and the recycling of CXCR4 receptor are unaffected. This inability is not due to an increase in adhesion properties or defects to migrate in general, as spontaneous motility are increased, but is associated with an alteration in phosphorylation of p38 mitogen-activated protein kinase (MAPK) and of ERK induced by CXCL12. The results have been confirmed in murine PreBI primary cells. *Conclusions.* These preliminary results indicate that, in our murine models, TEL-AML1 affects the cellular adhesive and migratory properties and we are now investigating how these alterations can give advantages to the pre-leukemic cells in the pathogenesis of TEL-AML1–expressing leukaemia.

Transplantation I

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THE MONITORING AND THE TREATMENT OF CYTOMEGALOVIRUS REACTIVATION AFTER AUTOLOGOUS STEM CELL TRANSPLANT FOR LYMPHOPROLIFERATIVE DISEASE: A PROSPECTIVE STUDY ON 92 PATIENTS

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

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TREATMENT OF IRON OVERLOAD AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION BY ERYTHROCYTAPHERESIS: CASE REPORT

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Introduction. In the patients undergoing allogeneic bone marrow transplantation (BMT) the iron overload (IO) remains an important cause of morbidity and mortality. The follow-up post-BMT, assessed by serum ferritin and SQUID technique, has shown that the IO decreases very slowly in the young patients and almost nothing in the elderly. Phlebotomy is the main treatment actually used to reduce the IO after BMT. In the present study we report a case of a patient affected by acute myeloid leukemia FAB M5b treated by Erythrocytapheresis (EA) in order to induce iron depletion post-transplantation. *Methods.* A 43-year-old man was hospitalized by our Division of Hematology with fever, dyspnea, and hematuria. The blood count showed the following values: WBC: 76.700/l , Hb 6.2 g/dl; PLT 21.000/l and the smear of peripheral blood was infiltrated by monoblastic, promonocytes and monocytes cells. The bone marrow aspirate showing a diagnosis of Acute Monocytic Leukaemia subtype FAB M5b characterised by >80% of monocytoid cells lysozyme +, CD68 $^{+}$, myeloperoxidase + and rare Auer rods. Typing immunology showed the following results: CD34 1%, CD 117 68%, MPO 16%, CD13 29%, CD33 80%, CD64 77%, CD68 72%, CD45 96%. The cytogenetic analysis was negative. The patient was then submitted to a cycle of chemotherapy according to the protocol GIMEMA/LAM 99P and obtained a complete remission (CR) after a month. After about 6 months the patient relapsed and received 2 cycles of the FLAD (Fludarabine / AraC / liposomal Danorubicine), obtaining a second CR after 1 month. The identification of a family haploidentical donor allowed, after 4 months, the BMT with conditioning regimen. The clinical course post-transplantation occurred in the absence of the acute and chronic graft-*versus*-host disease, but a slow recovery of the haematological parameters was observed and the patient was submitted to stimulation with G-CSF and 2 transfusions of red blood cells. Biochemical controls performed after 12 months of BMT revealed ferritin values> 800 ng/mL, with signs of iron overload and a therapeutic iron depletion was considered. Presenting the patient a good hematopoietic activity (Hb> 11, WBC> 5000, PLT> 100.000), an EA program was conducted with discontinuous flow cell separator MCS Plus (Haemonethics). *Results.* The protocol therapy was executed as follows: 1 treatment/week for 3 weeks, followed by a suspension of 15 days and a restarting during the first 3 months. One treatment/month for the next 3 months, and then 1 every 2 months for the remaining 6 months. At the end of the treatments, the patient showed a serum ferritin value of 75 ng/mL and normal values of ALT/AST. The controls after 1 EA year have shown the firmness of these values. *Conclusions.* The results of our study suggest that EA seems to be an advantageous treatment of the iron overload post-BMT, inducing quickly iron depletion with no side effects and long-term maintenance of remission of ferritin values.

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PROSTAGLANDIN-E2 CRITICALLY ENHANCES THE REGULATORY FUNCTION OF DENDRITIC CELLS VIA INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION

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Introduction. Indoleamine 2,3-dioxygenase (IDO1) and indoleamine 2,3-dioxygenase-like (IDO2), are enzymes involved in the tryptophan catabolism. It is well known that IDO1-expressing dendritic cells (DCs) contribute to tolerance in a number of biological settings, whereas the expression and function of IDO2 in DCs is under investigation. DCs can be generated *in vitro* to obtain immunogenic antigen-presenting cells (APCs) to be used as cellular vaccines. In the clinical setting, DCs are commonly matured with a cytokine cocktail (CC) which includes TNF-α, IL-1β, IL-6 and PGE2. In particular, PGE2 enhances APC function of DCs by increasing IL-12 production and facilitating DC migration to lymph-nodes. *Methods.* CD14^{+} were immunomagnetically isolated and cultured with GM-CFS and IL-4 in order to obtain DCs. DCs were matured with different maturation stimuli, including LPS, LPS + IFN-γ, CD40L and the CC. After culture, DCs were tested for the gene and protein expression of IDO1 and IDO2, kynurenine production, inhibition of T-cell proliferation and Tregs induction. *Results.* among the different maturation stimuli, PGE2-containing CC was the most effective in up-regulating IDO1 and IDO2, both at mRNA and protein level. Accordingly, DCs matured with PGE2-containing CC showed the highest kynurenine production and the highest generation of CD4^{+}CD25^{+}FOXP3^{+} Tregs. Interestingly, within the CC, the up-regulation of IDO1 and IDO2 was strictly associated with the presence of PGE2 in a dose-dependent manner. IDO1 and IDO2 induction of PGE2 corresponded to increased capacity of DCs to generate Tregs, which highly suppressed allogeneic and autologous T-cell proliferation. Such inhibitory effect was eliminated when both IDO1 and IDO2 inhibitor, 1-L-methyl tryptophan (MT) and 1-D-MT, respectively, were added to cell cultures. *Conclusions.* These data demonstrate that the exposure of DCs to PGE2 during the maturation phase results in the enhancement of important tolerogenic features, such as the capacity to induce Tregs, through the up-regulation of IDO gene and protein. From a clinical standpoint, the strong IDO1 and IDO2 up-regulation by PGE2 can limit the effectiveness of PGE2-matured DCs as cellular vaccines.

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HEMATOPOIETIC CD34+ CELLS GIVE RISE TO CD1A+CD14-CD103+, IDO-EXPRESSING LANGHERANS CELLS WITH TOLEROGENIC PROPERTIES

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Introduction. Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in tryptophan catabolism along the kynurenine pathway. IDO expressed by different cell subsets inhibits T-cell activation, proliferation and survival and induces regulatory T cells (Tregs), thus mediating immunological tolerance. Although human monocyte-derived dendritic cells have been shown to express IDO, little is known about its expression in other subsets of human DCs, including those generated from CD34+ hematopoietic progenitors (CD34+-derived DCs). In particular, no data are currently available for IDO expression in CD34+-derived DC subsets, such as dermal DCs and Langherans cells (LCs). *Methods.* CD34+-derived DCs were generated from healthy donors from purified CD34+ cells after 7 days of culture with GM-CSF and TNF-α. Then, DCs were separated into CD1a-CD14+ (dermal DCs) and CD1a+CD14- LCs. DCs subsets were analyzed for IDO expression by real-time PCR and western immunoblot, kynurenine production, inhibition of allogeneic proliferation and Tregs induction. *Results.* CD34+ cells did not express IDO mRNA expression regardless of the progenitor cell sources (cord blood, mobilized peripheral blood, bone marrow). During DC differentiation, IDO expression and function, evaluated by enzymatic and immunological tests, was markedly induced at day 7. Interestingly, the expression of IDO was shown to be 10 times higher in the LCs compartment as compared to dermal DCs. IDO expression by LCs resulted in increased production of kynurenine and in reduced allostimulatory capacity of T-cell proliferation. Moreover, LCs were shown to induce a population of CD4+CD25+Foxp3+ which acted as Tregs by inhibiting allogeneic T cell proliferation. This effect was abrogated by the addition of the IDO inhibitor 1-methyl tryptophan. Phenotypically, IDO-expressing LCs expressed CD103, which has been recently identified as a marker for tolerogenic DCs. *Conclusions.* DC differentiation of CD34+ cells results in the expression of a functionally active IDO protein in LCs. Given the role of IDO in regulating immune tolerance, a subset of LCs, such as those expressing CD103, may be intrinsically committed to function as regulatory DCs. These data point toward IDO expression as part of a tolerogenic signature during DC development.

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OUTCOME OF THERAPY-RELATED ACUTE MYELOGENOUS LEUKEMIA AND MYELODISPLASTIC SYNDROMES RECEIVING ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. Patients with therapy-related myelodisplastic syndromes (tMDS) and acute myelogenous leukemia (tAML) are not frequently cured by allogeneic stem cell transplantation (alloSCT). Since disease and transplant-related mortality are high, it is crucial to understand which patients may benefit from alloSCT. This retrospective study was aimed to assess which characteristics may predict the alloSCT outcome in this setting. *Methods.* Twenty-eight patients with tAML (86%) or tMDS (14%) allografted in 4 Italian centers between 1998 and 2009 were included. Patients' median age was 49 years (range 21-65), previous neoplasia was lymphoma (83%) or non-hematologic cancer (17%) and it was treated with chemotherapy (31%), radiotherapy (14%) or both (55%). 36% of patients received >2 therapy lines. tMDS/tAML occurred after a median time of 86 months (range 13-253) from previous cancer. Cytogenetic analysis was performed in 86% of patients, 33% of them had intermediate-risk and 67% of high-risk cytogenetics according to Medical Research Council AML10 Trial definitions. 82% of patients received induction and 71% consolidation, 18% received upfront alloSCT. Median time from tAML/tMDS diagnosis to alloSCT was 5.7 months (range 0-25). At transplant, 32% of patients had a Karnofsky Performance Status (KPS)≤80%, 40% of patients was in CR1, 3% in CR2, 14% in PR and 25% in PD. Patients underwent reduced intensity (RIC, 39%) or myeloablative (61%) alloSCT from HLA identical (32%) or mismatched siblings (3%), unrelated (54%) or haploidentical donors (11%). AlloSCT outcomes were analyzed by Kaplan-Meier method for overall (OS) and progression free survival (PFS) and by Cumulative Incidence method for non-relapse mortality (NRM) and relapse incidence (RI). *Results.* Twelve patients (43%) are alive at last follow-up, 7 (25%) died of disease, 9 (32%) died of NRM. Median follow-up of surviving patients was 528 days (55-1704). One- and 2-years OS was 50% and 36%, 1- and 2- years PFS was 42% and 38%. RI was 28% at both 1 and 2 years, NRM was 18% at 100 days, 30% at 1 year and 35% at 2 years. OS and PFS were impaired by high-risk cytogenetics (P=0.03 and P=0.01, respectively) and KPS≤80% (P=0.008 and P<0.001). RI was higher in patients with KPS≤80% (P=0.02), chemo-resistant disease (P=0.05) or high-risk cytogenetics (trend, P=0.08). Patients treated for previous neoplasia with >2 therapy lines had a worse NRM (P=0.005) and OS (P=0.05), regardless of treatment type (chemotherapy, radiotherapy or both). NRM was higher in patients who had infectious complications at induction (P=0.009), received consolidation (P=0.03) or allografted >180 days after tAML/tMDS diagnosis (P=0.05). Conditioning (RIC *vs.* myeloablative) did not affect survival, NRM and RI. *Conclusions.* AlloSCT is an effective procedure for few patients affected by tAML/tMDS, and patients' selection should be based on cytogenetics, KPS, and treatment history. Prospective trials are awaited in order to confirm these results.

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HEMATOPOIETIC, MESENCHYMAL AND IMMUNOCOMPETENT CELL PROFILE IN HAPLOIDENTICAL DONOR G-CSF PRIMED FOR NON T-CELL DEPLETED BONE MARROW TRANSPLANT

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Introduction. Granulocyte-colony stimulating factor (G-CSF) is widely used to mobilize hematopoietic stem cells (HSC) from bone marrow (BM) niche to the peripheral blood (PB) where they are harvested by leukapheresis. Although the hematopoietic and immunologic features of G-CSF–mobilized PB grafts have been extensively studied, less is known about the characteristics of the cells abided in BM after mobilization. *Study design.* Recently, the feasibility and clinical value of non T-cell depleted BM transplant from haploidentical family donor have been reported. The present study has been addressed to evaluate the modifications of hematopoietic and lymphoid donor cells in both compartments, either BM or PB, induced by G-CSF priming used in this particular transplant setting. *Methods.* After informed consent, BM and PB samples were obtained form donors before priming with G-CSF administered at dose of 4 μg/Kg/d for 7 days and at the day of transplant. The effects of G-CSF were evaluated by the following tests: clonogenic assay for the detection of hematopoietic and mesenchymal progenitors, cytofluorimetric evaluation of CD34+ cells, early and late CD34 subpopulations, immunophenotyping of lymphocyte cell fractions, cytofluorimetric determination of human Th1/Th2 cytokines. *Results.* The results of the modification induced by G-CSF priming on BM (G-BM) and PB (G-PB), expressed as increase over the baseline levels, are reported in the Table. The early CD34 cell fractions (CD34+38- and CD34+90+) were sig-

nificantly increased in G-BM, by contrast the fractions of mature hematopoietic progenitors increased significantly more in G-PB. The mesenchymal progenitors (CFU-F) increased in G-BM more than 10 fold compared to the steady state BM. With regard to the effect of G-CSF on the cells of the lymphoid compartment, a consistent increase of type-2 (CD40+) dendritic cells either in BM (2.5 fold) and in PB (5.5 fold) has been detected. The monocytes significantly increased in G-BM (P=0.044). The absolute number of T-reg cells was higher in G-BM than in G-PB ($CD4^+25^+$: median 3.55×10^5/mL *vs.* 1.37×10^5/mL). Finally, after G-CSF priming a significant increase (P=0.005) of the IL-10 cytokine was detected in the serum. *Conclusions.* These data are in agreement with our clinical experience in non T-cell depleted BM transplant from haploidentical donor: after G-CSF priming, the significant increase of the early HSC in BM ensure the long-term engraftment, which is conversely delayed (21 median day) by the migration of committed progenitors towards the PB. Furthermore the selective increase of DC2 cells, the lymphocyte polarization towards Th2 differentiation, the increase of BM mesenchymal progenitors and regulatory T cells might contribute to contain the risk to developping advanced grades of acute and chronic GVHD, which account for an incidence of only 7% and 5%, respectively, in a series of 55 patients receiving a non T-cell depleted BM transplant from a haploidentical donor.

Table.

	BM vs G-BM		*PB vs G-PB*	
	fold increase	P	fold increase	P
NC/mL	4.13	<0.001	5.9	<0.001
CFU-GM/mL	3.11	0.039	12	<0.001
BFU-E/mL	3.23	0.004	6.9	0.006
CFU-GEMM/mL	1.77	ns	12	0.002
CFUtot/mL	3.06	0.004	8.3	<0.001
CFU-F/mL	10.86	0.021		
$CD34^+$/mL	2.35	ns	3.4	ns
$CD34^+38^-$/mL	39.95	0.027	3.8	ns
$CD34^+90^+$/mL	20.55	0.012		
$CD34^+33^+$/mL	32.27	0.006	4.4	ns

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CYTOKINE INDUCED KILLER CELLS POSSESS BOTH T AND NK MARKERS AND FUNCTIONAL PROPERTIES

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Introduction. Cytokine Induced Killer (CIK) cell cultures are a heterogeneous population of effector cells activated and expanded *in vitro*. They show a potent non-MHC restricted cytotoxicity against tumor cells of several lineages, in particular hematologic neoplasms, but not against normal cells *in vitro* and *in vivo* in murine models. At maturity, three lymphocyte subsets constitute the bulk of the CIK cell culture: $CD3^+/CD56^-$, $CD3^-/CD56^+$ and $CD3^+/CD56^+$, the latter representing the predominant subset. We have characterised the double nature "T-" and "NK-like" of purified $CD3^+/CD56^+$ CIK cells. *Methods.* CIK cells were generated by stimulation of PBMCs from healthy donors or CMV-seropositive donors with 1000 U/mL IFN- (day 0), 50 ng/mL anti-CD3 antibody OKT3 (day 1) and subsequent expanded for 21 days in 500 U/mL IL-2. $CD3^+/CD56^+$ or anti-CMV specific subpopulations were purified by immunomagnetic beads. Their phenotype was determined by flow cytometry, while their cytotoxic effect was evaluated by calcein release assay. *Results.* $CD3^+/CD56^+$ CIK cells have retained their TCR/CD3 complex usage. Indeed, upon TCR/CD3 crosslinking in CIK cells, we observed ERK-1/2 phosphorylation, IFN-γ production (mean 32.6% of positive cells by intracellular staining), TNF-α production (mean 19.6%), increase in the percentage of cells undergoing degranulation (57.4±6.9 of $CD107a^+$ cells) and strong cytotoxicity in a reverse Ab-dependent killing assay (R-ADCC). The TCR/CD3 mediated activation is blocked by pre-treatment with cyclosporine A, confirming the role of calcineurin in the CD3-linked degranulation pathway. Interestingly, CIK cells retain functional activity as antigen-specific T cells. Indeed, in case of CIK cultures obtained from CMV-seropositive donors, CMV-specific $CD3^+/CD56^+$ CIK cells can be generated. CMV-specific CIK cells immunopurified by HLA-peptide tetramers are able to specifically recognise and kill autologous but not allogeneic PHA-blasts pulsed with CMV specific pp65495-503 but not with irrelevant peptide (average lysis 63±8%, E:T 10:1) and to produce IFN-γ following antigenic stimulation (26.4±7%). The same CMV-specific purified CIK population shows to posses non MHC-restricted cytotoxic properties against K562 leukemic targets (average lysis 31.1±2.8%, E:T 10:1). Regarding the activating receptors relevant for NK activity, CIK cells express NKG2D, DNAM-1 and also, in a small percentage, the NK-specific NKp30. Upon mAb cross-linking in a R-ADCC assay, these receptors could induce cytotoxicity. Blocking of NKG2D, DNAM-1 and NKp30 on CIK cells significantly reduced their non MHC-restricted cytotoxicity. *Conclusions.* These data show that $CD3^+/CD56^+$ CIK cells are activated T cells which have retained their TCR/CD3 complex usage and their antigen specificity but have acquired unrestricted anti-leukemic activity. NK receptors, including NKG2D, DNAM1and NKp30 could be involved in the non MHC-restricted recognition and killing of leukaemic target.

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908ARG VARIANT IN NOD2/CARD15 GENE IS ASSOCIATED TO GASTROINTESTINAL GRAFT VERSUS HOST DISEASE IN PATIENTS RECEIVING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Acute Graft-*vs.*-Host Disease (aGvHD), an immunological disorder in which the T cells present in the graft react against host tissues, is considered the main complication of allogeneic Hematopoietic Stem Cell Transplantation. Acute GvHD may affects many organ systems, including the gastrointestinal (GI) tract, liver, skin, and lungs. To date, this complication remains a source of significant morbidity and mortality in the setting of allogeneic HSCT. Moreover, while the absence of aGvHD is associated with a higher risk of relapse of the underlying malignant disease, severe aGvHD usually induces the occurrence of life-threatening complications such as severe infections. The NOD2 gene polymorphisms (SNPs)(Arg702Trp, Gly908Arg and L1007fsinsC), which characterizes Crohn's disease, have shown to be associated with the risk to develop GI aGvHD and further transplant-related complications. *Aims.* To assess a contribution of Arg702Trp, Gly908Arg and L1007fsinsC NOD2 polymorphisms and to search for likely correlation in clinical GvHD occurrence in patients affected by haematological malignancies receiving allogeneic HSCT in Italian population. *Materials and methods.* One hundred and six HLA-matched donors/recipients pairs (R/D) and 824 healthy controls (HC) were genotyped for Arg702Trp, Gly908Arg and L1007fsinsC polymorphisms by using the TaqMan allelic discrimination method. Genotype frequencies were compared in respect to III-IV GvHD, gastrointestinal GvHD, and TRM. *Results.* The investigated SNPs were in Hardy-Weinberg equilibrium in both controls, donors and recipients. Significant allele and genotype association with Gly908Arg and recipients group (P=0.02, OR=2.4, CI=1.1-5.1; P=0.008, OR=2.6; CI=1.2-5.3, respectively) was shown, with a subsequently significant difference of allele frequency only in GI GvHD group (P=0.03, OR=4.74, CI=1.4-16.29). After stratifying the R/D pairs on the basis of NOD2 genotypes ($NOD2^-$=no major variants; $NOD2^+$=at last one variant), only a trend towards NOD2+ pairs with GI GvHD (33%), III-IV GvHD (30%), and TRM (33%), compared with group without GvHD (23%), was also demonstrated. *Conclusion.* The 908Arg (risk allele) variation has shown to be mainly associated with recipients who developed GI GvHD. In addition, in $R^+D/NOD2^+$ pairs, a trend towards in GI GvHD, III-IV GvHD and TRM, was also demonstrated.

Transplantation II

P205

CHANGES IN CIRCULATING ENDOTHELIAL CELLS (CEC) DURING AUTOLOGOUS PERIPHERAL HSC INFUSION IN MYELOMATOUS PATIENTS

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Introduction. Circulating endothelial cells (CEC) concentration in bloodstream is a rough estimate of endothelial damage. We explored the reliability of this parameter in evaluating the endothelial distress caused by the conditioning regimen and the infusion of thawed DMSO containing suspensions of peripheral blood stem cells (PBSC). We measured the CEC levels in myeloma patients submitted to Melphalan 200 mg/m^2 regimen followed by autologous PBSC infused 24 hour after the chemotherapy. *Method.* we evaluated 19 myeloma patients (13 males and 6 females). From each patient we obtained 5 samples: (1) before chemotherapy, (2) 24 hours after therapy and before the PBSC infusion, (3) 48 hours, (4) 7 days and (5) 14 days from the chemotherapy. We also evaluated 21 samples from healthy donors as negative control. CECs measurement was performed using a combination of pre-enrichment of CD146$^+$ circulating cells and multiparameter flow cytometry measurement (FCM). CD146$^+$ cells were isolated using CD146-coated magnetic nanoparticles and labelled using CD45-fluorescein isothiocyanate and CD146-PE or isotype control antibody and propidium iodide before FCM. CECs are defined as CD146$^+$/CD45$^-$ nucleated cells. Results have been considered as non parametric variables and analyzed matched-pairs Wilcoxon test. We considered significant the difference when P values resulted lower than 0.05. *Results.* Comparing for each patient the CEC levels at each selected time with that measured before chemotherapy, we found a significant increase immediately after the PBSC reinfusion: p(t2-t0)=0.0156, while their values progressively reverted to basal levels near the 14th day after the treatment: p (t4-t0)=0.6359. No difference was found between males and females. *Discussion.* This data shows that CEC concentration has a typical trend in relation to the main events of the treatment: chemotherapy and infusion of staminal cells. The increase of CEC level could be related to the damage induced to the endothelium by chemotherapy the PBSC thawed DMSO-containing suspension by chemical toxicity and hypothermic shock .The following normalization of CEC levels in about 14 days reflects the fade of those injuries and the endothelium repair. Data repeatability suggests that a typical endothelial response happens after those therapeutic procedures and shows that the evaluation of CEC concentration could reliably monitor its entity and progression.

P206

LONG-TERM FOLLOW-UP OF AUTOLOGOUS STEM CELL TRANSPLANTATION IN RELAPSED/REFRACTORY HODGKIN'S LYMPHOMA AFTER THE BEAM REGIMEN: A RETROSPECTIVE ANALYSIS

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Introduction. Autologous stem cell transplantation (ASCT) is the standard therapy for patients with relapsed or refractory Hodgkin's lymphoma (HL). From 1994 to 2009, 138 patients were referred to the "Coordinamento Trapianti di Cellule Staminali Emopoietiche della Regione Lazio" and received ASCT after the BEAM conditioning regimen. The main objective of this final report is to analyze the long-term outcome of these patients and to identify prognostic factors that may have influenced progression-free survival (PFS). *Methods.* There were 62 females (45%) and 76 males (55%), with a median age at ASCT of 27 years (range 8-66). HL stage at diagnosis according to the Ann Arbor criteria was evaluable in 132 cases: 2 patients (1.4%) were in stage I, 55 (40%) in stage II, 31 (22%) in stage III and 44 (32%) in stage IV. Seventy-two % of patients had B symptoms and 47% had bulky disease. Disease status at transplant was Ist sensitive relapse in 70 patients (50%), ≥2 sensitive relapse in 1 (0.7%), primary refractory responsive to 2nd line treatment 43 (31%) or to more than 2 lines 19 (14%), progressive disease in 5 (4%). Median time from diagnosis to ASCT was of 18 months (range 2-188). For 56/70 patients in 1st relapse, median duration of 1st CR was 13 months (range 1-156), in 20/56 cases 1st CR duration was less than 12 months. All patients received the BEAM conditioning regimen (BCNU 300 mg/m^2 on day -6, Ara-C 200 mg/m^2 and Etoposide 200 mg/m^2 on days -5 to -2, Melphalan 140 mg/m^2 on day -1). *Results.* Stem cell source was bone marrow in 8 cases and peripheral blood in 130. The median number of CD34$^+$ cells infused in patients transplanted with peripheral blood stem cells was 5×10^6/Kg (range 1-37). The median time to >500 PMN/ L was 11 days (range 3-47) and to >50000 PLTS/ L 17 days (range 4-107). Mortality in the first 30 days was 1.4%. The percentage of patients free from progression at 180 days from ASCT was 88%; according to disease status at transplant, it was 87% for 1st sensitive relapse, 88% and 57%, respectively, for patients autografted in response after 2 or >2 lines of treatment, and 40% for progressive disease patients. The median follow-up of progression-free patients is of 52 months (range 1-172), which resulted in a Kaplan-Meyer estimate 10-year overall PFS of 62%; in particular: 72% for 1st relapse patients, 57% for primary refractory and 30% for patients with progressive disease. In a Cox regression model analysis, the only variable that significantly influenced PFS was disease status at transplant. For patients in 1st relapse, a 1st CR duration <12 months did not affect PFS (Log rank: P= 0.9). *Conclusions.* The results of this retrospective analysis on a multicentric patients' database are of interest, although pre-transplant PET data, which may allow a better classification of disease status at transplant, were not available for the majority of patients.

P207

STORE OPERATED CALCIUM ENTRY (SOCE) IS EXPRESSED IN HUMAN ENDOTHELIAL PROGENITOR CELLS

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Introduction. Circulating endothelial progenitor cells (EPCs) are mobilized from the bone marrow to the sites of tissue regeneration and sustain neo-vascularization after acute vascular injury. Moreover, they represent an appealing tool for cell-based therapy in regenerative medicine. Finally, they are thought to be involved in the early phases of cancer growth and dissemination by favoring tumor angiogenesis and establishment of sites of metastasis. An increase in Ca^{2+} concentration is the key signal in the control of a myriad of functions in mature endothelial cells (ECs), including the responses to VEGF and SDF-1. Ca^{2+} signals are sustained by a number of Ca^{2+} permeable ion channels within the plasma membrane. Among these channels, store-operated Ca^{2+} entry (SOCE) represents the main pathway for Ca^{2+} inflow and controls the genetic program underlying mature EC proliferation by engaging Ca^{2+} sensitive transcription factors such as NF-κB. Whereas SOCE and intracellular Ca^{2+} homeostasis have been extensively investigated in mature ECs, little is known about SOCE in circulating EPCs. The aim of this study was to investigate SOCE expression in EPCs harvested from both peripheral blood (PB) and umbilical cord blood (UCB). Methods. EPCs were grown *in vitro* either from human PB (n=6) or from human UCB (n=5) as endothelial colony forming cells (ECFCs), according to Ingram *et al.* (Blood 2004;104:2752) and their endothelial origin confirmed by phenotypic analysis of surface proteins. Presence of SOCE in ECFCs was investigated by employing Ca^{2+} imaging, whereas its putative mediators were assessed at molecular level by real time RT-PCR carried out on total RNA extracted from *in vitro* expanded ECFCs. Results. SOCE was induced both by pharmacological (i.e. cyclopiazonic acid) and by physiological (i.e. ATP) depletion of intracellular $Ca2^+$ pools. Furthermore, store-dependent $Ca2^+$ entry was inhibited by the SOCE inhibitor, BTP-2, thus confirming the existence of SOCE on EPC membrane. Notably, BTP-2 significantly reduced the rate of proliferation of EPCs isolated from PB. Pharmacological maneuvers demonstrated that, as in differentiated ECs, the signal transduction pathway leading to the depletion of intracellular Ca^{2+} pools impinged on the phospholipase C/InsP3 pathway. Real time

RT-PCR, finally, showed that both PB-EPCs and UCB-EPCs expressed all the molecular candidates that are known to mediate SOCE in mature ECs, including TRPC1, TRPC4, Orai1 and Stim1. Conclusions. These findings provide the first evidence that EPCs, both from PB and from UCB, express SOCE, and gain novel and crucial insights into the molecular mechanisms which govern EPC response to angiogenic stimuli. In addition, SOCE might be regarded as a novel target both to enhance the regenerative outcome of cell-based therapy and to modulate, upon its inhibition, EPCs trafficking in cancer patients.

P208

ELEVATED SERUM FERRITIN AND MAJOR TRANSPLANT-RELATED COMPLICATIONS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Iron overload (IO), mainly from red cell transfusions, is a frequent condition in hematopoietic stem cell transplantation (HSCT). IO is associated with free radical generation and tissue damage which can increase toxic and infectious events after HSCT. We retrospectively evaluated the clinical impact of pretransplantation iron status on early transplant related toxicity. *Patients and methods.* The charts of 224 (110 M, 114 F, median age 43 years, range 17-70 years) patients who underwent HSCT [142 autologous (auto) and 82 allogeneic (allo)] were reviewed. Serum ferritin, a surrogate marker of iron overload, was measured before the beginning of the conditioning regimen. The range of diagnoses included 83 patients with acute myeloid leukemia, 35 with acute lymphoblastic leukemia, 2 with myelodysplasia, 45 with myeloma, 59 with lymphoma. In the allo group, myeloablative conditioning was employed for 63 pts and reduced intensity conditioning for 20 pts. The median pretransplant serum ferritin of the 224 patients was 720 ng/mL with values ranging from 20 to 9255 ng/mL.We set 800 ng/mL as the cut-off value for pre-transplant serum ferritin, obtaining a group of 143 patients with ferritin levels <800 ng/mL (low ferritin group) and a group of 81 above the cut-off level (high ferritin group). The effect of elevated pretransplant ferritin on the incidence of mucositis, blood stream infections, day 100 mortality, acute GvHD and invasive fungal disease was assessed. Student's t-test or the Mann-Whitney test was performed for comparisons of means. *Results.* The high ferritin group showed a higher incidence of grade 3 and 4 mucositis than the low ferritin group after both auto (30 % *vs.* 15%, P=0.05) and allo transplantation (34% *vs.* 15%, P=0.03). At univariate analysis, the incidence of bloodstream infections was significantly lower in the low ferritin group after auto (15% *vs.* 30%, P=0.05) and allo HSCT (25% *vs.* 40%, P=0.05). In the autologous group, the high ferritin group had increased day 100 mortality (5% *vs.* 1%, P=0.06). In allogeneic group, the high ferritin group had increased day 100 mortality (due to hepatic veno-occlusive disease, pneumonitis and severe endothelial leakage syndrome) (20 *vs.* 8%, P=0.03), increased acute GvHD grade II-IV (57 % *vs.* 25%, P=0.02), increased invasive fungal disease (18% *vs.* 5 %, P=0.05). The time to engraftment was shorter in the low ferritin (median: 13 days, range: 11-16 days) than the high ferritin group (15 days, 13-23 days) after allogeneic HSCT (P=0.05). *Conclusions.* Pre-transplant serum ferritin was a predictor for major transplant-related complications, especially after allogeneic HSCT. Our results need to be validated by further prospective studies. Methods for modifying IO in candidate patients for HSCT should be developed.

P209

EFFECT OF DONOR REGULATORY T CELLS ON CMV-SPECIFIC CD8$^+$ T LYMPHOCYTES RECONSTITUTION AND ACUTE GVHD AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Backgound. Regulatory T-cells (T reg,CD4$^+$CD25^{high}Foxp3$^+$) are important regulators of allo-reactivity and may therefore represent an important predictor for the risk of acute graft versus-host disease (aGvHD) and immune reconstitution after allogeneic peripheral stem cell transplantation (allo-PBSCT). *Patients and methods.* To determine the clinical significance of T regs in peripheral stem cell grafts, we analyzed 34 myeloablative alloPBSCT and correlated the T regs in the donor graft with the aGvHD incidence and immunological recovery (evaluated on recovery of CMV-specific CD8$^+$ T lymphocytes by tetramer analysis). We used fluorochrome-conjugated tetrameric complexes of HLA-A101, HLA-A201, HLA-B702, HLA-B801, HLA B3501 to monitor recovery of CMV-specific CD8$^+$ according to the patient's HLA. Patients were transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=29) or an HLA identical unrelated donor (n=5). Median age was 32 years (range (r) 18-58); diagnoses were acute myeloid leukaemia (n=31) and acute lymphoblastic leukaemia (n=3). The median T regs dose administered was 5×10^6/Kg (r:1-20). The patients were divided into a high T regs group (T regs>5×10^6/Kg, n=14) and a low T regs group (T regs<5×10^6/Kg, n=20) according to the number of T regs in the grafts. *Results.* Median CMV-specific CD8$^+$ T lymphocytes were significantly higher in patients with high than with low T regs in the graft at 1(2 cells/mm^3 *vs.* 0, P<.001), 2(6 cells/mm^3 *vs.* 1, P<.001), and 3(15 cells/mm^3 *vs.* 3, P<.001) months. During the three months after transplantation, CMV infection/disease was observed in 2/13 (15%) patients with high T regs and in 12/20(60%) patients with low T regs (p=.015). Moreover, the median recovery of T regs after transplantation was significantly higher in patients with high T than with low T regs in the grafts at 2 (15 cells/mm^3 *vs.* 6, P<.001) and 3 months(23 cells/mm^3 *vs.* 8, P<.001). The incidence of aGvHD (grade II-IV) in the high infused T regs group was lower than in the low T regs group (1/13 or 7% *vs.* 11/20 or 55%, P=.009). *Conclusions.* We suggest that there is a good correlation between the number of T regs in the graft and the incidence of aGvHD after myeloablative allo-PBSCT. T regs mediate protective effects against aGvHD and the maintenance of an optimal microenviroment for the reconstitution of functional immunity. Our results support further consideration of T regs immunotherapy for clinical alloSCT.

P210

GENERATION OF A T-CELL CLONE DIRECTED AGAINST AML BLASTS THAT RECOGNIZES A TUMOR SPECIFIC ANTIGEN EXPRESSED ALSO BY SOLID TUMORS

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Introduction. The aim of this study was to generate cytotoxic T-lymphocytes (CTL) clones directed against AML cells and to identify new leukemia specific antigens. *Methods.* We loaded normal dendritic cells (DC) from healthy donors with apoptotic bodies from primary AML cells and used loaded DC to stimulate autologous lymphocytes. Donors were selected to be partially matched with the leukemic patient for HLA-class I. With this strategy, CTL lines were generated that specifically recognized both loaded DC and AML cells used for loading. CTL lines were cloned by limiting dilutions and 180 CD8$^+$ clones were screened by IFN-gamma elispot. *Results.* Two clones were selected that recognized 5/5 HLA-matched primary AML and 0/8 mismatched AML (clone 31D3 and clone 8F12). They did not recognize both resting and activated normal myeloid, lymphoid and CD34$^+$ cells expressing the proper HLA-restriction allele (HLA-B7 supertype for clone 31D3 and HLA-B44 supertype for clone 8E12). To confirm the lack of reactivity against normal tissues, we analyzed the elispot activity of both clones against HLA supertype matched or mismatched fibroblasts and we found that clone 8E12 displayed a low reactivity activity against normal matched fibroblasts, while clone 31D3 was not reactive. We therefore focused the analysis on clone 31D3 and tested whether it could also kill AML cells, and whether its cytotoxic activity was restricted to leukemia or also directed against a small panel of HLA-B7+ positive (N=3) or negative (N=5) solid tumors. Interestingly, we found that the clone 31D3 could recognize and kill not only AML cells but also one colon carcinoma and one melanoma cell line expressing the HLA-B7 supertype. In particular, the melanoma cell line (G4-mel) was HLA-B35$^+$ (HLA-B35 belongs to HLA-B7 supertype) and was recognized at very high levels. The availability of a tumor cell line expressing adequate amounts of antigen will make the generation of a cDNA library more feasible than with primary leukemic cells. Finally, to further confirm that HLA-B35 is

the proper MHC restriction element and to determine to what extent the antigen is shared among different tumors, we transduced 7 tumor cell lines (both solid and hematopoietic malignancies) and 3 normal lymphoblastoid B-cell lines with the HLA-B35 allele. Clone 31D3 efficiently recognized 5/7 HLA-B35 transduced tumor cell lines but none of the control B-cell lines. *Conclusions.* Clone 31D3 recognizes an antigen that is shared among tumors of different histology and is restricted by the HLA-B7 supertype. Considering the allogeneic model used for antigen presentation, we hypothesize that this clone recognizes a minor histocompatibility antigen (i.e. a polymorphic peptide) from a protein expressed by malignant cells and not by normal tissues, and therefore a protein that might play an important role in malignant transformation. We are now attempting to identify this shared tumor antigen.

P211

T-CELL BIOMARKERS OF CLINICAL OUTCOME AFTER T-CELL BASED HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION: AN APPRAISAL IN 135 CONSECUTIVE PATIENTS

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Introduction. Hematopoietic stem cell transplantation from a haploidentical family donor (haplo-HSCT) offers the chance of a cure to patients with high-risk leukemia who lack an HLA-compatible donor. In haplo-HSCT, however, the T-cell depletion required for avoid rejection and reduce the risk of graft-versus-host disease (GvHD) results in delayed immune reconstitution and associates with a high incidence of disease relapse and life-threatening infections. With the aim of speeding up immune reconstitution, we explored the possibility of "T-cell based" haplo-HSCT in a cohort of 135 consecutive patients (median age 48, advanced disease in 61%). Donor T cells were administered either in concomitance with the graft (10^6/Kg) followed by immune suppression with rapamycin (n=81) or as delayed infusions (10^7/Kg) after genetic modification with the tk suicide gene (n=54). The purpose of this study was to explore in retrospect different biomarkers of T-cell immune reconstitution and to find correlates with clinical outcome. Methods. At different time points after haplo-HSCT, we studied different biomarkers of T-cell immune reconstitution by phenotypic, molecular and antigen-specific techniques. Results. One-year non-relapse mortality was 31%, mainly due to infections and especially to CMV-reactivation syndromes. Grade II-IV GvHD incidence was 17%. Relapse mortality was 47%, and 5-years overall survival was 22%. At day 90 after haplo-HSCT, the median $CD3^+$ cell count/microl was 378. Higher early post transplant $CD3^+$ cell counts did not predict a better overall survival, nor lower relapse and non-relapse mortalities. However, an early-differentiated T-cell phenotype, including naïve and central memory T cells, was significantly associated with a lower risk of disease relapse (P less than 0,05), possibly reflecting a wider T-cell repertoire as demonstrated by TCR Vβ spectratyping. Interestingly, in patients treated with rapamycin, there was a substantial expansion of T cells with a regulatory phenotype ($CD4^+/CD25^+/Foxp3^+/IL\text{-}7R^-$), averaging 10.9%. Analysis of Foxp3 demethylation status confirmed the Treg nature of this sub population. At an antigen-specific level, the reconstitution of anti-CMV T-cell responses measured by IFN-γ ELISPOT inversely correlated with the risk of CMV reactivation (P<0.01). Conclusions. Altogether these results show that absolute T-cell counts are poor predictors of clinical outcome in haplo-HSCT and prompt the validation of novel candidate biomarkers for clinical purposes as well as for the implementation of targeted immunointervention.

P212

AN ALLO-RESTRICTED PEPTIDE-SPECIFIC $CD8^+$ T-CELL CLONE DIRECTED AGAINST THE UNIVERSAL TUMOR ANTIGEN SURVIVIN

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Introduction. High-avidity T cells specific for self-peptide/self-MHC ligands are eliminated in the thymus by negative selection. An attractive strategy to circumvent tolerance to self-protein is the generation of peptide-specific, allo-restricted cytotoxic T cells (CTL). Our aim was to isolate an allo-restricted CTL clone with high avidity against a modified, HLA-A2 restricted survivin epitope. Survivin is the prototype of "universal" tumor associated antigens, because it is expressed at high levels in nearly all tumor types and at low levels in normal tissues. *Methods.* A survivin specific CTL clone was generated by priming $CD8^+$ cells from an HLA-A2 negative donor with peptide-pulsed T2 cells. Primed $CD8^+$ cells were restimulated with peptide-pulsed PBMC from 2 different donors who were mismatched only for the HLA-A2 allele. One day after each restimulation, CD137 expression was evaluated on bulk cultured CTL and after the fourth cycle a significant CD137 upregulation was observed (8%). The $CD137^+$ fraction was purified by magnetic beads and cloned by limiting dilution. *Results.* A total of 135 clones were screened by IFN-gamma elispot using peptide-loaded T2 cells pulsed with the modified survivin peptide or with an irrelevant peptide. 109 clones were not reactive, 25 clones were reactive against both the relevant and irrelevant peptide and one clone (4C12) was highly reactive only against the survivin peptide. The activity of clone 4C12 was tested against T2 cells pulsed with escalating doses of the modified (LMLGEFLKL) and of the naïve (ELTGEFLKL) survivin peptide. The functional avidity against the modified peptide was higher than against the naïve peptide, and 4C12 activity against T2 cells pulsed with the naïve peptide was similar to that against $HLA\text{-}A2^+$ leukemic cells expressing endogenous survivin (THP-1). We have also tested clone 4C12 against $HLA\text{-}A2^+$ normal fibroblasts and EBV-transformed B-cells and found that it has a low level of reactivity against these targets. Real time PCR was performed on normal and malignant targets, which revealed that the degree of reactivity is strictly correlated with the amount of survivin transcript expressed by each cell type. After these experiments, clone 4C12 could not be further expanded *in vitro* probably due to proliferative exhaustion/cell senescence. Therefore, we have characterized and sequenced the V-a and V-b family of its T-cell receptor (TCR) and cloned them into a lentiviral backbone vector. Lentiviral transduction will allow to impose survivin specificity to recipient T-cells and to better characterize the antitumor activity and the safety profile of this TCR. *Conclusions.* If clone 4C12 will confirm its high degree of specificity and activity against $HLA\text{-}A2^+$ tumor cells overexpressing survivin, it will be used for redirecting the T-cell repertoire of tumor patients against survivin by TCR gene transfer. The final aim is to develop an effective cell therapy approach for AML and MDS patients.

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PU01

TREATMENT OF REFRACTORY PRURITUS IN A PATIENT WITH CHRONIC MYELOMONOCYTIC LEUKAEMIA BY PHLEBOTOMY

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Introduction. Generalized pruritus can often be the primary manifestation of systemic diseases of hematological origin such as lymphomas, leukemias and polycythemia vera. Chronic myelomonocytic leukemia (CMML) is a myelodysplastic/myeloproliferative disease affecting elderly people (>60 years), characterized by hepatosplenomegaly, serous effusions and a marked peripheral monocytosis (>1×10^9/L). The pruritus is a rare systemic symptom of disease, often refractory to common treatments (hydroxyzine, trimeprazina, cetirizine, etc.). In this paper we report the case of a patient with CMML suffering from years of systemic pruritus refractory to standard therapies, treated with phlebotomy therapy that has resulted in the total clinical remission of the symptoms. *Methods.* A 54-year-old man, with spread pruritus to upper and lower limbs, not sensitive to treatment with common anti-histaminic drugs , was observed by our Immunohematology Division. The clinical history revealed a gingival bleeding tendency, while the physical examination demonstrated hepatosplenomegaly, petechiae and a widespread organized pericardial effusion. The patient was then subjected to the following diagnostic tests: blood count (BC), biochemical profile, ECG and echocardiogram, chest X-ray and complete abdomen Eco. The BC showed the following values: Hb: 15.2 g/dl, Hct: 49.8 %, MCV: 89, WBC: 12.8×10^3/mm^3, N:45% M:20%, PLT: 100.000/mm^3. An echocardiogram and chest x-ray confirmed the presence of a no significant pericardial effusion; eco abdomen revealed a complete spleen diameter> 10 cm and a bilateral renal lithiasis. It was decided to perform a bone marrow aspirate showing a diagnosis compatible with CMML (blasts and promyelocytes <20%). It was begun thus a cycle of chemotherapy with hydroxyurea and, in parallel, it was programmed the cycles of phlebotomy (Hct: 49%), in order to control the refractory pruritus. *Results.* The patient was initially subjected to a phlebotomy of 200cc weekly for 2 consecutive weeks and at the end of the treatments he showed a Hb 13.5g/dl and Hct 45%, accompanied by a marked improvement in the pruritus symptomatology. It was therefore decided to continue with weekly phlebotomy at the following volumes (mL): 250, 300, 350. After the last phlebotomy, the patient showed a total disappearance of the pruritus for about 1 month. The reappearance of pruritus induced a pattern of a further cycle of phlebotomy at the following volumes (mL): 200, 250, 300, 350. At the end of this treatment the patient showed a complete remission of the pruritus symptomatology, which it was preserved over the time. *Conclusions.* The data of our study show that the phlebotomy therapy of tailored type for each individual patient seems to be a very good clinical practice in the control of refractory pruritus of the CMML, and it can be probably extended to other disorders of myeloproliferative and non origin, characterized by the same type of symptoms.

PU02

HIGH FREQUENCY OF PRIMARY THYROID TUMOURS (PTC) OCCURRING IN A GROUP OF 428 CONSECUTIVE LYMPHOMA PATIENTS OBSERVED IN A 5 YEARS PERIOD IN A SINGLE HAEMATOLOGICAL UNIT

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Background. The emergence of secondary neoplasia after treatment of a primary cancer is a more and more frequent event. The probability of a secondary cancer is due to the toxic effect of therapies and to genetic predisposition. Nonetheless epidemiological studies have highlighted that, despite previous therapies, several gene mutations and/or polymorphism are involved in the individual predisposition to multiple primary tumors. *Aims.* Evaluating the incidence of primary solid tumours in patients affected by lymphoma. *Materials and Methods.* The incidence of other primary cancers, was investigated in a series of 428 patients diagnosed with lymphoma between January 2003 and January 2010. Primary cancers were considered 1) cancer diagnosed at the same time of lymphoma in patients who had not previously received chemotherapy and/or radiotherapy 2) solid cancers that were not synchronous with the diagnosis of lymphoma but diagnosed in patients who were not treated with chemotherapy and/or radiotherapy, with the exception of those patients who received radiotherapy outside the field of tumor occurrence. Patients suffering from Sjogren syndrome were excluded from the analysis. *Results.* 21 patients (4.9%) out of 428 consecutive lymphoma patients, had a diagnoses of a primary solid tumour and overall 25 cancers were diagnosed. The relative frequency (RR) of the different tumours was the following: thyroid 32% (8 cases), renal 16% (4 cases), melanoma 12% (3 cases), breast 15% (2 cases), lung 8% (2 cases), uterus 15% (2 cases), liver 4% (1 case), colon 4% (1 case), myxofibrosarcoma 4% (1 case), tongue 4% (1 case). *Conclusions.* The RR of PTC (32%) in our group of lymphoma patients appears to be unexpectedly high compared to the RR of PTC in the Italian Register of Tumours (1.6%), suggesting possible common mechanism or similar pathogenetic pathways. This association deserves further studies to be elucidated.

PU03

TISSUE FACTOR PATHWAY INHIBITOR AND HOMOCYSTEINE: EVIDENCE FOR ENDOTHELIAL DAMAGE IN ALZHEIMER'S DISEASE PATIENTS

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Although Alzheimer's Disease (AD) is mainly considered a neuronal disease, much evidence points to a vascular pathogenetic involvement in its etiology. Many vascular risk factors have been associated to AD; i.e. Hyperhomocysteinemia (HHcy) is one of the strongest independent risk factors for vascular and cerebrovascular disorders and has recently been associated to the risk of develop AD in elderly people. Moreover, β-amyloid peptide (Abeta), which plays a central role in AD, not only exerts harmful effects on the vessel walls, increasing the risk of silent hemorrhagic and ischemic strokes, but also facilitates the ultrastructural degeneration of the vessels. Conversely, vascular damage can influence APP processing, modulating the expression of enzymes responsible for Abeta production. Based on these evidence, we investigated the possible involvement of vascular damage in the pathogenesis of AD, by assessment of plasma levels of tissue factor pathway inhibitor (TFPI), a serine protease inhibitor induced by endothelial injury; homocysteine (Hcy); and folate levels, the most important co-factors involved in methionine metabolism. Plasma levels of tissue factor (TF), and thromboxane B2 (TXB2) were also evaluated. 110 probable AD, 38 mild cognitive impairment, 31 patients affected by idiopathic Parkinson's disease (without dementia) and 100 healthy controls, who displayed no vascular disorders were enrolled. TFPI and Hcy were significantly higher in AD patients with respect to other groups. The levels of TFPI and Hcy were positively correlated in hyperhomocysteinemic AD and mild cognitive impairment subjects, and were negatively correlated with folate levels. Our findings suggest that an impairment of endothelial function associated with high Hcy levels may occur in AD patients, despite the absence of manifest cerebrovascular lesions. Therefore, TFPI may represent a candidate marker of endothelial damage in AD and might be used for the identification and monitoring of patients that would benefit from folate supplementation treatment.

PU04

OUR EXPERIENCE WITH DCR PROTOCOL IN WALDENSTRÖM MACROGLOBULINEMIA

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Introduction. Waldenström macroglobulinemia (WM) is a distinct entity restricted to patients with lymphoplasmacytoid lymphoma, which involves the bone marrow and causes the secretion of a monoclonal

immunoglobulin M (IgM) into the serum. The symptomatic patients require treatment to control symptoms and invalidate or prevent complications of the disease. Alkylating agents and the anti-CD20 monoclonal antibody rituximab are the primary treatment of symptomatic patients with WM that induce at least a partial response in 30% to 50% of patients. It has been difficult to select the most active treatment for the rarity of this disease, the different diagnostic and response criteria, and the small size of most trials. The combination DCR had been used in WW with good response. *Patients and Disease Characteristics.* 3 patients with WM were treated with DCR protocol. The diagnosis of WM was established by the presence of lymphoplasmacytoid lymphoma involving the bone marrow and by the detection of serum monoclonal IgM. All patients had $CD20^+$ tumor cells, determined by either bone marrow immunohistochemistry and flow cytometry. The patients had a clear indication to start treatment and we applied strict criteria to assess response based on the international recommendations. The median time to response was about 4 months. Characteristic of patients: 3 male patients, median age 68 years, with splenomegaly (all), hyperviscosity syndrome (one), lymphadenopathy (two), hemoglobin <10 g/dL (all), platelets $<100\times10^9$/L (all), serum M protein >4 g/dL (all), Albumin <3.5 g/dL (all), β2-microglobulin >4.0 mg/dL (all), bone marrow lymphocytes >50% (all). The reasons for begin treatment were anemia, hyperviscosity, splenomegaly and lymphadenopathy, weight loss, night sweats. All patients didn't develop neutropenia or thrombocytopenia or adverse effects related to infusion of rituximab. We found in all patients a slow PR response after eight DRC that continues for about a year. *Discussion.* The DRC regimen, designed in 2002, consist of dexamethasone 20 mg i.v. followed by rituximab 375 mg/m^2 i.v. on day 1 and cyclophosphamide 100 mg/m^2 orally bid on days 1 to 5, every 21 days for 6 months; it is poorly myelotoxic therefore advisable for the patients with cytopenia. For the patients who need rapid disease control CHOP may be more appropriate. Furthermore, the infectious complications after DRC were not pronounced. The cases we reported are too small to make an assessment of efficacy as well as the time elapsed since the end of CHT is short; DCR protocol causes remission soft slowly and small without side effects also for the high monoclonal IgM. The RP of the patients is maintained for long. *Conclusion.* We conclude that DRC regimen is an effective and well tolerated treatment, administered without difficulty, convenient, and active for symptomatic and untreated patients with WM but the response is slow.

PU05

PRIMARY NON-HODGKIN'S LYMPHOMA OF THE ORAL TONGUE: CASE REPORT AND REVIEW OF LITERATURE

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Introduction. Primary non-Hodgkin's lymphomas of the oral region are extremely rare and only few cases involving the oral tongue have been reported in the literature. We report a case of oral tongue non Hodgkin's lymphoma diagnosed in our hospital. *Case Report.* We describe a 57 year old woman who presented with a mass lesion primarily involving the oral tongue and diagnosed as diffuse large B cell non-Hodgkin's lymphoma. She presented with a history of a slowly growing painless swelling on right side of the tongue for many months without other symptoms. Local examination revealed a nodular hard lesion, involving the left margin of half of the oral tongue. The mobility of the oral tongue was unaffected. On examination, he was pale, with upper cervical non-tender lymphadenopathy. Examination of oral cavity revealed proliferative growth on the left and posterior part of the tongue. Margins were indistinct. Her routine investigations were normal. She was HIV negative. Biopsy of the lesion on histopathological examination demonstrated a diffuse B non-Hodgkin's lymphoma. Immunohistochemical evaluation showed positive immunoreactivity for LCA (leukocyte common antigen), $CD3^-$ and $CD20^+$, KI67 70%, BCL2+-, $BCL6^+$. Bone marrow aspiration, CSF examination, total body CT scan and ultrasonography were performed. No other sites in the body were found to be involved by the disease. Thus, a final diagnosis of the primary non-Hodgkin's lymphoma, diffuse large cell type, B cell of the oral tongue was established and her disease was staged as I-E. The patient started chemotherapy with R-CHOP and after the first cycle is in partial response. The Radiotherapy is programmed after the end of the chemotherapy. *Discussion.* 20-30% of non-Hodgkin's lymphoma arise from extranodal sites. Involvement of various parts of the oral cavity is very uncommon. The gingiva and the hard palate are the most often involved intraoral sites. Involvement of buccal mucosa, oral tongue, floor of mouth and lip(s) has been reported quite infrequently. To date, only 12 cases of the non-Hodgkin's lymphoma of the oral tongue has been mentioned in the literature. Little is known about the etiological factors for primary lymphoma of the oral region. Few cases of oral lymphomas have been reported in association with AIDS. It generally affects the elderly. The oral non-Hodgkin's lymphoma may mimic more commonly benign oral and dental pathologic conditions . Thus, these lesions may be easily misdiagnosed. Most of the head and neck non-Hodgkin's lymphomas including oral lesions are of B-cell origin and diffuse large cell type being the most common. Some tumors of oral region can be confused with lymphomas. Conclusion: non-Hodgkin's lymphoma involving oral region is uncommon, it should always be considered in differential diagnosis of various benign and malignant lesions in this region, because the treatment and prognosis for these conditions are quite different.

PU06

THE TREATMENT WITH AZACITIDINA IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA: OUR EXPERIENCE

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Introduction. The management of elderly patients with acute myeloid leukemia (AML) is more than ever a challenge. Results associated with intensive chemotherapy remain disappointing, at least, in non selected patients. On the contrary, there are still no real standard alternatives even if some new approaches seem promising. 5-Azacytidine (AZA) is a pyrimidine nucleoside analog that has been discovered more than 40 years ago and approved in 2004 by the US FDA for the treatment of all subtypes of myelodysplatic syndromes (MDS). Complete remission rates ranged between 10-17%, and more recently, a significant survival benefit for MDS patients treated with 5-AZA could be established. The antineoplastic activity is due to incorporation into RNA with disruption of RNA metabolism, and inhibition of DNA methylation. Methods and *Results.* During the year 2009 eight patients of our institution received AZA as front-line treatment because they were considered not elegible for intensive chemotherapy due to age, co-morbidities or poor performance status. Median age of patients was 75 years (range 63-90) , 3 were male and 5 were female. 1 case was post chronic LMMO, 5 cases were post MDS, 1 case was post essential thrombocythemia and 1 case was a LMA without hematologic diseases in the past. A seven day-per month schedule was employed in all patients. The median number administered of cycles of AZA was six (range1-15). 4 patients received AZA at the fixed dose of 100 mg /day (one single vial) s.c., 4 patients received a dose of 75 mg^2/day s.c.. Five patients received AZA alone and three patients in combinations with valproic acid and ATRA. AZA was associated with few total days in hospital. Median remission duration was 24 weeks, and median survival has not been reached. The most side effects were myelosuppression, infections and gastro-intestinal adverse events. Eight partial response were observed. Three patients died and five patients are at moment in continuous partial remission with monthly therapy with AZA. The standard dose of 75 mg^2/ seem to more effective than 100 mg but it is more toxic. The survival was good as the quality of life and the need for transfusion were decreased. Conclusion: Older patients with AML have today limited options of treatment because of the lack of effectiveness of them and for the toxicity of available therapies. We investigated in our division the efficacy and toxicity of the hypomethylating agent azacitidina as initial therapy in older patients with AML de novo or after MDS. The AZA prolongs the OS with significant improvements in elderly patients with WHO AML with low marrow blast counts; it might be considered an effective and relatively safe drug, and

may have a contribute to improving the quality of life and delaying the progression of the leukemia. AZA has good and significant responses in old patients with LMA as front- line treatment.

PUB07

RICHTER'S SYNDROME BEGAN WITH SEVERE HYPERCALCEMIA AND EXTRA NODAL INVOLVEMENT: CASE REPORT

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Introduction. Richter syndrome (RS) is well known as a secondary high-grade lymphoma, developed in patients with B-cell chronic lymphocytic leukemia (B-CLL). RS occurs in 3-10% of B-CLL cases; it is often characterized by the abrupt development of systemic symptoms (fever without infection, night sweats, weight loss), progressive lymphadenopathy, hepatosplenomegaly and high serum lactate dehydrogenase. RS frequently arises in the lymph nodes or bone marrow. Extra nodal RS has also been reported to occur in the central nervous system, eye, gastrointestinal system, nose, kidney, skin, face, testis, bone and bronchus. *Case report.* A 55 years old man affected by B-CLL - diagnosed about eight years ago - developed extranodal non Hodgkin lymphoma, large B cell, six months after the start of alemtuzumab used as consolidation for a partial response after R-FC chemotherapy. The patient was treated in the past with steroids, also for AEA, and with splenectomy. After few months of the end of alemtuzumab he had pain at the jaw and at the all bone, drowsiness and nausea. The clinical examination shows no lymph nodes. Laboratory tests showed a hypercalcemia and he was treated with zoledronic acid, steroids and diuretics with normalization of the test. A bone scan showed spread osteolytic lesions. After eight days progressive exophthalmos and right nodules head to the heap. CT scan demonstrated a mass to develop intra and extraorbital right region with osteolysis of the roof of the orbit. Other lesions in ipsilateral temporal muscle and the skull bilaterally and at left front-temporal region of the cranial with osteolysis. Biopsy of the temporal lesion, bone marrow aspiration and biopsy procedures were performed. After seven days there was an enlargement of lymph nodes in the neck; a CT scan revealed multiple lymphadenopathy, nodules of the liver, adrenal, multiple swellings at the coast. Biopsy specimens of the mass revealed a peripheral lymphoma diffuse large B cell. Liquor examination was negative. Bone marrow biopsy revealed total infiltration of CLL. Coexisted thus, two clones, one of CLL in peripheral and bone marrow and one of the large cell NHL at extra nodal sizes. The patient received one cycle of R-ICE protocol and died 2 weeks later. No autopsy was performed. *Discussion.* Diffuse large B cell lymphoma is the most common histology seen in RS. Abnormalities of chromosomes 11 and 14 are most frequently involved in RS. Immunosuppression related to treatment with fludarabine is suggested to promote RS. The therapeutic options include chemo-immunotherapy, followed by allogeneic bone marrow transplant but the patients are resistant to tretment and died within months of diagnosis. *Conclusion.* We hypothesize that alemtuzumab treatment promoted the uncontrolled growth of the latest clone of the initial B-CLL and inducing a strong T cell depletion with consequent impairment of the immunosurveillance.

PU08

USE OF COMBINED ORAL ADMINISTRATION OF ANALGESIA AND ANXIOLYSIS FOR PAIN ASSOCIATED WITH BONE MARROW ASPIRATION AND BIOPSY IN CHRONIC MPN AFFECTED PATIENTS

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Introduction. Bone marrow aspiration and biopsy (BMAB) is central to the diagnosis and management of many haematological disorders. In adults, the infiltration of local anaesthesia at the biopsy site is the main type of analgesia used for BMAB; unfortunately this is often insufficient, especially during aspiration of bone marrow. In addition, pain is likely to contribute to the anxiety the patient may already be experiencing. In this study we assessed the use of oral administration of analgesia (fentanil) and anxiolysis (midazolam) combined. *Patients and methods.* One hundred and seven consecutive adult Patients referred for bone marrow examination to our day hospital unit were enrolled, following their informed consent to the procedure. All Patients received an injection of local anaesthesia (LA) with 10 mL 2% lignocaine. Patients were then randomized into two groups, 52 who received LA alone (group A), and 55 who received LA plus 5 mg midazolam (oral administration) and 200 mcg of fentanyl trans-mucosal (group B), 30 min before the procedure. The pain experienced was assessed by the Numeric Rating Scale which distinguishes ten levels of pain, from 0 to 10 at five time points during the procedure (baseline T0, start LA T1, aspiration T2a, biopsy T2b, five minutes after the end of the procedure T3). At the end, each patient was given a questionnaire about efficacy, satisfaction, and comfort, according to three scores (1/low - 2/medium - 3/high). All biopsies were performed by the same junior experienced staff member. *Results.* At time T1 the medium level of pain was 0.87 in group A *vs.* 0.88 in group B; at time T2a it was 3.63 in group A *vs.* 3.54 in group B; at time T2b it was 4.63 in group A *vs.* 4 in group B ($P<0.05$); at time T3 it was 0.41 in group A *vs.* 0.16 in group B ($P<0.05$). Moreover, the 21 patients, who had previously undergone the procedure without sedoanalgesia, claimed to prefer the new approach. *Conclusions.* Our data confirm other reports showing no substantial differences in pain relief in patients undergoing BMAB with or without sedoanalgesia. To the best of our knowledge, this is the first study assessing a combination of 5 mg midazolam and 200 mcg of fentanyl as pain reliever. In addition, given the lack of side effects in group B, we underline the safety of administering this drug combination. Based on our results and those in the literature, it may be preferable to involve the Patient in the option of administering sedo-analgesia, avoiding deciding a prioristically on the basis of someone else's pain.

PUB09

ITP-LIKE SYNDROME IN CANCER PATIENTS

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Introduction. Thrombocytopenia in cancer patients is more often related to chemo- and/or radiotherapy, especially with elevated doses of chemotherapeutics or when the cumulative dose (radiotherapy) is high. A thrombocytopenia resembling idiopathic thrombocytopenic purpura (ITP-like syndrome), instead, is rarely associated with solid tumors. Only about 40 cases are reported in the English literature. We here describe 4 cases of ITP-like syndrome observed in our Division of Hematology between 1999 and 2008. *Patients and Methods.* Four patients (2 M, 2 F; age range: 62-74 years) were diagnosed as having ITP according to standardized criteria. Anti-platelet antibodies determination (on serum and elute) was carried out by ELISA in all patients, except for patient #1 in which cytofluorimetry was used instead. In patient #4, immunohistochemistry with monoclonal antibodies versus CD62P was performed on biopsy samples of the sarcomatous nodules, in order to detect P-selectin on the surface of activated platelet in the neoplastic vessels. *Results and Conclusions.* (Table 1 and Legend) In 3/4 patients, thrombocytopenia was diagnosed before than the solid neoplasm. Only in 1 case they were discovered concomitantly. Patient #1 initially achieved a complete response to therapy but he relapsed after prednisone discontinuation. Subsequently, he was only partially responsive to the treatment with the same corticosteroid. Also in patients #2 and #3 only a partial response was achieved. In the fourth patient, instead, a treatment to increase the platelet number was not needed. Anti-cancer therapy (surgery +/- chemotherapy or chemotherapy alone) was followed by platelet number normalization in the first three patients. In the patient affected by Kaposi's sarcoma (pt #4), treated only with pegylated liposomal doxorubicin, a partial response was obtained. In patients #1, #2, and #3, the sequence of events suggests a causal relation between the tumor removal, the stable increase in platelet number, and the disappearance of anti-platelet antibodies. It also indicates a possible immunologic

mechanism: neoplastic antigens may stimulate the production of antibodies that cross-react with platelet glycoproteins. In patient #4, likely, the thrombocytopenia was due also to platelet activation (and subsequent adhesion-aggregation) into the abnormal tumor vessels, in addition to the dysimmune state. P-selectin is a protein of -granules expressed by activated platelet during platelet secretion. On biopsy samples of the sarcomatous nodules, it was possible to demonstrate the presence of P-selectin on the platelet surface (into neoplasm vessels).

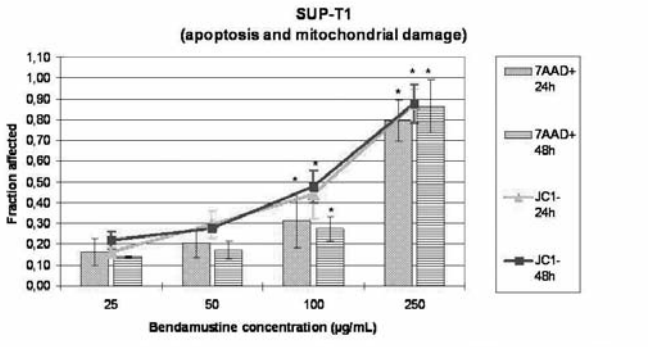

Figure 1. Apoptosis induced by Bendamustine after 24 and 48 hours of incubations on SUP-T1 cells measured with 7-AAD (columns) and disruption of mitochondrial membrane potential evaluated with JC-1 (lines). * p<0.05 compared to control (0µg/mL).

PU10

IRON OVERLOAD IN TRANSFUSION DEPENDENT MYELODYSPLASTIC SYNDROMES (MDS)

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Introduction. Blood transfusion is the only supportive therapeutic chance in MDS patients refratory to other treatments. Repeated transfusions always cause an iron overload with an elevated associated comorbidity and mortality risk independently from their primitive hematological disease. Several studies have demonstrated that patients with "good prognosis" (Refractory Anemia, Acquired Idiopathic Sideroblastic Anemia, and 5q- Syndrome) have an elevated morbidity and mortality risk after the transfusion of more than 100 units of blood red cells. Deferasirox is a new, convenient, once-daily oral iron chelator that has demonstrated in various clinical trials good efficacy and acceptable safety profile in adult and pediatric patients affected by transfusion-dependent thalassemia major and by different chronic anemias. *Methods.* We have treated 10 patients affected by MDS with deferasirox (4 AISA, 4 RA, 2 5q- Syndrome) refractory to any treatment modality and blood transfusion dependent form at least 1 year. All patients (6 male and 4 female, median age 69 years) showed before the beginning of the iron chelator treatment more than 2000 ng/mL of ferritinemia and a mean blood transfusion request of 1 unit of red blood cells every week in order to maintain Hgb levels higher than 8 g/dL. All patients received deferasirox 10 mg/kg p.o. once-a-day. A dose escalation to 20 mg/kg p.o. once-a-day was performed after one month from the beginning of the treatment. *Results.* After 6 months from the beginning of the therapy with deferasirox all the patients showed a reduction of ferritinemia (an about 64% decrease, r: 61-68%). Interestingly, after 6 months from the beginning of deferasirox therapy, a reduction of the transfusion request (50%) was recorded in four out of ten patients. Until to-day (24 months after the beginning of the therapy) we have not recorded either toxicity or adverse events. *Conclusions.* Our results confirm the effectiveness of the therapy with deferasirox in reducing the iron overload in polytrasfused MDS patients with acceptable toxicity profile. Moreover, recent studies seem to suggest a therapeutic role of deferasirox in MDS, independently of its iron chelating action: deferasirox seems to act as a potent NFkB inhibitor and this property could explain the activity in MDS, which results in the improvement of the Hgb level. However, further studies are warranted to define the role of deferasirox in reducing the blood transfusion request in a subset of patients affected by MDS refractory to any other kind of conventional therapeutic strategy.

PU11

VALUTATION OF T-LYMPHOCYTES CD200+ AND T-REGULATORY AFTER *IN VITRO* TREATMENT WITH ACTIVE DRUGS AGAINST CLL

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Background. Purine analogues- based regimens, and particularly those containing fludarabine, are considered the gold standard for CLL therapy. However, fludarabine treatment is sometimes complicated by autoimmune haemolytic anaemia (AHA). The mechanism of this side effect is not clear but it is conceivable that a fludarabine-induced suppression of some regulatory systems, including T-reg, is responsible for this phenomenon. In addition, we have observed that patients affected by autoimmune diseases such as AHA or PTI have a reduced number of T lymphocytes bearing the CD200 antigen that is considered a tolerogenic molecule. In this perspective, we evaluated the variation of T-reg and $CD200^+$ T lymphocytes induced by incubating *in vitro* peripheral blood mononuclear cells (PBMC) of CLL patients and normal subjects with purine analogues and other drugs active against CLL. *Methods.* PBMC obtained from patients with chronic lymphocytic leukaemia (CLL) (n=12) and from normal adult (n=8) were isolated by density gradient and cultured in RPMI supplemented with 10% FBS and 1% of penicillin streptomicyn. Cells were then incubated for 24 hours with drugs at two concentrations: bendamustine (1 and 50 µg/mL), prednisone (1 and 10 nM), fludarabine (1 and 10 µg/mL) pentostatin (10 and 60 µg/mL). The cytotoxicity was evalutated after 24 hours by trypan Blue and flow cytometry. T-reg cells were identified as $CD4^+/CD25^+/FoxP3^+$ T cells and expressed as a percentage of the $CD4^+$T-cell population. *Results.* Although all of these drugs induced lymphocytes cytotoxicity, fludarabine reduced also the percentage of T-reg and $CD200^+$ T -lymphocytes, while bendamustin and pentostatin induced the same cytotoxicity but spared T-reg populations and CD200+ T-lymphocytes. The results are shown in Table 1. In conclusion, pentostatin and bendamustine seem to be active drugs against CLL and their usage should not be complicated by autoimmune phenomena.

Table 1.

	$\%CD3^-CD200+$		$\%CD4^+CD25^+FOXP3^+$		% Lymphocytes	
	N.H n=8 means	*CLL n=12 means*	*N.H n=8 means*	*CLL n=12 means*	*N.H n=8 means*	*CLL n=12 means*
No Drugs	100	100,00	100	100	100	100
Bendamustin 1 µg/mL	107,3*	104,9*	101	107,3*	96,5*	85,7*
Bendamustin 50 µg/mL	122,6*	129,3*	121,4*	130,1*	79*	56,8*
Prednisone 1 nM	101,8	100,4	100	88,9*	81,5*	79,0*
Prednisone 10 nM	90*	98,4	90,1	86*	62,5*	68,4*
Fludarabin 1 µg/mL	93,5*	95,7*	86	81,8*	77*	80,7*
Fludarabin 10 µg/mL	82*	77,6*	74,7	78,4*	67,5*	62,2*
Pentostatin 10 µg/mL	118*	108,7*	105,3*	111	80,5*	80,7*
Pentostatin 60 µg/mL	140,5*	133,0*	124,3*	119,8*	75*	75,4*

**P < .05 by t test.*

PU12

SAFETY AND EFFICACY OF RITUXIMAB-SUPPLEMENTED HIGH DOSE CHEMOTHERAPY SUPPORTED BY AUTOLOGOUS PERIPHERAL STEM CELL TRANSPLANTATION AS *IN VIVO* PURGING FOR RELAPSED OR HIGH-RISK NON HODGKIN'S LYMPHOMA

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Introduction. The concept of *in vivo* purging represents one of the most attractive topics of Rituximab, however clinical impact of multiple doses of its before or concurrent to ASCT is still pending. On this basis we have conducted a prospective study with the objectives of evaluating feasibility, safety and efficacy of adding Rituximab both during mobilizing therapy and ASCT. *Methods.* Study included patients aged 20-65 years with FL or DLBCL with relapsed/refractory disease, first PR or first CR but at high risk (IPI >3 or FLIPI >2, and/or bulky disease at onset of disease). Patients were given IEV regimen (Ifosfamide, VP16, Epirubicin)

supplemented with Rituximab 375 mg/mq on day 0 and +7. Patients were planned to receive one or two cycles before ASCT. Before and after cryopreservation leukapheresis have been characterized for level of $CD34^+$ cells and MRD by PCR. Preparative regimen was BEAM (Carmustine, AraC, VP16, Melphalan) supplemented with Rituximab 375 mg^2 on day -8 and +1. Primary end points included: $CD34^+$ count, CFU assay and vitality of collected PBSCs, time to hematopoietic recovery, rate of infections or any adverse event. Secondary end points included: OS and PFS estimated using the Kaplan-Meier method. *Results.* 38 consecutive patients have been enrolled in this study: M/F ratio 17/21. Mean age was 50 years (30-65). Histology was FL in 15 patients and DLBCL in 23 (11 high-risk disease, 11 PR, 14 relapsed disease and 2 refractory disease). All patients collected adequate amounts of PBSC with a mean value of 11.23×10^6/kg (2.1-32.2). Evaluation of MRD performed on PBSC was negative in all patients but one. A mean of 10 (6-14) doses of Rituximab were infused: a cumulative dose of 3.750 mg/mq/patient. Patients received a mean number of $CD34^+$ of 6.04×10^6/kg (2.16-12.88). All patients engrafted (mean time to >500/mcl neutrophils and to >20.000/mcl platelets respectively 11 days and 12 days). A mean of 2 RBC and 3 PLT transfusions were required. No patient died of TRM. During neutropenia, 21 infectious events have been recorded: 9 FUO, 6 septicaemias, 2 interstitial pneumonias, 2 CMV and 1 HHV6 reactivations and one probable aspergillosis. The infusion of Rituximab did not have any significant impact in terms of time to start leukapheresis, time to engraftment, number of transfusions and outcome, nor on CFU assay and vitality. The mean follow-up was 30 months (range 6-58). Mean OS and PFS of the entire cohort were respectively 86.8% and 73.4%. At mean follow up OS and PFS for patients with FL and B-DLCL were 92.3%/58.7% and 82.9%/82.1% respectively. *Discussion.* Our data show that Rituximab-supplemented high dose chemotherapy supported by ASCT does not interfere with PBSC collection and hematological reconstitution. Our study is not powered enough to detect any significant improvement in OS and PFS, however the clinical outcome of our cohort seems very promising.

PU13

PRIMARY PROPHYLAXIS WITH PEGFILGRASTIM IN ELDERLY PATIENTS WITH LYMPHOMA OR BREAST CANCER TREATED WITH CHEMOTHERAPY: A MULTICENTER STUDY

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Introduction. Cancer is a disease of the elderly, and its incidence and mortality increases with age. However, elderly patients exhibit a greater risk of bone marrow suppression by chemotherapy and chemotherapy-induced neutropenia and its complications are more likely in these patients. Also age, comorbidities, stage of disease, treatment with highly myelosuppressive chemotherapy could influence the outcome of these patients and therefore led to a higher incidence of febrile neutropenia. The benefit of treating elderly patients with colony-stimulating factors is well established, with their use beginning in the first cycle of chemotherapy being crucial for minimizing neutropenia and its implications. *Aims.* This study evaluated the incidence of febrile neutropenia and related events in elderly cancer patients receiving pegfilgrastim beginning with cycle 1 (proactive) in primary prophylaxis. *Methods.* 200 patients (≥65 years of age, median age 75 years, 45 male, 155 female) with breast cancer (n=104, 30 subject with disease stage 2 and 74 with a disease stage of 3-4) and Non-Hodgkin's lymphoma (NHL, n=96, 13 subject with disease stage 2 and 83 with a disease stage of 3-4). 52 breast cancer patients and 74 NHL patients have an eastern cooperative oncology group (ECOG) performance status score of 2. CIRS comorbidities scale was grade II in 62 breast cancer and in 80 NHL. All the patients received pegfilgrastim 6 mg from cycle 1 chemotherapy. *Results.* Number of courses of Pegfilgrastim administered: 1080 (median 6 at patient); number of chemotherapy delivered: 6-8 cycles. Grade 4 neutropenia occurred in 16/200 patients (8%, 5 patients with breast cancer and 11 patients with lymphoma): 10 patients with neutrophils count between 100-400 mm^3 and 6 patients with neutrophils count about 0 mm3 at nadir (all lymphoma patients). About 39.5% of the patients with G4 neutropenia, were hospitalized for 15 days. No patient died for Neutropenia or its consequences. Grade 3 neutropenia (3 events for patients, with absolute neutrophil count 800-900/mm^3) occurred in 24/104 patients with stage III breast cancer (23,7%) and 60/96 patients with stage III and IV lymphoma (62,5%). No one patient with G3 neutropenia has been hospitalized. All the subjects completed the planned cycles without dose reductions or delays. 20 subject died because progression disease. 27 patients (13.5%) experienced grade 5 pain (according the VAS scale). *Conclusions.* Our data suggest that in 200 elderly patients with advanced solid and hematologic malignancies with ECOG 2, the primary prophylaxis with pegfilgrastim compares favorably with our experience using the same chemotherapy regimens with daily G-CSF support in term of incidence of neutropenia, grade 3 (42%) and grade 4 (8%) and hospitalizations for fever and infections. No patient died for febrile neutropenia, according with literature review (Balducci, The oncologist 2007;12:1416-24).

PU14

CLOFARABINE, CYTARABINE AND GEMTUZUMAB OZOGAMICIN (CLAC-MYL) IN REFRACTORY/ RELAPSED AML PATIENTS

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Introduction. Clofarabine has been shown to be effective in AML patients, especially in association with cytarabine. On the basis of these reports, we conducted a preliminary study combining clofarabine, intermediate dose cytarabine and gemtuzumab ozogamicin (Mylotarg) in AML patients who relapsed or failed to respond to at least two induction therapies. *Methods.* We treated 24 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/m^2 on day 6 (CLAC-Myl). Seven patients received a further consolidation cycle with clofarabine at 22.5 mg/m^2 and cytarabine at 1 gr/m^2 day 1-4. *Results.* Among the 24 patients, nine were in first relapse, seven in second relapse, eight with resistant disease. The mean age was 51 years (range 33-71 years), the white blood count at the accrual was 31.500×10^9/L (range 2140-153.000). 12/24 patients achieved a complete remission, 3/24 a partial response, 7/24 had resistant disease, 2/24 died of complications during the aplastic phase (a case of multiorgan failure an a septic shock caused by Pseudomonas Aeruginosa). The most frequent non haematologic adverse events were vomiting, diarrhea, transient liver toxicity (2/24 grade 3-4), febrile neutropenia (9/24), infections microbiologically documented (2/24 Pseudomonas Aeruginosa sepsis). 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 31.5 days), except in a patient (female, 34 years old, relapsed after ABMT) that experienced an unexpected, irreversible aplasia after the consolidation course, complicated by an unusual HHV6 reactivation. Among the twelve responding patients, seven eligible for transplant procedures underwent allogeneic bone marrow transplantation, three relapsed after 6, 7 and 12 months, one patient is still in complete remission with a follow up of 18 months.*Conclusions.* These 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.

PU15

CASE REPORT: ABERRANT EXPRESSION OF THE CD4 ANTIGEN IN A DIFFUSE LARGE B CELL LYMPHOMA

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Introduction. Aberrant expression of T-cell antigens on B-cell non Hodgkin lymphomas (B-NHL) is a known but uncommom phenomenon. B-NHLs with aberrant expression of CD2, CD3, CD7, CD8 are previously reported,[1-4] aberrant expression of CD4 however is rarely described by the literature.[5,6] We report here a case of CD4 positive diffuse large B cell lymphoma (DLBCL). *Methods.* A 55-year-old Caucasian female with an abdominal mass was referred to our Instution for clini-

cal evaluation. The abdominal mass was surgically removed and investigated for routine histologic, immunophenotypic and molecular analysis. *Results.* Six color flow cytometry revealed the presence of a large neoplastic B population with atypical coexpression of CD19/CD4 antigens. No other T-cell antigens were displayed by the tumour cells. Histology evidenced a diffuse infiltrate consistent of medium to large lymphoid cells with roundish nuclei, prominent nucleoli and moderate amount of clear cytoplasm; immunohistochemistry confirmed the CD4 expression on the neoplastic B-cells. Molecular analysis documented the immunoglobulin heavy chain rearrangements; the TCR was germ line. *Conclusions.* The CD4 antigen is normally expressed on immature thymocytes, mature T-lymphocytes, monocytes and macrophages; the CD4 expression on a B-cell is a rare event. The significance and the nature of this aberration has not yet been fully determined, although several mechanisms have been proposed for expression of T-antigens on neoplastic B-cells such as the derugulated control of gene expression in malignant B-cells, leading to activation of some silent or repressed gene of T-cell differentiation.[1] The detection of aberrant phenotypes emphasizes the importance of an integrated diagnostic approach combining clinical, morphologic, immunophenotypic and molecular studies and may be diagnostically useful in the detection of post-therapy minimal residual disease.

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PU16

COMPLETE RESPONSE TO SECOND GENERATION TYROSINE KINASE INHIBITORS IN TWO PATIENTS WITH PH-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA RELAPSING AFTER ALLOGENEIC STEM CELL TRANSPLANT

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Philadelphia chromosome (Ph) is the most frequent karyotypic aberration in adults with acute lymphoblastic leukaemia (ALL) and is traditionally related with a poor prognosis. Introduction of tyrosine-kinase inhibitors (TKIs) has led to a significant improvement in treatment outcome of this leukemia. In patients relapsing after allogeneic stem cell transplant (SCT), however, prognosis is still dismal. We report two cases of Ph+ALL relapsing after allo-SCT who obtained a complete response with II generation TKIs. *Case 1.* A 21-year-old man was diagnosed as p190 Ph+ALL in March 2006. He was administered Hyper-CVAD alternating with MTX/HIDAC and Imatinib, obtaining a complete hematological response (CHR) and complete cytogenetic response (CCyR). In November 2006 the patient was submitted to a myeloablative SCT from unrelated donor (MUD). CHR and complete engraftment were observed at month 3, but at month 4 the patient had a hematologic relapse associated with meningeal infiltration. Treatment with Dasatinib was started at 70 mg bid and associated with intratechal chemotherapy. Blast clearance in PB was obtained in 5 days. CHR was achieved after 1 month, and complete molecular response (CMR) was obtained after 4 months. Donor Lymphocyte Infusions (DLI) were administered every 6 weeks for a total of 5 infusions. Due to a grade 2 diarrhea, Dasatinib dosage was reduced to 120 mg/day. The patient is in good clinical conditions and bone marrow is persistently PCR negative 36 months after relapse. *Case 2.* A 18-years-old boy was diagnosed as Ph^+ ALL, p190, in December 2008. He was treated with high dose chemotherapy associated with Imatinib and obtained a CHR with persistence of molecular disease. In May 2009 he underwent a allogeneic MUD transplant. In spite of complete engraftment and BCR/ABL transcript reduction, he relapsed in October 2009, with 88% blasts in BM and 9% donor cells. Mutational analysis showed a 5 aminoacid insertion in BCR/ABL. He started treatment with Dasatinib 70 mg bid, resulting in blast clearance and recovery of 93% chimerism at day 15. After 3 months a full donor chimerism with less than 0.1% blasts was observed in the bone marrow. The patient however complained of diarrhea G3, and Dasatinib was stopped at month 4, when Nilotinib 400 mg bid was started. Diarrhea resolved and the patient is well and in CMR six months after relapse. Few cases of successful therapy with II generation TKIs in Ph+ALL relapsing after BMT have been reported, either in adults or pediatric patients. In children, dasatinib therapy has been used as a bridge to a second transplant. Our patients showed a prompt response to Dasatinib treatment. In one case, it was associated with DLI infusion, and CMR is stable 36 months after relapse. In the second case, Dasatinib therapy was accompanied by gastrointestinal toxicity which resulted in change to Nilotinib, with persistent disease response. Second generation TKIs can rescue Ph^+ ALL patients after SCT failure.

PU17

PSEUDOHYPONATRIEMIA IN A PATIENT WITH NEWLY DIAGNOSED MULTIPLE MYELOMA: DESCRIPTION OF A CASE

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Introduction. Pseudohyponatremia is a rare condition characterized by decrease in plasma sodium concentration associated with normal or increased effective plasma osmolarity. A case with polifactorial pseudohyponatremia is discussed. *Methods.* A 71-years-old woman was admitted to our hospital presenting fatigue, peripheral edema, mild dispnea, dizziness and diarrhoea with vomiting. Physical examination indicated pale face, bilateral edema of lower extremities and few crackles in the bilateral lower lobes. She reported hypertension and anxiety in therapy. Chest x-ray revealed bilateral pleuric effusion, abdominal ultrasound was negative. Echocardiogram showed hypertensive cardiopathy with mild systolic dysfunction. Brain computed tomography was negative. Laboratory examinations revealed: haemoglobin 10.6 grams per deciliter, serum creatinine level 0.65 milligrams per deciliter, gammaglutamyltransferase 91 unit per liter, serum aspartate aminotrasferase 49 unit per liter, serum alanine aminotransferase 56 unit per liter, glycemia 189 milligrams per deciliter, serum sodium level 104 millequivalent per liter, serum cloreum level 72 millequivalent per liter, serum calcium level 7.8 milligrams per deciliter, serum potassium level 3.6 millequivalent per liter. Serum albumin 3.67 grams per deciliter, urine protein 3 grams per liter. The anionic gap was –8 millequivalent per liter, serum osmolality was 226 milliOsm per killogram. PO2, pCO2 and PH were normal. Urine sodium level 11 millequivalent per 24 hours, urine cloreum level 12.10 millequivalent per 24 hours, urine potassium level 31.52 millequivalent per 24 hours. Immunoglobulines M 19 milligrams per deciliter, immunoglobulines G 6217 milligrams per deciliter, immunoglobulines A 7 milligrams per deciliter. Monoclonal protein G 5.7 grams per decilliter. The serum kappa/lambda ratio free light-chain assay 2383/17 milligrams per deciliter, β2microglobulina 3.48 milligrams per liter. Urine monoclonal protein was G kappa and the urine kappa/lambda ratio free light-chain assay were 78.5/0 milligrams per liter. Skeletal radiogram showed diffuse lytic lesions. Bone marrow documented a multiple myeloma. *Result.* We found a case of severe hyponatremia that could be the consequence of secondary hyperaldosteronism (low urine sodium level), cardiac failure, infection with diarrhoea and vomiting, polidipsy, psycosis drug use, multiple myeloma. The patient was treated with intake reduction of drink water, diuretic drugs and insulin therapy with increase in plasma sodium until 130 mEq/l with restore of good clinical condition. She started treatment with bortezomib and dexamethasone and after first cycle of chemothrapy the serum sodium level was normal (137 milliequivalent per liter). *Conclusion.* Considering that the multiple myeloma treatment improved the plasma sodium level, we can say that paraproteinemia should be o could have played a role in the occurrence of hyponatremia.

PU18

INTRA BONE MARROW TRANSPLANTATION OF UMBILICAL CORD BLOOD HEMATOPOIETIC STEM CELL: A REPORT OF TWO CASES

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Introduction. Umbilical cord blood (UCB) has gradually emerged as an alternative source of hematopoietic stem cells in patients who need allo-SCT. The advantages of UCB transplantation are: quick availability of CB, absence of donor risk, tolerance of 1-2 antigen HLA mismatch and low incidence of severe acute GVHD (aGVHD). One of the major limiting factors is the low number of hematopoietic stem cells in a CB unit. Direct intra-bone UCB injection has been suggested to partially overcome this limit. *Methods.* We report here the outcome of intra-bone UCB transplantation in two patients. The first with AML secondary to MDS in second complete remission, the second with refractory Hodgkin's Lymphoma either mismatched for one HLA antigen (locus B). A reduced intensity conditioning regimen consisted of cyclophosphamide (100 mg/kg for 2 days), fludarabine (100 mg/sqm for 5 days), thiotepa (10 mg/kg for 1 day) and thymoglobulin (0.5 mg/kg day -3, 2 mg/kg day -2, 2.5 mg/kg day -1). GVHD prophylaxis was given with cyclosporin A (1 mg/kg) from -7 to +120 day and mycophenolate (30 mg/kg) from +1 to +27 day. The first patient received 1.82×10^7/kg nucleated cells (1.25×10^5/kg $CD34^+$) and the second one received 1.85×10^7/kg nucleated cells (0.43×10^5/kg $CD34^+$). *Results.* The neutrophil (>500/mm^3) and platelet (>50.000/mm^3) recovery occurred on day +36 and +31 for the first patient, respectively. Neutrophil recovery occurred on day +33 for the second patient, for whom platelet recovery has not been achieved yet. The first patient's transplant related complications were Gram negative sepsis on +4 day, CMV infection on day +5, Coombs positive hemolytic anemia on day +71 that was successfully treated with i.v. immunoglobulin. Guillain Barre syndrome developed on day +97 and treatment with i.v. immunoglobulin was given. The second patient's transplant related complications were respiratory failure secondary to fungal pneumonia on day +19 requiring ICU admission and assisted mechanical ventilation, CMV infection and Gram negative sepsis on day +32 , CyA related microangiopathy on day +37 and BK polyomavirus hemorrhagic cystitis on day +56 treated with bladder irrigation and i.v. cidofovir. No aGVHD was documented in our patients. Haematological assessment on day +90 showed mixed chimerism in the first patient and complete donor chimerism in the second patient. *Conclusions.* Preliminary data about our two cases suggest intra-bone UCB transplant is a choice in patients who need an allo-SCT and don't rapidly find a HLA compatible donor. Direct intra bone UCB transplantation is a safe medical procedure that overcomes the problem of graft failure even when low numbers of nucleated cells are transplanted. The mechanisms of UCB engraftment and the low incidence of aGVHD require further intriguing studies. *Acknowledgment.* Supported by "Progetto Regione Lombardia", COFIN 2007, FONDI 60%.

PU19

MULTIPARAMETER FLOW CYTOMETRY (MPFC) 8 COLOURS: COMPARISON BETWEEN MONOCLONAL GAMMOPATHIES OF UNCERTAIN SIGNIFICANCE (MGUS) AND MULTIPLE MYELOMA (MM) PATIENTS

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Introduction. MGUS is a group of diseases in which is present a monoclonal component in serum, while the hallmark of MM is an uncontrolled proliferation of malignant plasma cells localized mainly in the bone marrow. MM and MGUS diseases are characterized, both, by the presence like marker of $CD38^{+bright}$ $CD138^+$ on plasma cells. The aim of this work is to perform 8 colour multiparametric analysis (MPFC) in MGUS and MM patients, comparing to literature results with four and six colours. *Methods.* Bone marrow samples of 14 patients with MM and 17 with MGUS were analysed, respectively. The antibody panel utilized is: CD27 FITC/ CD56 PE/ CD45 PerCP-Cy5.5/ CD117 PE-Cy7/ $CD38^+$CD138 APC/ CD20 APC-Cy7/ CD19 Pacific Blue. One thousand events were acquired on the gate $CD38^{+bright}CD138^+$. The analysis permitted to discriminate different subpopulations of the $CD38^{+bright}CD138^+$ plasma cells. We considered significative only the subpopulations with a percentage of events ≥10%. Acquisition of the samples were performed with BD FACSCantoIITM (BD Biosciences, San Jose, CA, USA) and CyAN ADPTM (Beckman Coulter, Miami, FL, USA), cytometers and analyzed with analysis software Kaluza 1.0 (Beckman Coulter, Miami, FL, USA). *Results.* Contrary to the literature, where the analysis are most performed with four or six colours MPFC, in our work we used an eight colours analysis. This type of analysis was performed in order to identify the distribution CD45 CD19 CD20 CD27 CD56 CD117 antigens relatively to the $CD38^+$bright$CD138^+$ plasma cells population. MM plasma cells population present a value range 7.5-73% (Median=26.85) of bone marrow populations, value range of MGUS is 0.1-4% (Median=1). The MPFC analysis permitted to discriminate 35 subpopulation in the 14 MM cases and 39 subpopulation in the 17 MGUS cases examinated. Our data show that CD20 and CD27 antigens are always expressed in both MM and MGUS cases; CD19 and CD56 don't show difference in their expression contrary to what is reported in literature. The difference we noticed, between MM and MGUS phenotype, is the expression pattern of CD45. (see Table 1) Data relatively to the expression of CD117 antigen need further analysis. *Conclusions.* These preliminary data suggest that eight colours analysis, performed with the Kaluza software, permit the identification of all the subpopulations of CD38+brightCD138+plasma cells, according to CD45 CD19 CD20 CD27 CD56 CD117 expression pattern. We notice that the only antigen that assumes variability within the two diseases is CD45.

Table 1. In the table is reported the percentage of antigen positivity referred to MM and MGUS subpopulations.

	MGUS	*MM*
$CD45^+$	72%	57%
$CD19^+$	36%	34%
$CD56^+$	85%	86%
$CD20^+$	82%	74%
$CD27^+$	82%	80%

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